

Non-overlapping functions of polX family DNA polymerases, pol μ , pol λ and TdT
during immunoglobulin V(D)J recombination *in vivo*

Barbara Bertocci^{1,2}, Annie De Smet^{1,2}, Jean-Claude Weill^{1,2*} and Claude-Agnès Reynaud^{1,2*}

¹ INSERM U783 Paris, France

² Université Paris Descartes, Faculté de Médecine René Descartes, Site Necker-Enfants
Malades, Paris, France
156 rue de Vaugirard
75730 Paris Cedex 15

*Correspondence: reynaud@necker.fr; weill@necker.fr

*These authors share senior authorship

INTRODUCTION

The antigen receptor expressed on B cells is assembled by the rearrangement of separate coding elements in the genome, V (variable), D (diversity) and J (joining) segments for the heavy chain gene, V and J for the kappa and lambda light chain genes. This assembly is mediated by the lymphoid-specific RAG-1 and RAG-2 proteins that recognize and cleave specific signal sequences flanking the coding elements. Recombination is completed by the cellular machinery responsible for double strand break (DSB) DNA repair, i.e. the DNA-PK complex (Ku80, Ku70 and the catalytical subunit, DNA-PKcs) together with the endonuclease Artemis, and the religation complex composed of XRCC4-ligase IV/Cernunnos-XLF (Buck et al, 2006; Ahnesorg et al., 2006; reviewed by Hesslein and Schatz, 2001; Dudley et al., 2005).

The DNA breaks created during V(D)J recombination and their repair by the non-homologous end-joining (NHEJ) pathway differ markedly from other types of double-strand breaks, for example those induced by ionizing radiations. First, they result from a concerted DNA cleavage at two different sites in the genome, frequently located widely apart. These two domains are brought together in a paired complex in which DNA cleavage is completed, generating four DNA ends. Two of them (the signal ends) will be rejoined without nucleotide loss. The two coding ends in contrast are first generated as closed hairpin structures. This unusual configuration will be subject to end-opening, with, in contrast again to most DSB rejoining events, considerable end modification by nucleotide addition or nucleotide loss which represents, at the heavy chain locus at least, a

major contribution to the diversification of the pre-immune repertoire. Moreover, the protein complex comprising the two coding ends must provide enough stability to avoid deleterious transposition events, and sufficient accessibility to allow for the processing of DNA ends. Accordingly, mutations in both the RAG-1 protein and the DNA-PKcs have been described that modify, one way or the other, the accessibility of the coding ends (Lee et al., 2004b; Ding et al., 2003).

Hairpin opening is mediated by Artemis, which displays not only endonucleolytic activity for these specific structures, but also nicking activity at various DNA conformations displaying double-strand to single strand transitions (Ma et al., 2002; Ma et al., 2005). Other exonucleases are probably involved in the processing of DNA ends, although their nature, and the possible fluctuation of their activity along the lymphoid differentiation pathway, are unknown. Polymerase activities at the coding junctions are of several types. Random nucleotide addition during heavy chain gene rearrangement is performed by the lymphoid-specific enzyme, terminal deoxynucleotidyl transferase (TdT) (Desiderio et al., 1984). But the completion of V gene segment ligation most likely involves templated polymerization, by the filling of 3'-recessed DNA ends, bridging of 3'-protruding end across the two coding parts, or simply by antagonizing exonucleolytic degradation.

We have reported previously that DNA polymerase mu, a close homologue of TdT that displays template-dependent polymerase activity, participates in the end-processing of immunoglobulin (Ig) light chain, but not of heavy chain junctions (Bertocci et al., 2003). This study seemed to imply unexpectedly that another polymerase should be involved in

heavy chain gene rearrangement. Pol μ belongs to the polX family that includes two other members, a structurally similar enzyme, pol λ (Aoufouchi et al., 2000; Dominguez et al., 2000; Garcia-Diaz et al., 2000; reviewed by Ramadan et al., 2004), and the enzyme involved in base excision repair, pol β , which, contrarily to the other three, lacks a BRCT-domain involved in the binding of phospho-proteins (Manke et al., 2003; Yu et al., 2003). Pol λ appears therefore as an obvious candidate for participating in the junctional process at the heavy chain locus. A thorough study of an *in vitro* biochemical reconstitution of the NHEJ process has indeed described the participation of TdT, pol μ and pol λ in the formation of DNA junctions starting from incompatible ends (Ma et al., 2004). Their recruitment, mediated through DNA/Ku70-Ku80 complexes, appears to proceed through competition between the three polymerases, in a strict hierarchical order that vary according to the combination of the different partners present in the reaction. More recently however, Ramsden and colleagues have proposed, based on *in vitro* biochemical and cellular rearrangement assays, that only pol μ can participate in V(D)J recombination, through a unique biochemical ability to bridge 3'-protruding DNA ends without any base homology (Nick McElhinny et al., 2005).

We report here the role of pol λ on V(D)J recombination through the study of pol λ -deficient mice, and we address the question of the possible overlapping/competitive function of the three related pol X polymerases by studying mice that harbor a combined inactivation of two of these enzymes. We show that, while pol μ is responsible for the restricted processing of light chain junctions, pol λ participates in heavy chain gene rearrangement, at a step that likely precedes the action of TdT. Collectively, the data

obtained with double deficient animals suggest that the specific function of these different polymerases during V(D)J recombination results from their regulated recruitment and not from a competition between enzymes that harbor defined enzymatic specificities.

RESULTS

Pol μ and pol λ compete with TdT in an *in vitro* recombination assay

In order to examine the possible competition between the various pol X DNA polymerases, we used a transient VDJ recombination assay. We chose to follow the displacement of TdT by the other polymerases through the monitoring of N-nucleotide additions. Pol μ -deficient fibroblasts were transfected with the recombination substrate pJH290 (Lieber et al., 1988), full length RAG1, RAG2 and equimolar ratio of TdT together with the other pol X family members (pol μ , pol λ , pol β). When TdT was the only polymerase added, 70% of the substrate joints had N-nucleotide additions (Supplementary Table 1). As previously reported, the cotransfection of pol μ or pol λ decreased their frequency respectively to 39% and 42%, while the average number of N-nucleotides added per sequence was unchanged (Supplementary Table 1 and Bertocci et al., 2003). In contrast, pol β , as the unrelated protein EGFP, did not modify this value (80% and 67% of sequence with N additions respectively, Supplementary Table 1). Thus, this cellular recombination assay, performed with an extrachromosomal substrate, showed a competition between TdT, pol μ and pol λ but not with pol β at coding junctions.

V(D)J coding junctions in pol μ /TdT-deficient mice: no role for pol μ in heavy chain gene rearrangement or for TdT isoforms in the processing of light chain junctions

We have shown previously that light chain but not heavy chain gene rearrangement was affected in pol μ -deficient mice (Bertocci et al., 2003). We asked therefore whether pol μ

might be prevented from participating in heavy chain gene rearrangement by the presence of TdT at the pro-B cell stage.

To answer this question, heavy chain gene rearrangement was studied in pol μ /TdT-double deficient mice. VDJ_{H4} rearrangements were amplified from B220⁺ IgM⁻ bone marrow cells using either a V_H consensus primer or a primer specific for the V_H81X D-proximal gene. The analysis of these coding junctions showed no difference between TdT- and pol μ /TdT-deficient mice (Table 1, Supplementary Fig.1). In both genotypes the average CDR3 length is 32 base pairs (bp), showing a 7 bp reduction compared to heavy chain joints from wild type animals (Table 2) and varies within a nucleotide range of 14 to 48 bp. Thus, pol μ appears not to be recruited by the rearrangement machinery at heavy chain junctions even in the absence of TdT.

Rare untemplated nucleotides (12 cases out of 201 sequences or 329 if V_H81XDJ_{H4} sequences are included, see Supplementary Fig. 1) are still observed in both TdT^{-/-} and pol μ /TdT^{-/-} backgrounds, suggesting that a low level of error-prone DNA synthesis takes place during the heavy chain joining process. Six of these 12 events correspond to a slippage event on a T dinucleotide at the 5' side of the Df16.1 gene. This might correspond to the specific catalytic properties of pol λ , which, as shown below, appears to contribute an average synthesis of 5 bp at heavy chain junctions. This misincorporation frequency appears however lower than anticipated from *in vitro* or structural assays (Lee et al., 2004a; Garcia-Diaz et al., 2006).

Different forms of TdT produced by alternative splicing have been reported in various species (Thai and Kearney, 2005). In mice, the major splicing variant of TdT

(TdTL) contains a 20 amino acid insertion near the C-terminus of the protein (Doyen et al., 1993). A 3'-5' exonuclease activity has been described for TdTL, and because this specific isoform is still present in pre-B cells, it has been suggested that it could play a role in the processing of light chain junctions during V-J recombination (Thai et al., 2002). Since extensive light chain end processing is observed in pol μ -deficient mice, we asked whether isoforms of TdT could be responsible for this degradation. $V\kappa$ -J κ 1 and $V\kappa$ 41-J κ 1 rearrangements were amplified from bone marrow B220⁺ IgM⁻ cells of pol μ /TdT double-deficient mice. The same degradation of light chain coding ends described in pol μ -deficient mice was observed in pol μ /TdT double knockouts (Fig. 1 and Table 1). In conclusion, TdT or its splicing variant does not seem to contribute to the end-nibbling activity at Ig light chain junctions.

Altered heavy chain but normal light chain coding junctions in pol λ -deficient mice

We generated pol λ -deficient mice in which exons encoding the catalytic domain have been deleted; they did not reveal any specific phenotype (Bertocci et al., 2002). No quantitative cellular modification was observed in bone marrow, spleen or thymus after FACS analysis with B cell- and T cell-specific markers (data not shown). Since biochemical studies pointed to the participation of pol λ in various DNA repair processes, in particular in NHEJ (Lee et al. 2004a; Ma et al., 2004), we therefore investigated whether pol λ participates in V(D)J recombination. Rearrangements were amplified from bone marrow B220⁺ IgM⁻ cells using V_H consensus primers and primers for two different J_H (J_{H1} and J_{H4}). CDR3 of rearranged VDJ_{H1} and VDJ_{H4} genes from control mice showed an average length

of 41 and 39 nucleotides respectively (Fig. 2A, 2B and Table 2). Their size varied over a large range from 10 to 54 bp, this variability being created by the process of N-nucleotide addition and the nibbling of the four coding ends (J_H , both sides of D, and to a much lesser extent the V_H side).

Heavy chain gene rearrangement in pol λ -deficient mice displayed a reduction of 5 bp in the average CDR3 length for either $V_H D J_{H1}$ or $V_H D J_{H4}$, which is respectively 36 and 34 bp, compared to 41 and 39 bp for controls (Fig. 2A, 2B and Table 2). CDR3 varied from 12 to 54 bp in length. This reduction in size, although moderate, was systematically associated with pol λ deficiency, and resulted in a clear shift in the overall size distribution of CDR3 regions (Fig. 2A). With 6 contiguous bp taken as the criterion for assessing a rearranged D region to a specific D gene, 29% of rearranged D genes were too short to be identified in pol λ -knockout versus 18% in control mice (Supplementary Fig. 2A). The average number of N/P additions, whether estimated for both V-D and D-J junctions, or for D-J junctions only, for which there is no ambiguity in the attribution of germline sequences, did not differ in pol λ -deficient mice (6.8 bp and 3.0 bp respectively) from that in wild type mice (7.7 bp and 3.2 bp) (Supplementary Fig. 2B). Pol λ , therefore, most probably exerts its activity before the insertion of N-nucleotides by the TdT.

The analysis of $V\kappa$ - $J\kappa 1$ light chain rearrangements from bone marrow B220⁺ IgM⁺ cells did not show any alteration in the average and size distribution of CDR3 lengths in pol λ knockouts compared to control mice (Table 2). Thus, pol λ , in contrast to pol μ , does not contribute to light chain end-processing.

Pol μ and pol λ do not show a cooperative effect for immunoglobulin gene rearrangement

The participation of pol μ in the DNA end processing of light chain and that of pol λ in heavy chain gene rearrangement raises the question whether the two DNA polymerase activities might partially compensate for each other during V(D)J recombination. Pol μ /pol λ double knockout mice are viable and fertile, showing no obvious phenotype. At the lymphoid cellular level, they display the phenotype of pol μ -deficient mice: an abnormal B cell differentiation in bone marrow, in which the transition from IgM⁻ to IgM⁺ is impaired, together with a variable peripheral B cell deficiency (spleen of double knockouts have 47% B cells on average versus 68% in wild type mice, data not shown). Heavy and light chain recombination was studied from B220⁺ IgM⁻ bone marrow cells as described above. V_HDJ_{H1} and V_HDJ_{H4} rearrangements were affected to the same extent in pol μ /pol λ - as in pol λ -deficient mice. CDR3 showed an average length of 35-36 bp, 4-5 bp shorter than controls (Table 2 and Fig. 2A, B). Accordingly, the percentage of sequences in which the D sequence was unidentifiable was similar to that observed in the absence of pol λ (Supplementary Fig. 2A). N and P additions are, as for pol λ -deficient animals, comparable to controls (Supplementary Fig. 2B).

Shortening of light chain CDR3 was observed as in pol μ knockout mice, with an average of 9 base pair loss among V κ -J κ 1 junctions (Table 2). This size reduction is slightly more pronounced in double knockouts than in pol μ -deficient mice (9 bp loss versus 6 bp), a difference which is not statistically significant ($p=0.053$ using the nonparametric Wilcoxon Rank Sum test, *vs.* $p<10^{-5}$ for the same comparison between pol μ ^{-/-} and controls, Bertocci et al., 2003). It was moreover not observed for the junctions of a specific V κ gene

(V κ 41) (8 bp loss in double knockouts versus 10 in pol μ -deficient mice; Table 2). Therefore, we conclude that the alterations observed in pol μ /pol λ -deficient mice reflect the strict addition of the individual phenotype of the single deficient animals.

Pol λ and TdT act independently at heavy chain junctions during V(D)J recombination

To address the question whether pol λ and TdT might cooperate or compete during V(D)J recombination, we analyzed heavy chain junctions assembled in the absence of pol λ and TdT. Pol λ and TdT are located 5.5 Mb apart on chromosome 19, which made a double knockout impossible to obtain by animal breeding. We therefore chose to study heavy chain gene rearrangements in fetal liver from day 17 embryos of the different genotypes since TdT is not expressed during embryonic development (Li et al., 1993).

The CDR3 average length of V_H(D)J_{H4} in pol λ -deficient and control embryos was respectively 28 bp and 33 bp (Table 3 and Supplementary Fig. 3), thus showing the same 5 bp reduction observed in bone marrow B220⁺ IgM⁻ cells for adult animals of the same genotype. As shown for TdT- and pol μ /TdT-double knockouts (Table 1), heavy chain CDR3 length was unchanged in pol μ -deficient embryos (Table 3 and Supplementary Fig. 3) compared to that of controls (33 bp for both). Pol μ /pol λ -deficient embryos, in which the three polymerase activities are absent, displayed a heavy chain CDR3 average length with a reduction in size similar to that observed in pol λ -deficient embryos (Table 3). In conclusion, the activity of pol λ on heavy chain junctions is neither dependent nor modulated by the presence of TdT.

The relative expression of pol μ and pol λ varies during B cell development

To better understand the specific recruitment of pol μ and pol λ during V(D)J recombination, their relative level of expression was determined during B cell development in IgM⁺CD43⁺ (pro-B), IgM⁺CD43⁻ (pre-B) and IgM⁺CD43⁻ (immature) fractions as well as in B220⁺ cells from day 17 fetal liver by Real Time Quantitative PCR. When compared to TdT, pol μ or pol λ expression in pro-B cell is about 300-400 times lower. Interestingly, pol μ expression undergoes a 10-fold increase in the pre-B cell fraction, before decreasing in immature B cells (Fig 3). Pol λ expression varies within a limited range in the three bone marrow compartments with the lowest level in pre-B cells (Fig. 3). In fetal liver, in contrast, its expression is 2.5-fold higher than that of pol μ . Thus, the B cell compartment in which pol μ activity is manifest on Ig rearrangement corresponds to the one in which pol μ expression peaks during B cell differentiation.

No increased X-ray sensitivity in pol λ and pol μ/λ -double deficient mice

Pol μ and pol λ are recruited by NHEJ factors (Ku, XRCC4-ligase IV) in order to participate in DNA end repair during this process (Mahajan et al., 2002; Ma et al., 2004; Nick McElhinny et al., 2005). We have shown previously that pol μ is dispensable for double-strand break repair in mouse embryonic fibroblasts (MEFs) (Bertocci et al., 2003). We analyzed here the X-ray sensitivity of pol λ - and pol μ/λ -deficient fibroblasts. Sensitivity to ionizing radiations was not significantly increased in pol λ - or pol μ/λ -deficient MEFs (Fig 4A). Since Artemis-deficient ES cells are more sensitive to bleomycin than to γ -rays (Rooney et al., 2003), we investigated the sensitivity of pol λ - or pol μ/λ -

deficient MEFs to this radiomimetic drug. Neither pol λ nor pol μ /pol λ -deficient MEFs exhibited an increased sensitivity to bleomycin compared to that observed in wild type fibroblasts (data not shown). Thus, pol μ and pol λ appear not to contribute to cell survival after DNA double-strand break damage.

Growth defect and premature senescence in primary fibroblasts lacking pol λ

Pol λ -deficient fibroblasts showed a significantly reduced proliferation rate when compared with wild type MEFs (Fig.4B). To monitor replicative capacity, MEFs from pol μ -, pol λ -deficient and control mice were cultured according to a 3T3 protocol (Todaro and Green, 1963). Pol λ -deficient MEFs showed a reduced number of cells entering into S phase with a decrease of 2.6 times compared to pol μ -deficient MEFs derived from embryos of the same gestation (Fig. 4C). When cultured in 20% O₂, control and pol μ -deficient MEFs continued to proliferate through several rounds of passaging after pol λ -deficient MEFs have entered a growth-arrested state (Fig. 4D, left part). The number of cells expressing the senescence-associated β -galactosidase activity at passage 6 was 3 times more elevated in pol λ -deficient than in control MEFs indicating a premature entry into senescence (Fig. 4E). It has been shown that oxygen sensitivity increases chromosomal instability in NHEJ-deficient cells and limits the replicative lifespan of murine fibroblasts (Karanjawala et al., 2002; Parinello et al., 2003). In 3% O₂, a condition corresponding to the physiological cellular oxygen tension, pol λ -deficient MEFs displayed normal replicative growth (Fig. 4D, right part). This result suggests a role for pol λ in the protection against oxidative DNA damage.

DISCUSSION

We studied in this work the impact on V(D)J recombination of the three DNA polymerases of the polX family, TdT, pol μ and pol λ , in single and double-deficient animals. TdT is a well-known contributor to the junctional diversity of heavy chain genes, and pol μ and pol λ appear as plausible partners of the rearrangement machinery as well, since they are able to interact with proteins involved in the NHEJ repair process (Mahajan et al., 2002; Lee et al., 2004a; Ma et al., 2004).

We reported previously that pol μ is necessary for the correct end-processing of light chain junctions, which show abnormal nibbling in its absence. This molecular defect was reflected at the cellular level in the impairment of the pre-B to immature B cell transition in the bone marrow. In contrast, no impact of pol μ deficiency was observed at the Ig heavy chain rearrangement level (Bertocci et al., 2003).

We therefore wanted to ask whether pol μ was prevented from interacting at the heavy chain locus by the presence of TdT, whose expression is largely restricted to the pro-B differentiation stage. Heavy chain CDR3 of bone marrow B220⁺ IgM⁻ cells from pol μ /TdT-deficient animals show the same 7 bp reduction as TdT knockouts do, a reduction caused by the absence of N additions. The lack of contribution of pol μ during heavy chain gene rearrangement, although surprising, is thus more likely to result from a regulated restriction of its recruitment rather than from a direct competition between enzymes of similar structure.

TdT isoforms have been described, which consist in the mouse in the co-expression, with the normal form of TdT, of a splicing variant in which 20 amino acids are inserted

between the penultimate and the last exon (Doyen et al., 1993). This insertion has been proposed to endow the molecule with an exonuclease activity, constituted by three distantly located motifs that would then be allowed, possibly through an altered folding of the molecule, to assemble as a new catalytic site (Thai et al., 2002). This TdTL variant, expressed up to the pre-B cell stage, would either process light chain coding extremities, or counter-balance the activity of TdT to restrict the length of N additions. *In vitro* primer extension and V(D)J recombination assays have been performed that support this proposition (Thai et al., 2002, 2004). However, conflicting data have been reported by two other groups that question the reality of the exonuclease activity of TdTL (Doyen et al., 2004; Repasky et al., 2004). We therefore investigated whether the extensive degradation of light chain junctions observed in pol μ -deficient animals could be contributed by an isoform of TdT, an activity that would be competed out in the normal situation. The nibbling of κ light chain coding ends is identical between pol μ - and TdT/pol μ -deficient mice, showing the same widespread sizes of CDR3 sequences, suggesting that activities other than those contributed by TdT isoforms are responsible for this degradation.

Since pol μ does not participate in the heavy chain joining process, we wondered whether another polymerase involved in NHEJ could be recruited during this event. Pol λ appears as a logical candidate for this task. Pol λ -deficient mice do not display any obvious phenotype, in particular in the lymphoid compartment (Bertocci et al., 2002). It should be mentioned that inactivation of this gene has been described by Kobayashi et al. (2002) as resulting in a strong phenotype corresponding to the primary cilia dyskinesia syndrome: this syndrome, corresponding to an abnormal motility of ciliated cells results in

male infertility, left-right inversion of organs, sinusitis and hydrocephaly. Genomic analysis however predicts the existence of a divergently transcribed gene, located less than 100 bp from the 5' end of pol λ , a sequence showing strong conservation with a predicted *Drosophila* gene (57% homology), and which would have been inactivated simultaneously with pol λ (Zariwala et al., 2004). Such closely linked gene configurations have been described as a frequent occurrence in the mouse genome (Adachi and Lieber, 2002).

No alteration in B cell differentiation was observed in pol λ -deficient animals neither was any modification of light chain junctions. In contrast, shortening of heavy chain CDR3 was consistently observed in the pool of V_H rearrangements. Five bp were lost on the average, a size reduction that had a visible impact on the rearranged D sequences, whose germline counterpart became less readily identifiable. The shift in CDR3 sizes was also observed in peripheral B cells, as heavy chain sequences from Peyer's patch B cells showed a similar size difference between wild-type and pol λ -deficient animals (not shown). This lack of counter-selection at the periphery, as well as the lack of effect of pol λ deficiency on B cell development, suggests that such modifications of the heavy chain repertoire have no major impact on the B cell homeostasis of an unchallenged laboratory mouse. TdT deficiency, which results in an approximate 7 bp CDR3 size reduction that generates major changes in the B cell repertoire, has similarly little impact on the B cell compartment (Gilfillan et al., 1993; Komori et al., 1993).

The impact of pol λ on heavy chain CDR3 and the one of pol μ on light chain junctions, although not very different in terms of size effect, differ totally when the resulting distribution of CDR3 sequences is considered. For light chains, the impact of pol

μ deficiency represents a change from a rather homogeneous distribution, varying within a 10 bp range, to a widespread distribution varying over 30 nucleotides (with a few sequences totally lacking CDR3). For the heavy chain, the already large, bell-shaped distribution of sizes over a 30-40 bp range observed in the normal situation, is shifted toward a similar bell-shaped array, 5 bp shorter on average in pol λ -deficient animals. Interestingly, the frequency of N additions was not altered, strongly suggesting that pol λ acts before TdT at the heavy chain locus, by filling a 3'-recessed DNA end or by antagonizing an exonuclease activity. It should be therefore noted that pol λ deficiency not only results in a CDR3 size reduction, but that it generates a different balance between the germline encoded elements (that are the sole affected) and the untemplated N additions (whose length are unchanged) in their relative contribution to CDR3 sequences.

Analysis of pol μ /pol λ double-deficient mice showed a strict addition of their respective phenotype: a similar 5 bp-shift was observed for heavy chain CDR3 sequences and a widespread dispersion of CDR3 sizes for κ light chains. Moreover, B cell development in the bone marrow showed a similar impairment of the IgM⁻ to IgM⁺ transition as described in pol μ -deficient animals with no increased severity of the phenotype. These data collectively argue in favor of a compartmentalization of the function of pol μ and pol λ , which, as opposed to previous propositions (Nick McElhinny et al., 2004), show neither competition nor redundancy for V(D)J recombination.

The specific recruitment of pol λ during the processing of heavy chain coding ends was also not affected by the absence of TdT. As the close genetic linkage between TdT and pol λ precludes that their combined deficiency be obtained by simple animal breeding, the

study of heavy chain gene rearrangement in fetal liver B220⁺ cells, a developmental stage at which TdT is not expressed, was taken as a surrogate approach for TdT inactivation in animals with pol μ and/or pol λ deficiency. The alteration in heavy chain CDR3 sizes did not differ between fetal liver and adult bone marrow of the same genotype, i.e. pol λ - and pol μ /pol λ -deficient mice show a similar size shift compared to wild-type or pol μ -deficient animals. These results appear thus coherent with a function of pol λ taking place before the process of N-nucleotide addition.

Whereas embryonic development is unaltered in pol λ -deficient mice, a striking sensitivity to oxidative damage is observed for embryonic fibroblasts cultured at environmental O₂ concentrations, evidenced by premature senescence and impaired cell growth. Normal proliferation was recovered by culture in 3% O₂. This defect is so far, with heavy chain rearrangement, the only “spontaneous” phenotype, i.e. observable without addition of DNA damaging or toxic agents, attributable to pol λ deficiency. This sensitivity to oxidative stress (which nevertheless comprises a complex array of lesions, including base damage and DNA breakage, Karanjilawa et al., 2002; Parrinello et al., 2003) correlates with the H₂O₂ and hydroxymethyldeoxyuridine sensitivity described by Braithwaite et al. (2005) for these cells.

Using a cellular V(D)J recombination assay, we could show that pol μ and pol λ can reduce considerably the frequency of N-additions at the coding junctions of an extrachromosomal substrate. Moreover, Ma et al. (2004) showed in their sophisticated biochemical reconstitution of the NHEJ process that pol λ can compete out TdT in the presence of pol μ , whereas pol μ is more efficient than pol λ in the absence of TdT. The

differences between these *in vitro* data and the one seen *in vivo* suggest a tight regulation involving a differential temporal expression and a specific targeting linked to each one of these polymerases. Interestingly enough, quantitative RT-PCR analysis revealed wide differences in the relative expression of pol μ , pol λ and TdT during B cell differentiation. Pol μ expression undergoes a large increase in pre-B cells, the stage at which it participates in Ig gene rearrangement, before decreasing in immature B cells. Pol λ mRNA varies within a limited range in the three compartments with its lowest level in pre-B cells. In contrast, TdT expression in pro-B cells is 300- to 400-fold higher than that of pol μ and pol λ , respectively. Such a molar ratio (if reflected at the protein level) obviously argues against a regulation by direct competition between the different enzymes. Since pol λ has binding affinity for DNA complexed with Ku70/Ku80, it might be recruited at an earlier step in the rearrangement process than TdT that requires in addition XRCC4-ligase IV for binding (Ma et al., 2004). Pol μ on the other hand might be only recruited when it reaches a threshold expression level, a situation that would occur at the pre-B cell stage.

Ramsden and colleagues have recently proposed that the participation of pol μ in V(D)J recombination is due to its specific property of bridging 3' protruding ends without any base pairing (Nick McElhinny et al., 2005). Although this proposition is a possible scenario for the action of pol μ on light chain junctions, the possibility remains, as we first proposed, that pol μ could be also acting by antagonizing exonuclease activities involved in the nibbling of the coding ends and/or by filling 3'-recessed extremities (Supplementary Fig. 4). The difference between the pol λ and the pol μ phenotype would then be in the strength of their action. Pol λ would be less potent in antagonizing heavy chain coding

end processing, thus keeping the large size diversity characteristic of heavy chain CDR3s. More robust protection/end-filling/bridging activity would be provided by pol μ , resulting in the striking homogeneity of κ light chain junctions. It should be recalled accordingly that the last proline amino acid encoded in $V\kappa$ genes represents a structural invariant (Tomlinson et al., 1995), which is therefore only conserved when nibbling is prevented.

In conclusion, we show here that the immune system has evolved two different DNA polymerases, in addition to TdT, to regulate its heavy and light chain gene rearrangements, each one of these enzymes playing an independent role at specific steps of the process. The contrasted function of each heavy and light chain CDR3 in the generation of a healthy B cell repertoire may explain this rather complex configuration.

EXPERIMENTAL PROCEDURES

Animals

Pol μ and pol λ -deficient mice have been described previously (Bertocci et al., 2002, 2003). Pol μ /pol λ double knockouts were obtained by breeding heterozygous mice for pol μ and pol λ . Pol μ and TdT double-deficient mice were also generated from heterozygous mice for both enzymes. TdT knockout mice (Gilfillan et al., 1993) were kindly provided by J. Kanellopoulos. Two to four month-old mice were used in this study with age-matched littermates as control. Pol μ and pol λ heterozygous mice were selected for the absence of the pol ι mutant allele from the 129 background (McDonald et al., 2003).

Transient V(D)J recombination assay

Mouse embryonic fibroblasts (MEFs) from pol λ - and pol μ /pol λ -deficient mice were generated from days 13.5 embryos as reported for pol μ knockouts (Bertocci et al., 2003). The rearrangement substrate pJH290 (Lieber et al., 1988) was kindly provided by F.W. Alt; the complete RAG-1 and RAG-2 cDNA expression vectors (Oettinger et al., 1990) by J. P. de Villartay. The test was performed as previously described (Taccioli et al., 1993; Gu et al., 1997). The full length mouse cDNA of pol μ , pol λ , pol β , TdT and EGFP was cloned in eukaryotic expression vectors under the control of CMV promoter. pJH290 rearranged clones selected by ampicillin and chloramphenicol resistance were sequenced.

Analysis of immunoglobulin gene rearrangements

Genomic DNA was isolated from sorted bone marrow B220⁺/IgM⁻ cells and from fetal liver. V_HDJ_{H4}, V_κ-J_{κ1}, V_{κ41}-J_{κ1} rearrangements were amplified by PCR as described previously (Bertocci et al., 2003). PCR amplification of V_HDJ_{H1} was performed with a 3' primer downstream of J_{H1}, CTGCAGCATGCAGAGTGTGGC and with the consensus V_H primer used for V_HDJ_{H4} PCR amplification which amplified altogether the V_{H1} (V_HJ558), V_{H2} (V_HQ52), V_{H4} (V_HX24) and V_{H5} (V_H 7183) families. Heavy chain gene rearrangements in TdT- and pol μ/TdT-deficient mice were amplified as described, or with a primer for the V_{H1} family (Gilfillan et al., 1993) and with a specific primer for the V_{H81X} gene (Komori et al., 1993). Sequence of the PCR products was accomplished after Zero Blunt PCR cloning (Invitrogen), using the BigDye Terminator sequencing kit and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Analysis of V(D)J junctions

D elements were compared to the 15 functional D segments of the 129/Sv strain (that shares the IgH^a haplotype of the 129/Ola background, Genbank AJ851868) and to the 10 functional D of the C57BL/6 strain (IgH^b haplotype, AC073553). D specific to the 129 background and found in our sequence sample are Dfl16.2, Dsp2.7, Dsp2.8 and Dsp2.11, and for the C57BL/6 strain, Dsp2.x. On the V_H side, sequences were attributed to germline encoded V_H genes after search in the IMGT database (<http://imgt.cines.fr>), or in the V_H contig assembly performed by the group of Roy Riblet for the C57BL/6 strain (<http://riblet.tpims.org>).

Analysis of pol μ , pol λ and TdT expression in bone marrow

Bone marrow cells were purified by cell sorting into B220⁺IgM⁻CD43⁺, B220⁺IgM⁻CD43⁻ and B220⁺IgM⁺CD43⁻ fractions from 3-4 month-old wild type mice. B220⁺ fetal liver cells were isolated from 3 embryos at day 17 of gestation. The cell staining was performed with R-phycoerythrin (R-PE)-conjugated B220 (clone RA3-6B2), biotin-conjugated CD43 (clone S7) rat anti-mouse monoclonal antibodies from BD Biosciences and fluorescein isothiocyanate(FITC)-conjugated goat anti-mouse IgM antibodies from Southern Biotechnology Associates. RNA was extracted from 3x10⁵ cells using the RNeasy kit (Qiagen) and cDNAs were synthesized with ProSTAR First-Strand RT-PCR kit (Stratagene). Real-time Quantitative PCR for mouse pol μ , pol λ , TdT and beta-2 microglobulin was performed using the specific gene expression assay designed by Applied Biosystems (pol μ : Mn00450512_m1, pol λ : Mn00451420_m1, TdT: Mn00493500_m1, B2m: Mn00437762_m1). The results were obtained from two separated cell sortings (except for fetal liver) and two to four independent PCR performed in triplicate. They were analyzed according to the comparative method (Livak and Schmittgen, 2001), normalizing to 1 the expression of pol μ in the IgM⁻CD43⁻ fraction.

Radiation sensitivity assay

Survival assay was performed as previously described (Bertocci et al., 2003).

Cell growth, cell cycle and senescence analyses

MEFs from *pol* μ -, *pol* λ -, and *pol* μ /*pol* λ -deficient mice were prepared as described above. Cells, cultured for 72 h after embryo dissection, were considered at passage 0. To establish the growth curve, 15×10^4 or 30×10^4 cells were plated in duplicate into 60 mm or 100 mm petri dishes and cell numbers were determined at various time points. For cell cycle analysis, 10^6 MEFs were incubated with 10 μ M bromodeoxyuridine (BrdU) for four hours. Then the cells were harvested and fixed in 70% ethanol before treatment with 1 ml of 2 N HCl/0.5% Triton-X. Following neutralization with 0.1 M sodium tetraborate and staining with an anti-BrdU-FITC-conjugated antibody (clone PRB-1, Alexis), the cells were incubated in PBS containing 5 μ g/ml of propidium iodide. Analysis was carried out on a FACStar apparatus with the Cell-Quest software (BD Biosciences).

To follow the lifespan of MEFs with different genotypes, cells were plated at the density of 30×10^4 per petri dish, counted after 3 days of culture and replated at the same initial density. Population doubling (PD) was calculated as described (Rubio et al., 2002). Replicative senescence of primary fibroblast cultures was determined by senescence-associated β -galactosidase activity at pH 6.0 (Dimri et al., 1995) using the Senescence Detection Kit (Biovision). The experiments were made using two independent MEF preparations of each genotype.

Acknowledgments

We thank F. Delbos for the quantitative PCR analysis, J. Mégret for performing cell sorting and C. Zober for excellent technical assistance. We acknowledge the contribution of J. Rouiller in initiating the analysis of pol λ -deficient mice and B. Ranque in performing tests of statistical significance. We thank F.W. Alt, M. Gellert, J.-P. de Villartay for the gift of vectors for the *in vitro* recombination assay and J. Kanellopoulos for providing TdT-deficient mice. We thank A. S. Chaplault, Y. Moreau and the personnel of the LEAT (Faculté de Médecine Necker-Enfants Malades) for mouse breeding and handling. This project was supported by the A.C.I. "Biologie du Développement et Physiologie Intégrative" of the Ministère de la Recherche and by the Fondation Princesse Grace de Monaco.

References

- Adachi, N., and Lieber, M.R. (2002). Bidirectional gene organization: a common architectural feature of the human genome. *Cell* 109, 807-809.
- Ahnesorg, P., Smith, P., and Jackson, S.P. (2006). XLF interacts with the XRCC4-DNA ligase IV complex to promote DNA nonhomologous end-joining. *Cell* 124, 301-313.
- Aoufouchi, S., Flatter, E., Dahan, A., Faili, A., Bertocci, B., Storck, S., Delbos, F., Cocea, L., Gupta, N., Weill, J.C. and Reynaud, C.A. (2000). Two novel human and mouse DNA polymerases of the polX family. *Nucl. Acids Res.* 28, 3684-3693.
- Bertocci, B., De Smet, A., Flatter, E., Dahan, A., Bories, J.C., Landreau, C., Weill, J.C., and Reynaud, C.A. (2002). Cutting edge: DNA polymerases mu and lambda are dispensable for Ig gene hypermutation. *J. Immunol.* 168, 3702-3706.
- Bertocci, B., De Smet, A., Berek, C., Weill, J.C., and Reynaud, C.A. (2003). Immunoglobulin kappa light chain gene rearrangement is impaired in mice deficient for DNA polymerase mu. *Immunity* 19, 203-211.
- Braithwaite, E.K., Kedar, P.S., Lan, L., Polosina, Y.Y., Asagoshi, K., Poltoratsky, V.P., Horton, J.K., Miller, H., Teebor, G.W., Yasui, A., and Wilson, S.H. (2005). DNA polymerase lambda protects mouse fibroblasts against oxidative DNA damage and is recruited to sites of DNA damage/repair. *J. Biol. Chem.* 280, 31641-31647.

Buck, D., Malivert, L., de Chasseval, R., Barraud, A., Fondaneche, M.C., Sanal, O., Plebani, A., Stephan, J.L., Hufnagel, M., le Deist, F., Fischer, A., Durandy, A., de Villartay, J.P., and Revy, P. (2006). Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. *Cell* 124, 287-299.

Daley, J.M., Laan, R.L., Suresh, A., Wilson, T.E. (2005). DNA joint dependence of pol X family polymerase action in nonhomologous end joining. *J. Biol. Chem.* 280, 29030-29037.

Desiderio, S.V., Yancopoulos, G.D., Paskind, M., Thomas, E., Boss, M.A., Landau, N., Alt, F.W. and Baltimore, D. (1984). Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. *Nature* 311, 752-755.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., and Campisi J. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* 92, 9363-9367.

Ding, Q., Reddy, Y.V., Wang, W., Woods, T, Douglas, P., Ramsden, D.A., Lees-Miller, S.P., and Meek, K. (2003) Autophosphorylation of the catalytic subunit of the DNA-dependent protein kinase is required for efficient end processing during DNA double-strand break repair. *Mol. Cell. Biol.* 23, 5836-5848.

Dominguez, O., Ruiz, J.F., Lain de Lera, T., Garcia-Diaz, M., Gonzalez, M.A., Kirchhoff, T., Martinez-A, C., Bernad, A. and Blanco, L. (2000) DNA polymerase mu (Pol μ),

homologous to TdT, could act as a DNA mutator in eukaryotic cells. *EMBO J.* 19, 1731-1742.

Doyen, N., d'Andon, M.F., Bentolila, L.A., Nguyen, Q.T., and Rougeon, F. (1993). Differential splicing in mouse thymus generates two forms of terminal deoxynucleotidyl transferase. *Nucleic Acids Res.* 21, 1187-1191.

Doyen, N., Boule, J.B., Rougeon, F., and Papanicolaou, C. (2004). Evidence that the long murine terminal deoxynucleotidyltransferase isoform plays no role in the control of V(D)J junctional diversity. *J. Immunol.* 172, 6764-6767.

Dudley, D.,D., Chaudhuri, J., Bassing, C.H, and Alt, F.W. (2005). Mechanism and Control of V(D)J Recombination versus Class Switch Recombination: Similarities and Differences. *Adv. Immunol.* 86, 43-112.

Garcia-Diaz, M., Bebenek, K., Sabariego, R., Dominguez, O., Rodriguez, J., Kirchhoff, T., Garcia-Palomer, E., Picher, A.J., Juarez, R., Ruiz, J.F., Kunkel, T.A., and Blanco, L. (2002) DNA polymerase lambda, a novel DNA repair enzyme in human cells. *J. Biol. Chem.* 277, 13184-13191.

Garcia-Diaz, M., Bebenek, K., Krahn, J.M., Pedersen, L.C., and Kunkel, T.A. (2006). Structural analysis of strand misalignment during DNA synthesis by a human DNA polymerase. *Cell* 124, 331-342.

Gilfillan, S., Dierich, A., Lemeur, M., Benoist, C., and Mathis, D. (1993). Mice lacking TdT: mature animals with an immature lymphocyte repertoire. *Science* 261, 1175-1178.

Gu, Y., Sidi, K.J., Rathbun, G.A., Zhu, C., Manis, J.P., van der Stoep, N., Davidson, L., Cheng, H.L., Sekiguchi, J.M., Frank, K., Stanhope-Baker, P., Schlissel, M.S., Roth, D.B. and Alt, F.W. (1997). Growth retardation and leaky SCID phenotype of KU70-deficient mice. *Immunity* 7, 653-665.

Hesslein, D.G., and Schatz, D.G. (2001). Factors and forces controlling V(D)J recombination. *Adv. Immunol.* 78, 169-232.

Karanjawala, Z.E., Murphy, N., Hinton, D.R., Hsieh, C.L., and Lieber, M.R. (2002). Oxygen metabolism causes chromosome breaks and is associated with the neuronal apoptosis observed in DNA double-strand break repair mutants. *Curr Biol.* 12, 397-402.

Kobayashi, Y., Watanabe, M., Okada, Y., Sawa, H., Takai, H., Nakanishi, M., Kawase, Y., Suzuki, H., Nagashima, K., Ikeda, K., and Motoyama, N. (2002). Hydrocephalus, situs inversus, chronic sinusitis, and male infertility in DNA polymerase lambda-deficient mice: possible implication for the pathogenesis of immotile cilia syndrome. *Mol. Cell. Biol.* 22, 2769-2776.

Komori, T., Okada, A., Stewart, V., and Alt, F.W. (1993). Lack of N regions in antigen receptor variable region genes of TdT-deficient lymphocytes. *Science* 261, 1171-1175.

Lee, J.W., Blanco, L., Zhou, T., Garcia-Diaz, M., Bebenek, K., Kunkel, T.A., Wang, Z., and Povirk, L.F. (2004a). Implication of DNA polymerase lambda in alignment-based gap filling for nonhomologous DNA end joining in human nuclear extracts. *J. Biol. Chem.* 279, 805-811.

Lee, G.S., Neiditch, M.B., Salus, S.S., and Roth, D.B. (2004b). RAG proteins shepherd double-strand breaks to a specific pathway, suppressing error-prone repair, but RAG nicking initiates homologous recombination. *Cell* 117, 71-84.

Lieber, M.R., Hesse, J.E., Lewis, S., Bosma, G.C., Rosenberg, N., Mizuuchi, K., Bosma, M.J. and Gellert, M. (1988). The defect in murine severe combined immune deficiency: joining of signal sequences but not coding segments in V(D)J recombination. *Cell* 55, 7-16.

Li, Y.S., Hayakawa, K., and Hardy, R.R. (1993). The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. *J. Exp. Med.* 178, 951-960.

Livak, K.J. and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ Method. *Methods* 25, 402-408.

Ma, Y., Pannicke, U., Schwarz, K., and Lieber, M.R. (2002). Hairpin opening and overhang processing by an Artemis/DNA-dependent protein kinase complex in nonhomologous end joining and V(D)J recombination. *Cell* 108, 781-794.

Ma, Y., Lu, H., Tippin, B., Goodman, M.F., Shimazaki, N., Koiwai, O., Hsieh, C.L., Schwarz, K., Lieber, M.R. (2004). A biochemically defined system for mammalian nonhomologous DNA end joining. *Mol. Cell.* 16, 701-713.

Ma, Y., Schwarz, K., and Lieber, M.R. (2005). The Artemis:DNA-PKcs endonuclease cleaves DNA loops, flaps, and gaps. *DNA Repair* 4, 845-851.

Mahajan, K.N., Nick McElhinny, S.A., Mitchell, B.S., and Ramsden, D.A. (2002). Association of DNA polymerase mu (pol mu) with Ku and ligase IV: role for pol mu in end-joining double-strand break repair. *Mol. Cell. Biol.* 22, 5194-5202.

Manke, I.A., Lowery, D.M., Nguyen, A., and Yaffe, M.B. (2003). BRCT repeats as phosphopeptide-binding modules involved in protein targeting. *Science* 302, 636-639.

McDonald, J.P., Frank, E.G., Plosky, B.S., Rogozin, I.B., Masutani, C., Hanaoka, F., Woodgate, R., and Gearhart, P.J. (2003). 129-derived strains of mice are deficient in DNA polymerase iota and have normal immunoglobulin hypermutation. *J. Exp. Med.* 198, 635-643.

Nick McElhinny, S.A., and Ramsden, D.A. (2004). Sibling rivalry: competition between Pol X family members in V(D)J recombination and general double strand break repair. *Immunol. Rev.* 200,156-64.

Nick McElhinny, S.A., Havener, J.M., Garcia-Diaz, M., Juarez, R., Bebenek, K., Kee, B.L., Blanco, L., Kunkel, T.A., and Ramsden, D.A. (2005). A gradient of template dependence defines distinct biological roles for family X polymerases in nonhomologous end joining. *Mol. Cell* 19, 357-366.

Oettinger, M.A., Schatz, D., Gorka, C., and Baltimore, D. (1990). RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* 248, 1517-1522.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. (2003). Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat. Cell Biol.* 5, 741-747.

Ramadan, K., Shevelev, I., and Hubscher, U. (2004) The DNA-polymerase-X family: controllers of DNA quality? *Nat. Rev. Mol. Cell Biol.* 5,1038-1043.

Repasky, J.A., Corbett, E., Boboila, C., and Schatz, DG. (2004). Mutational analysis of terminal deoxynucleotidyltransferase-mediated N-nucleotide addition in V(D)J recombination. *J. Immunol.* 172, 5478-5488.

Rooney, S., Alt, F.W., Lombard, D., Whitlow, S., Eckersdorff, M., Fleming, J., Fugmann, S., Ferguson, D.O., Schatz, D.G., and Sekiguchi, J. (2003). Defective DNA repair and increased genomic instability in Artemis-deficient murine cells. *J. Exp. Med.* 197, 553-565.

Rubio, M.A., Kim, S.H., and Campisi, J. (2002). Reversible manipulation of telomerase expression and telomere length. Implications for the ionizing radiation response and replicative senescence of human cells. *J. Biol. Chem.* 277, 28609-28617.

Taccioli, G.E., Rathbun, G., Oltz, E., Stamato, T., Jeggo, P.A. and Alt, F.W. (1993). Impairment of V(D)J recombination in double strand break repair mutants. *Science* 260, 207-210.

Thai, T.H., Purugganan, M.M., Roth, D.B., and Kearney, J.F. (2002). Distinct and opposite diversifying activities of terminal transferase splice variants. *Nat. Immunol.* 3, 457-462.

Thai, T.H., and Kearney, J.F. (2004). Distinct and opposite activities of human terminal deoxynucleotidyltransferase splice variants. *J. Immunol.* 173, 4009-4019.

Thai, T.H., and Kearney, J.F. (2005). Isoforms of terminal deoxynucleotidyltransferase: developmental aspects and function. *Adv. Immunol.* 86, 113-136.

Todaro, G.J., and Green, H. (1963). Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell. Biol.* 17, 299-313.

Tomlinson, I.M., Cox, J.P., Gherardi, E., Lesk, A.M., and Chothia, C. (1995). The structural repertoire of the human V kappa domain. *EMBO J.* 14, 4628-4638.

Yu, X., Chini, C.C., He, M., Mer, G., and Chen, J. (2003). The BRCT domain is a phospho-protein binding domain. *Science*. 302, 639-642.

Zariwala, M., O'Neal, W.K., Noone, P.G., Leigh, M.W., Knowles, M.R., and Ostrowski, L.E. (2004). Investigation of the possible role of a novel gene, DPCD, in primary ciliary dyskinesia. *Am. J. Respir. Cell. Mol. Biol.* 30, 428-434.