Contribution of DNA polymerase \( \eta \) to immunoglobulin gene hypermutation in the mouse

Frédéric Delbos, Annie De Smet, Ahmad Faili, Said Aoufouchi, Jean-Claude Weill, and Claude-Agnès Reynaud

Institut National de la Santé et de la Recherche Medicale U373, Faculté de Médecine Necker-Enfants Malades-Université Paris V, 75730 Paris Cedex 15, France

The mutation pattern of immunoglobulin genes was studied in mice deficient for DNA polymerase \( \eta \), a translesional polymerase whose inactivation is responsible for the xeroderma pigmentosum variant (XP-V) syndrome in humans. Mutations show an 85% G/C biased pattern, similar to that reported for XP-V patients. Breeding these mice with animals harboring the stop codon mutation of the 129/Olain background in their DNA polymerase \( \iota \) gene did not alter this pattern further. Although this G/C biased mutation profile resembles that of mice deficient in the MSH2 or MSH6 components of the mismatch repair complex, the residual A/T mutagenesis of pol\( \eta \)-deficient mice differs markedly. This suggests that, in the absence of pol\( \eta \), the MSH2–MSH6 complex is able to recruit another DNA polymerase that is more accurate at copying A/T bases, possibly pol\( \iota \), to assume its function in hypermutation.

Somatic hypermutation of immunoglobulin genes is initiated by activation-induced cytidine deaminase (AID; reference 1). Most experimental evidences suggest that AID initiates this process by deaminating cytidines into uracils in DNA (2). Accordingly, overexpression of AID in fibroblasts is sufficient to induce a G/C-targeted mutagenesis on a highly transcribed reporter gene (3). This mutagenesis is supposed to occur by saturation of the repair capacity of the cell, generating transition mutations by replication over the uracils created by deamination. Transversion mutations are likely generated by one or several translesional polymerases that ensure the replication bypass of abasic sites that failed to be corrected by the classical base excision repair pathway after excision of uracils by uracil glycosylases.

In contrast with this “passive” mutagenic process, hypermutation in activated B cells generates mutations at all four bases at the Ig locus (4). This mutagenesis is entirely dependent on the initial handling of the lesion by only two pathways, one dependent on the uracil glycosylase UNG, the other one upon a subset of the mismatch repair complex, its DNA binding moiety MSH2–MSH6, together with exonuclease 1 (Exo1; references 5–13). The UNG pathway appears necessary for generating most transversion mutations at G/C bases, whereas the MSH2–MSH6 pathway is required for a large part of the mutagenesis at A/T positions.

Translesional DNA polymerases are specific enzymes, recruited at replication forks stalled in front of noninstructional DNA lesions, and able to bypass them with various specificities and efficiencies (for review see reference 14). Moreover, based on in vitro assays, many of them are inherently error prone when copying undamaged DNA, again with various intrinsic misincorporation specificities, which made them prime candidates for the Ig gene mutational process, and several experimental data have indeed documented their involvement. The Ig gene mutation profile of xeroderma pigmentosum variant (XP-V) patients, deficient in DNA polymerase \( \eta \) (15), has suggested that this enzyme is a major contributor of the MSH2–MSH6 driven A/T mutagenesis (16–18). The DNA polymerase \( \iota \), which colocalizes with pol\( \eta \) at replication foci (19), has been implied in the Ig gene mutagenesis induced in a Burkitt’s lymphoma cell line (20). However, inactivation
of this enzyme in the mouse, by a stop codon present as a natural mutation of various 129 substrains, failed to show any phenotype on hypermutation (21). DNA polymerase η is proposed to contribute to the overall quantitative efficiency of the process, but not to any specific pathway (22, 23).

To look for possible differences in the various DNA polymerases involved between mouse and humans, we inactivated polη in the mouse, and analyzed the Ig mutation pattern in the context of the polη mutation of the 129/Ola strain. This analysis confirms the phenotype observed for polη deficiency in humans, which is not altered further in the presence of the polk mutation of the 129 background.

RESULTS AND DISCUSSION

Inactivation of the mouse Polh gene was performed by a conditional knock-out strategy that introduces loxP sites flanking exon 4, an exon containing the DE polymerase motifs conserved in all Y-family polymerases and indispensable for polη catalytic function (Fig. 1 and references 15, 24). This strategy was designed with the aim of crossing the animals obtained with mice harboring other DNA repair defects, allowing the follow-up of the B cell lineage in case of lethality of their combined deficiencies. Deletion of exon 4 was performed here in the targeted embryonic stem (ES) cell clone, leaving in place a single loxP site and, therefore, minimizing disturbance around the gene. The Xpo5 gene, encoding exportin-5 involved in the nuclear export of microRNAs, indeed initiates 200 bp upstream from the first Polh exon in opposite transcriptional orientation; affecting expression of this gene is, therefore, likely to result in an early embryonic lethal phenotype (25).

The heterozygotes obtained by breeding chimeras with C57Bl/6 mice were crossed together and screened for the presence of the stop codon mutation born by the Poli gene from the 129/Ola genetic background of the ES cells. Wild-type, homozygous polη-deficient, and double polη-polk mutant mice were selected for analysis. Deletion of exon 4 of polη resulted at the transcriptional level in the direct splicing of exons 3–5, an out-of-frame junction that introduces a stop codon 11 amino acids downstream in exon 5 and generates a truncated polη protein of 102 amino acids (Fig. 1 D). Apart from deletion of exon 4, such a truncated protein would lack domains involved in nuclear localization and repair foci formation (19).

Wild-type, polη-deficient, and double polη-polk mutant mice were analyzed for the mutation pattern of their Ig locus. Different types of sequences were analyzed: intronic sequences flanking rearranged J4 sequences (“J4 intronic sequences”) and a 560-bp region upstream from the repeats of the Sμ core sequence (hereafter referred to as “pre-switch”), both from Peyer’s patch PNAhigh B cells (Table I).

No B cell anomalies were observed in either type of polymerase-deficient animals. Ig sequences from polη−/− PNAhigh B cells displayed the same specific pattern as those

| Number of sequences | Controls | Polη−/− | Polη−/− × polk−/− | Controls | Polη−/− | Polη−/− × polk−/− |
|---------------------|----------|---------|-----------------|----------|---------|-----------------|
| Total length sequenced (bp) | 10,290 | 34,790 | 25,970 | 53,760 | 66,320 | 71,120 |
| Unmutated sequences (%) | 14 | 17 | 25 | 42 | 46 | 57 |
| Number of mutations | 124 | 241 | 129 | 147 | 160 | 122 |
| Number of deletions and insertions | 2 | 4 | 0 | 4 | 3 | 2 |
| Mutation frequency per total sequences (per 100 bp) | 1.2 | 0.7 | 0.5 | 0.3 | 0.2 | 0.2 |
| Mutation frequency per mutated sequences (per 100 bp) | 1.4 | 0.8 | 0.7 | 0.5 | 0.4 | 0.4 |
obtained from memory B cells of XP-V patients; i.e., a marked reduction of mutations at A/T base pairs. 86.1% mutations at G/C base pairs for the J₄ intron and 88.6% for the pre-switch sequence (vs. 89.3 and 89.8% for the corresponding Ig gene sequences in XP-V patients; Figs. 2 A and 3 A). Mutation frequencies were somewhat lower for Pol- deficient animals compared with wild type when J₄ intronic sequences are considered, but similar for the pre-switch sequences. Such a quantitative dissociation has been described for MSH6- and for UNG/Exo1-deficient animals (5, 13).

We next compared the mutation profile of mice harboring both Pol- and Pol+ inactivation. These profiles appear extremely similar, either in their distribution along the sequence (shown for the pre-switch sequence in Fig. 3 C), or in their base substitution characteristics (Figs. 2 A and 3 A). The lack of phenotype of Pol- deficient mice on Ig gene hypermutation is, therefore, not due to a compensatory role of Pol+ in this specific strain.

All further analyses were, therefore, performed on pooled data from both types of mutant mice, allowing us to compare larger databases (366 mutations for the J₄ intron and 277 for the pre-switch sequence). The targeting was slightly more pronounced for C than for G, in particular for the pre-switch sequence. Such an increase has been described in switch junctions from XP-V patients (18). However, among the three types of sequences that we have analyzed in similar patients, we could not observe this bias in either switch junctions or J₄ introns, the specific increase in C mutations being only significant in pre-switch sequences from XP-V individuals (17). The general relevance of this observation is, therefore, difficult to assess at this stage. Moreover, among G/C mutations, no difference from wild type is noticeable in the relative proportion of transitions and transversions (Table II).

MSH2 deficiency, together with defects in MSH6 and Exo1, results in a similar bias toward G/C mutagenesis (7–13). We, therefore, wanted to compare the mutation pattern in the J₄ intron in the Msh2- and Exo1-deficient mice, as well as MSH2-ATPase mutant mice (326 mutations), taking into account only data obtained using Pfu polymerase, to exclude any contribution of less accurate enzymes whose intrinsic error spectrum might specifically impinge on the A/T pattern. At G/C base pairs, these mutant mice show a strong bias toward transversion.

Table II. Pattern of nucleotide changes in J₄ intron sequences of normal, Pol+, and MSH2-deficient mice

| Mouse   | Controls 1⁺ | Controls 1⁻ | Polh⁻⁻ | Polh⁻⁺xPolh⁻⁻ |
|---------|-------------|-------------|--------|----------------|
| GC/AT   | Within G/C | Within A/T  | Trans. | Transv. |
| Trans.  | Trans.     | Trans. C/T  | A/G   | A/T   | A/C   |
| Transv. | Transv.    | Transv C/T  | T/C   | T/A   | T/G |
| Mouse   | Controls 1⁺ | 44.3:55.7   | 53.7:46.3 | 61.4 | 18.5 | 20.1 | 47.6 | 26.2 | 26.2 |
| Controls 1⁻ | 49.9:50.1  | 53.3:46.7   | 54.5 | 16.8 | 28.7 | 52.1 | 25.9 | 22.0 |
| Polh⁻⁻  | 85.5:14.5  | 51.3:48.7   | 57.1 | 12.7 | 30.2 | 17.2 | 26.2 | 56.2 |
| Msh2⁻⁻⁻⁻ | 87.0:13.0  | 71.2:28.8   | 74.0 | 14.0 | 20.0 | 52.7 | 25.7 | 21.6 |
| Human   | Controls 1⁺ | 46.0:54.0   | 51.2:48.8 | 55.4 | 16.5 | 28.1 | 47.6 | 25.0 | 27.4 |
| XP-V⁻⁻⁻⁻ | 89.3:10.7  | 46.6:53.4   | 51.1 | 17.1 | 31.8 | 9.4  | 36.4 | 54.2 |

Values in bold represent significant differences from controls discussed in the text.

1This study (122 mutations).
2From a larger sample (334 mutations; reference 36).
3This study, data from all deficient mice together (366 mutations).
4Data from mice deficient in the MSH2-dependent pathway (Msh2⁻⁻⁻⁻, Exo1⁻⁻⁻⁻, Msh2⁻⁻⁻⁻Exo1⁻⁻⁻⁻) were pooled (326 mutations obtained by Pfu polymerase; references 8, 11, 12).
5From ref. 17 (168 mutations).
tions, which has been interpreted as an increased replicative bypass of uracils, the absence of the mismatch-binding complex leading to an increased fraction of deaminated bases escaping detection and repair. Polh /H9257/ animals, in contrast, show a transition/transversion distribution at G/C bases similar to controls. Strikingly, the mutation pattern at A/T bases pairs shows the reverse situation. MSH2 deficiency shows a distribution similar to wild type, whereas pol /H9257/ deficiency results in a strong transversion bias. This transversion bias is consistent enough to emerge from both types of sequences analyzed (JH4 and pre-switch), and in both mouse and human polh deficiencies (Table II). Moreover, among transversions at A/T bases, most of them are A to C or T to G changes, which represent more than half of all A/T mutations. Such a mutation bias has been described for DNA polymerase κ (26, 27). It is thus possible that, in the absence of polh, the mismatch binding complex is able to recruit a different translesional enzyme, which, due to its specific misincorporation pattern, would be less mutagenic at A/T base pairs, leading to a lower frequency of A/T mutations with a specific transversion bias. Because polh-deficient animals do not harbor the slightest alteration of their A/T mutation pattern (28), it is probable that this enzyme is not a normal actor of the Ig gene mutation process, being on duty only in case of absence of the regular partner of the MSH2–MSH6 complex (i.e., polh).

![Figure 3. Distribution of mutations in the Sμ core upstream region (pre-switch) of Peyer's patches PNA^high B cells from polh^- and polh^-polh^-deficient mice.](image-url)

(A) Pattern of nucleotide substitution from the same control and mutant animals described in Fig. 2. Base composition: 560 bp; A, 33.2%; C, 16.4%; G, 27.5%; T, 22.9%. (B) Accumulation of mutations in individual pre-switch sequences. Same representation as in Fig. 2 B. (C) Distribution of mutations along the pre-switch sequence. Mutations from polh^-deficient mice are listed above the sequence, and mutations from double-deficient mice are listed below the sequence. "G" within RGYW motifs and "C" within WRCY motifs are underlined.

**Figure 3.** Distribution of mutations in the Sμ core upstream region (pre-switch) of Peyer's patches PNA^high B cells from polh^- and polh^-polh^-deficient mice. (A) Pattern of nucleotide substitution from the same control and mutant animals described in Fig. 2. Base composition: 560 bp; A, 33.2%; C, 16.4%; G, 27.5%; T, 22.9%. (B) Accumulation of mutations in individual pre-switch sequences. Same representation as in Fig. 2 B. (C) Distribution of mutations along the pre-switch sequence. Mutations from polh^-deficient mice are listed above the sequence, and mutations from double-deficient mice are listed below the sequence. "G" within RGYW motifs and "C" within WRCY motifs are underlined.
Polh is another enzyme with such a transversion bias (29). However, contrary to Polh and POLH, its preferred misincorporation is at copying A, and not T. It is therefore unlikely, in the mouse at least, that Polh could generate the A over T bias of mutations in JH4 sequences, present in control mice and conserved in polh-deficient animals.

UNG and MSH2–MSH6 are the only two repair pathways handling uracils generated by AID during Ig gene hypermutation. The model of Neuberger et al. (5) posits that G/C mutations are introduced by replication over the uracils or the abasic sites generated by uracil glycosylase, both events occurring on the DNA strand opposed to the lesion and without repair. Replication over the abasic site would involve translesional DNA polymerases in their “classical” role of damage bypass. Effectively, in the AID-dependent mutations observed in the chicken cell line DT40 that are almost entirely restricted to G/C bases, Rev1 has been proposed to be the major enzyme involved (30). As the mutation pattern of DT40 is more biased toward G to C and C to G transversions (the hallmark of Rev1) than it is in the mouse, it is so far unclear how many translesional polymerases are involved in the G/C mutation pattern in mouse (and human) B cells. Mutations at A/T bases are more difficult to explain on a strict replication mode within this model.

In another scenario, the MSH2–MSH6 complex would recruit Polh for an error-prone repair of the lesion (i.e., on the same DNA strand) that would remove the uracil or the abasic site and create mismatches at nearby A/T bases because this enzyme is inherently more mutagenic at copying Ts. Along this line, specific modifications of AID in hypermutating B cells might also actively recruit UNG at the site of the lesion (31), driving the G/C mutagenesis to proceed differently from a strict saturation of the normal repair of uracils. In such a scheme, the dichotomy between translesion bypass and error-prone repair for the G/C versus A/T mutations might have to be reassessed.

The absence of phenotype of the polh mutation of the 129 strains obviously questions the relevance of the polh-dependent mutagenic process that we described in the BL2 Burkitt’s lymphoma cell line (20). In fact, it has been reported that such a polh-dependent mutagenesis can be induced at the Ig locus in B cell lines infected by the hepatitis C virus (32). These mutations would be related to the specific metabolic alterations brought upon infection by this oncoviral virus, alterations that may be shared by many B cell lymphomas (33). Alternatively, inactivation of polh in the 129/Ola mouse strain might show leakiness, in particular in activated B cells, by either read-trough of the stop codon or alternative splicing. A classical inactivation of the Polh gene and the analysis of mice cumulating several deficiencies in the activities involved in hypermutation might contribute to address these issues.

MATERIALS AND METHODS

Construction of targeting vectors. Three fragments from the POLH locus were amplified and cloned in the pFlox vector (34): fragment 1, 5’ flanks; fragment 2, exon 4; and fragment 3, 3’ flanks. Construction of the targeting vector and screening of recombinant clones are described in supplemental Materials and methods (available at http://www.jem.org/cgi/content/full/jem.20050292/DC1). Transfection of E14.1 ES cells was performed as described (35). One recombinant clone was obtained out of 287 ES clones, and subjected to Cre-mediated excision to generate exon 4–deleted clones.

Analysis of gene-targeted mice. Screening of gene targeted mice was performed by PCR around exon 4 (described in Fig. 1) using the following primers: 5’-screen, 5’-GTCTCCCTTGGAGTTATTGCC-3’, and 3’-screen, 5’-GCTTCTGACCTGTGTTGTC-3’ (45 s at 94°C, 30 s at 59°C, 3 min at 72°C, 40 cycles with Taq polymerase; Bioslab). The polh mutation of the 129/Ola background was screened by direct sequencing of a 540-bp genomic fragment encompassing exon 2 (amplified by iota-exon 2-5’, 5’-TTA-AAGCGGACTGAGAAC-3’; iota-exon 2-3’, 5’-CACATTTATCTCAGTTGTC-3’, 15 s at 98°C, 30 s at 60°C, 15 s at 72°C, 40 cycles with Plussion DNA polymerase; Finnzymes). Polh mRNA expression was analyzed by RT-PCR (Stratagene; Stratagene), using splenic RNA extracted by the QIAGEN RNaseasy kit and PCR primers located in exons 2 and 6 (exon 2, 5’-GGCCGAGAATCTGAGTGGTC-3’; exon 6 reverse, 5’-GGCGGTGGGCTTATTTAGTCC-3’, 30 s at 95°C, 90 s at 68°C, 40 cycles with Advantage II polymerase mix; CLONTECH Laboratories, Inc.).

Analysis of mutations in the Ig locus. B220+ PNA<sup>hi</sup> B cells from Peyer’s patches were isolated from 3–4-mo-old animals as described previously (8). Amplification of the JH<sub>4</sub> intronic sequence flanking rearranged VH<sub>4</sub> genes was performed using a mixture of primers amplifying most V<sub>H</sub> gene families described (compiled from the mouse V gene IMGT database, http://imgt.cines.fr): V1-FR3, 5’-GAAGCTTCTGCGRGATTTGC-3’; V5-FR3, 5’-GAAGACACRCGCGATTACTAGTCG-3’; V3-FR3, 5’-GAAGACACACCCATAATTACTGTCG-3’; V7-FR3, 5’-GAAGACCAGCTCCTTATTACTGTCG-3’; and V9-FR3, 5’-ATGAGGACTGACCCCATATTTCT-3’, respectively, in a 6:3:1:1:1 ratio, and J<sub>H</sub>3, 5’-TGAGACCAGGAGCTAGATGCC-3’. PCR was performed on five aliquots of 500 or 1,000 cells (15 s at 98°C, 30 s at 64°C, 30 s at 72°C for 30 cycles using Plusion DNA polymerase) and 480 bp sequences were determined using the J<sub>H</sub>3 primer. A 735-bp fragment upstream from the Sp core repeat sequence was amplified using primers Sp-5’, 5’-GGTGAAGGACTGATGCTGTGC-3’; Sp-3’, 5’-CCAGCTGATGTTAGCTTTAGC-3’ (45 s at 94°C, 30 s at 58°C, 2 min at 72°C, 40 cycles with Pfu Turbo), and a 560-bp sequence was determined using primer Sp-5’-CTATTCTGGCTCTTCTTAAC-3’. Sequences were obtained with an ABI Prism 3100 Genetic Analyzer after cloning in the Zero Blunt vector (Invitrogen).

Online supplemental material. Construction of the targeting vector for inactivation of the Polh gene, as well as conditions for analysis of the recombinant ES clones are described online. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050292/DC1.

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