Somatic hypermutation is a specific mutagenic process that can diversify the Ig genes at several stages of the B cell differentiation pathway: either outside an immune response, leading to repertoire diversification, or during antigen driven responses, leading to affinity maturation through the selection of B cells displaying better antigen-binding capacities (1).

Hypermutation is initiated by activation-induced cytidine deaminase (AID) through an enzymatic process that deaminates cytidines into uracils and is essentially, but not exclusively, targeted at the heavy and light chain V genes of the Ig locus (reference 2; for review see references 3, 4). However, mutations at the Ig locus are equally distributed at G/C and A/T bases, which requires that specific error-prone repair pathways process this C-focused lesion to broaden its mutation spectrum (5). In spite of the large number of repair factors able to handle uracils within the eukaryotic cell, only two of them have been shown to contribute to hypermutation: uracil N-glycosylase (UNG) and the DNA-binding moiety of the mismatch repair complex (MSH2–MSH6) (6–14). When these two factors are missing, the mutation pattern becomes the imprint of the sole AID action, the replication of uracils generated on both DNA strands resulting in C to T and G to A transitions at the sites of AID deamination (14).

Error-prone DNA polymerases, in particular those involved in the replicative bypass of DNA lesions, have been considered as prime candidates to generate mutations at A/T base pairs from the initial deamination event. The Ig gene mutation pattern of patients affected with the variant form of the xeroderma pigmentosum syndrome, a genetic disease corresponding to inactivation of DNA polymerase η (pol η) (15), was shown to be markedly, albeit...
RESULTS AND DISCUSSION

The residual A/T mutagenesis observed in MSH2-deficient animals harbors the same overall pattern as the one observed in Ig genes of normal mice, which raises the possibility that it could be generated by pol η, recruited outside the mismatch repair pathway.

We therefore generated Polh<sup>−/−</sup>/Msh2<sup>−/−</sup> animals and collected a large mutation database from four to seven mice of the single- or double-deficient genotypes. We observed rather large interindividual variations in the mutation frequency, notably between two groups of wild-type mice originating from two separate rooms of the same animal facility (Table I and Fig. 1 A). Taking into account the mutation frequency of the pool of mice constituted by the wild-type littermates of the Polh heterozygous breeding, the mutation frequency was reduced at least two times in either MSH2- or pol η–MSH2–deficient mice. This reduction is mainly caused by the absence of highly mutated sequences (Fig. 1 C).

Previous studies have concluded, based on [3H]thymidine incorporation after different mitogenic stimulations, that MSH2 deficiency did not result in a significant in vitro proliferative defect of splenic B cells, although isotype switching was reduced by two thirds (7, 27–30). Using carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling during in vitro stimulation of splenic B cells with either LPS or LPS plus IL-4, we observed no difference in proliferation of pol η–deficient B cells compared with controls, and only a marginal effect of MSH2 deficiency, which was, however, not manifest in the double Pol η–Msh2<sup>−/−</sup> context (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20062131/DC1).

pol η–MSH2–deficient mice show the same altered distribution of mutation along the Ig sequence as Msh2<sup>−/−</sup> mice do, with a striking clustering at a few G/C hotspot positions: seven hotspots concentrate more than half of the mutations in a sequence of 490 bp (Fig. 2). Most of them (except one, TGTT, at position 38 in the J<sub>H</sub><sup>4</sup> intronic sequence, whose targeting is, however, less pronounced in the double-deficient background) correspond to a WGCG sequence, as described previously (W = A or T) (12), with either or both internal G and C bases targeted. One major hotspot present in wild-type mice (AGTT, at position 46) disappears in the absence of MSH2. Similar again to the MSH2–deficient genotype, a strong increase in transitions within G/C mutations is observed in the double-deficient context (Table II).

The main difference between MSH2 and pol η–MSH2–deficient animals resides in A/T mutations: whereas they represent 10% of mutations in Msh2<sup>−/−</sup> mice, they are totally absent in the double knockout mice, with the 4 A/T changes collected in a sample of 310 mutations corresponding to the background of the enzyme used for amplification (Fig. 1 B). It should be noted that the low mutation frequency linked with the MSH2 genetic defect (in the 2 × 10<sup>−5</sup> range)

| Table I. | Somatic mutations in J<sub>H</sub><sup>4</sup> intronic sequences (490 bp) from normal and mutant mice |
|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Controls       | pol η<sup>−/−</sup> | Msh2<sup>−/−</sup> | pol η<sup>−/−</sup> × Msh2<sup>−/−</sup> |
| Number of sequences | 112 (4 mice) | 193 (7 mice) | 208 (4 mice) | 331 (4 mice) |
| Total length sequenced (bp) | 54,880 | 94,570 | 101,920 | 162,190 |
| Unmutated sequences (percentage) | 25 | 31 | 50 | 60 |
| Total number of mutations | 624 | 508 | 242 | 310 |
| Number of deletions and insertions | 8 | 6 | 4 | 5 |
| Mutation frequency per total sequences (per 100 bp) | 1.15 | 0.54 | 0.24 | 0.19 |
| Mutation frequency per mutated sequences (per 100 bp) | 1.54 | 1.13 | 0.78 | 0.48 | 0.48 |
Figure 1. Analysis of mutations in rearranged JH4 intronic sequences isolated from Peyer’s patches of controls and pol η–, MSH2– and pol η–MSH2–deficient mice. (A) Average mutation frequency per individual mouse, expressed relative to total sequences (left) or to mutated sequences (right). The mean values are represented by a horizontal bar. Controls (2) represent wild-type littermates of the Polh heterozygous breedings, whereas controls (1) come from a different module from the same animal facility (the dotted bar is the mean between the two sets of controls). These mean values differ slightly from the ones listed in Table I, which represent the average mutation frequency of pooled sequences. (B) Pattern of nucleotide substitution in the four different genotypes of mice. Values are expressed as the percentage of total mutations after correction for base composition. (C) Accumulation of mutations in individual JH4 intronic sequences. The number of sequences harboring a defined number of mutations relative to the total number of sequences is represented. MSH2-proficient (top) and MSH2-deficient (bottom) backgrounds are shown. All mutations are listed along the JH4 intronic sequence in Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20062131/DC1.
obviously requires high fidelity enzymes to collect mutation samples in which the contribution of the amplification step does not exceed a few percent (2–3% in this experiment, with $\sim 60\%$ of them being As or Ts) (31). It thus appears that pol $\eta$ is responsible for the residual A/T mutagenesis observed in MSH2-deficient animals by being involved in a mutagenic DNA synthesis that now appears to be driven by the UNG pathway. Taking into account our previous data suggesting that, in pol $\eta$-deficient mice, the A/T mutations observed are likely contributed by pol $\kappa$ recruited in a back-up function, we therefore conclude that, in the normal physiological situation, pol $\eta$ is the sole polymerase required for A/T mutagenesis at the Ig locus. Pol $\eta$ probably generates some G/C mutations as well, as its mutation spectrum in vitro affects G/C bases in a proportion of $\sim 20\%$, with two thirds of them being transitions and one third being transversions (32). These numbers fit remarkably well with the 3% of transversion mutations at G/C positions observed in UNG-deficient mice (6); indeed, this suggests that 10% of G/C mutations could be generated by pol $\eta$ via the MSH2 pathway, with one third of them being discernable as transversions in the UNG-deficient context.

Is the small contribution of pol $\eta$ via the UNG pathway observed in MSH2-deficient animals a physiological process? Although this question might not be easily answered, we would like to argue that the overall mutation pattern of MSH2-deficient animals corresponds to a major alteration of the repair pathways involved, a situation for which, surprisingly, no comprehensive explanation has been brought so far. The seminal work of Rada et al. (14) has shown that the mutation pattern of UNG–MSH2–deficient animals reflects the simple footprint of AID deamination and that it is close, in terms of targeting, to the G/C mutation pattern of wild-type animals. It is also quantitatively similar, at least at the V locus, and this striking observation that suggests that both UNG and MSH2 pathways are processing uracils mainly in an error-prone mode. It thus follows that the large decrease in mutation frequency, as well as the increased targeting of specific hotspots observed in the MSH2-deficient background, must be caused by an increased error-free repair of deaminated cytosines by the UNG pathway rather than by

Figure 2. Hotspot clustering of mutations in MSH2-deficient backgrounds. The distribution of mutations at G/C bases along the Jh4 intronic sequence is represented for the four different genetic backgrounds analyzed. The percentage of total mutations represented by the seven major hotspots observed in the Msh2$^{-/-}$ background (defined arbitrarily as a position mutated in 5% or more of sequences) is calculated for each genotype (marked by asterisks). These seven mutation hotspots occur in the following sequence context (described in their 5’ to 3’ order along the Jh4 sequence, with the mutated base underlined and the position of the first base of the motif (numbered from the Jh4 intronic border): TGTT (position 38), AGCA (position 55), TGCA (position 60), TGCT (position 251), and AGCA (position 362). One hotspot marked with an open triangle (AGTT, at position 46) is restricted to the MSH2-proficient background.
modulation of AID targeting by the MSH2–MSH6 complex, as proposed (12, 33). The mutation hotspots that emerge could correspond to deamination sites at which the residence time of AID would be increased (possibly through their symmetrical WGCW structure that would result in a stronger binding and/or an inefficient displacement by the sole UNG enzyme), thus preventing repair at those sites. The AGTT hotspot whose occurrence is restricted to the MSH2-proficient background would not provide such strong binding and would be more easily repaired. Error-free repair or ignorance would then be the two major outcomes of AID-induced cytidine deamination in the MSH2-deficient background, resulting in the drop of mutation frequency and the increase in transitions at G/C bases. A small fraction of lesions handled by the UNG pathway would generate abasic sites, leading to transversions when replicated over, or would recruit pol η in an error-prone short patch repair (Fig. 3).

Collectively, these data emphasize the very dissymmetrical role that the UNG and MSH2 pathways play in the hypermutation process. UNG deficiency affects mainly the nature of G/C mutations, which are biased almost exclusively toward transitions, and have only a moderate impact on the A/T mutagenesis driven by MSH2 (6, 34) and none on the mutation frequency. In contrast, the absence of mismatch repair leads to a stronger modification of the Ig gene mutation pattern, affecting not only mutations at A/T bases, as would be expected from a function symmetrical to the one of UNG, but also the overall mutation frequency and, within G/C mutations, the proportion of transitions as well as their distribution along the Ig sequence. Overall, this suggests that, although the function of MSH2–MSH6 is relatively independent from UNG, the mismatch repair complex strongly impinges on the role of UNG during hypermutation by preventing it to perform its natural error-free repair function.

The behavior of UNG in the mismatch repair–deficient context strikingly mirrors the effect of the deliberate overexpression of another uracil glycosylase, SMUG1, in a UNG–MSH2–deficient background, which results in a major error-free repair accompanied by a smaller error-prone activity generating A/T mutations and G/C transversions (35).

In conclusion, the exclusive formation of A/T mutations by pol η allows the formulation of a simplified scheme for the role of translesional polymerases in hypermutation (Fig. 3). pol η would be recruited by the MSH2–MSH6 complex outside of the S phase to permit U/G mismatch recognition, and would mostly generate A/T but also a small amount of error-prone short patch synthesis of the uracil-containing strand, most likely in G1 (reference 42). (B) Outline of a possible altered behavior of UNG in the absence of MSH2 and of its consequences on hypermutation in Msh2−/− mice. (Left) Increase of error-free repair (resulting in a reduced mutation frequency). (Middle) Recruitment of pol η for an error-prone short patch repair (residual A/T mutagenesis). (Right) Inefficient displacement of AID (increase in transitions at G/C), in particular at WGCW sites (increased focusing of mutations at specific hotspot positions).

Table II. Pattern of nucleotide changes in JH4 intronic sequences of normal and mutant mice

|                  | Within G/C | Trans. | Transv. |
|------------------|------------|--------|---------|
|                  | GC:AT      |        |         |
| Controls         | 49.3:50.7  | 54.3:45.7 | 59.1  |
| pol η−/−         | 84.4:15.6  | 53.0:47.0 | 93.3  |
| Msh2−/−          | 89.7:10.3  | 75.1:24.9 | 79.6  |
| pol η−/− × Msh2−/−| 99.2:0.8   | 84.6:15.4 | 85.3  |

Figure 3. Impact of the MSH2–MSH6 complex on UNG activity during Ig gene hypermutation. (A) A simplified scheme of hypermutation. UNG would be prevented from performing error-free repair in the presence of MSH2–MSH6 and would generate mainly abasic sites upon uracil recognition. These DNA lesions would be copied by a set of translesional DNA polymerases (among which are Rev1, Rev3, and possibly pol θ [references 36–40], albeit the contribution of this latter enzyme was recently shown to be less likely [reference 41]), acting in S phase in their function of lesion bypass. MSH2–MSH6 would recruit pol η in an
G/C mutations in an error-prone short patch repair process that appears thus far restricted to B cells. In contrast, most G/C mutations would be generated by several translesion DNA polymerases (33–38), whose exact number remains to be established, acting during replication in their function of lesion bypass by copying abasic sites generated by UNG, which is diverted by MSH2 from its error-free function. In such a model of hypermutation, and in contrast to their physiological repair function, UNG would trigger a translesional process at the replication fork, whereas MSH2–MSH6 would induce a base excision repair outside replication, most probably in the G1 phase of the cell cycle. The involvement of pol η in an error-prone process, whether driven by MSH2 during physiological hypermutation or by uracil glycosylases in specific experimental settings, thus appears to be a B cell–specific event whose biochemical basis remains to be established.

MATERIALS AND METHODS

Generation of gene-targeted mice. Polh-Msh2 double knockout mice were obtained by breeding the previously described Polh−/− mouse strain (19) with Msh2−/− animals provided by Hein te Riele (The Netherlands Cancer Institute, Amsterdam, Netherlands). Both genes are located distinctly enough on chromosome 17 to allow the efficient recovery of their combined inactivation by mouse breeding. All MSH2-deficient animals selected for analysis were devoid of overt tumors. Generation of gene-targeted mice and breeding was performed by the Service d’Expérimentation Animale et de Transgénèse. Experiments were performed according to the Institut national de la santé et de la recherche médicale guidelines for laboratory animals and were approved by the Scientific Committee of the Necker Animal Facility.

B cell proliferation assays. Splenic B cells were isolated from individual 2–3-mo-old mice by negative selection using the Mouse B Cell Isolation Kit (Miltenyi Biotec). Purified B cells were labeled with 5 μM CFSE, according to the manufacturer’s instructions (Vybrant CFDA SE Cell Tracer Kit; Invitrogen), before stimulation with either 20 μg/ml Escherichia coli O157:H7 (serotype O55:B5; Sigma-Aldrich) or 20 μg/ml LPS plus 10 ng/ml IL-4 (Preprotech). CFSE-labeled cells were analyzed after 3 d of stimulation, with forward scatter gating on live cells.

Sequence analysis. B220+/PNAhigh B cells were isolated from Peyer’s patches of 4–6-mo-old animals as previously described (8). The JH4 intron flanking rearranged VH sequences was amplified using a mixture of five VH primers designed to amplify most of the mouse VH families and a downstream primer allowing the determination of 490 bp of noncoding sequences, as reported previously (19).

Online supplemental material. Fig. S1 shows in vitro proliferation of splenic B cells from wild-type, Polh−/−, Msh2−/−, and Msh2−/−Polh−/− mice. Fig. S2 depicts the distribution of mutations along the JH4 intronic sequence in Peyer’s patch PNAhigh B cells from wild-type, Polh−/−, Msh2−/−, and Msh2−/−Polh−/− mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20062131/DC1.

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References

1. Longo, N.S., and P.E. Lipsky. 2006. Why do B cells mutate their immunoglobulin receptors? Trends Immunol. 27:374–380.

2. Muramatsu, M., K. Kinoshita, S. Fagarasan, S. Yamada, Y. Shinkai, and T. Honjo. 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 102:553–563.

3. Longenich, S., U. Basu, F.W. Alt, and U. Storb. 2006. AID in somatic hypermutation and class switch recombination. Curr. Opin. Immunol. 18:164–174.

4. Odegard, V.H., and D.G. Schatz. 2006. Targeting of somatic hypermutation. Nat. Rev. Immunol. 6:573–583.

5. Reynaud, C.-A., S. Aoufouchi, A. Faili, and J.-C. Weill. 2003. What role for AID: mutator, or assembler of the immunoglobulin mutasome? Nat. Immunol. 4:631–638.

6. Rada, C., G.T. Williams, H. Nilsen, D.E. Barnes, T. Lindahl, and M.S. Neuberger. 2002. Immunoglobulin isotype switching is inhibited and somatic hypermutation perturbed in UNG-deficient mice. Curr. Biol. 12:1748–1755.

7. Rada, C., M.R. Ehrenstein, M.S. Neuberger, and C. Milstein. 1998. Hot spot focusing of somatic hypermutation in MSH2-deficient mice suggests two stages of mutational targeting. Immunity. 9:135–141.

8. Frey, S., B. Bertocci, F. Delbos, L. Quint, J.-C. Weill, and C.-A. Reynaud. 1998.Mismatch repair deficiency interferes with the accumulation of mutations in chronically stimulated B cells and not with the hypermutation process. Immunity. 9:127–134.

9. Phung, Q.H., D.B. Winter, A. Cranston, R.E. Tarone, V.A. Bohr, R. Fuhel, and P.J. Gearhart. 1998. Increased hypermutation at G and C nucleotides in immunoglobulin variable genes from mice deficient in the MSH2 mismatch repair protein. J. Exp. Med. 187:1745–1751.

10. Wiesendanger, M., B. Kneitz, W. Edelmann, and M.D. Scharff. 2000. Somatic hypermutation in Msh5 homologue (MSH3−, MSH5−, and MSH3/MSH6-deficient mice reveals a role for the MSH2-MSH6 heterodimer in modulating the base substitution pattern. J. Exp. Med. 191:579–584.

11. Bardwell, P.D., C.J. Woo, K. Wei, Z. Li, A. Martin, S.Z. Sark, T. Parris, W. Edelmann, and M.D. Scharff. 2004. Altered somatic hypermutation and reduced class-switch recombination in exonuclease 1-mutant mice. Nat. Immunol. 5:224–229.

12. Martomo, S.A., W.W. Yang, and P.J. Gearhart. 2004. A role for Msh6 but not Msh3 in somatic hypermutation and class switch recombination. J. Exp. Med. 200:61–68.

13. Li, Z., S.J. Scherer, D. Ronai, M.D. Iglesias-Ussel, J.U. Peled, P.D. Bardwell, M. Zhuang, K. Lee, A. Martin, W. Edelmann, and M.D. Scharff. 2004. Examination of Msh6− and Msh3-deficient mice in class switching reveals overlapping and distinct roles of MutS homologues in antibody diversification. J. Exp. Med. 200:47–59.

14. Rada, C., J.M. Di Nota, and M.S. Neuberger. 2004. Mismatch recognition and uracil excision provide complementary paths to both Ig switching and the A/T focused phase of somatic mutation. Mol. Cell. 16:163–171.

15. Mautani, C., R. Kusumoto, A. Yamada, N. Dohmae, M. Yosoi, M. Yuasa, M. Araki, S. Iwai, K. Takio, and F. Hanaoka. 1999. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase e. Nature. 399:700–704.

16. Zeng, X., D.B. Winter, C. Kasmir, K.H. Kraemer, A.R. Lehmann, and P.J. Gearhart. 2001. DNA polymerase e is an A-T mutator in somatic hypermutation of immunoglobulin variable genes. Nat. Immunol. 2:537–541.

17. Faili, A., S. Aoufouchi, S. Weller, F. Vuillier, A. Stary, A. Sarasin, C.-A. Reynaud, and J.-C. Weill. 2004. DNA polymerase η is involved in hypermutation occurring during immunoglobulin class switch recombination. J. Exp. Med. 199:265–270.

18. Zeng, X., G.A. Negrete, C. Kasmir, W.W. Yang, and P.J. Gearhart. 2004. Absence of DNA polymerase η reveals targeting of C mutations on the nontranscribed strand in immunoglobulin switch regions. J. Exp. Med. 199:917–924.

19. Delbos, F., A. De Smet, A. Faili, S. Aoufouchi, J.-C. Weill, and C.-A. Reynaud. 2005. Contribution of DNA polymerase η to
