

Examination of Msh6- and Msh3-deficient Mice in Class Switching Reveals Overlapping and Distinct Roles of MutS Homologues in Antibody Diversification

Ziqiang Li,¹ Stefan J. Scherer,¹ Diana Ronai,¹ Maria D. Iglesias-Ussel,¹ Jonathan U. Peled,¹ Philip D. Bardwell,¹ Min Zhuang,² KyeRyoung Lee,¹ Alberto Martin,¹ Winfried Edelmann,¹ and Matthew D. Scharff¹

¹Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461

²Department of Oncology, Albert Einstein Cancer Center, Montefiore Medical Center, Bronx, NY 10467

Abstract

Somatic hypermutation and class switch recombination (CSR) contribute to the somatic diversification of antibodies. It has been shown that MutS homologue (Msh)6 (in conjunction with Msh2) but not Msh3 is involved in generating A/T base substitutions in somatic hypermutation. However, their roles in CSR have not yet been reported. Here we show that *Msh6*^{-/-} mice have a decrease in CSR, whereas *Msh3*^{-/-} mice do not. When switch regions were analyzed for mutations, deficiency in Msh6 was associated with an increase in transition mutations at G/C basepairs, mutations at RGYW/WRCY hotspots, and a small increase in the targeting of G/C bases. In addition, *Msh6*^{-/-} mice exhibited an increase in the targeting of recombination sites to GAGCT/GGGGT consensus repeats and hotspots in S γ 3 but not in S μ . In contrast to *Msh2*^{-/-} mice, deficiency in Msh6 surprisingly did not change the characteristics of S μ -S γ 3 switch junctions. However, *Msh6*^{-/-} mice exhibited a change in the positioning of S μ and S γ 3 junctions. Although none of these changes were seen in *Msh3*^{-/-} mice, they had a higher percentage of large inserts in their switch junctions. Together, our data suggest that MutS homologues Msh2, Msh3, and Msh6 play overlapping and distinct roles during antibody diversification processes.

Key words: isotype switching • mismatch repair • switching junction • switch region mutation • somatic hypermutation

Introduction

In mammals, the antibody response is diversified by somatic hypermutation (SHM) and class switch recombination (CSR) to produce high affinity antibodies of all possible isotypes (1). SHM introduces point mutations in the variable region that encodes the antigen binding sites at a rate that is one million times higher than the mutation rate of other genes. During CSR, the μ constant region (C μ) is replaced by downstream C γ , C ϵ , or C α genes through region-specific intrachromosomal recombination between the μ switch region (S μ) and one of the downstream γ , ϵ , or α switch regions that are 5' to these constant region

genes (2). Although SHM is primarily a mutational event and CSR requires recombination, both are initiated by the B cell-specific protein, activation-induced cytidine deaminase (AID) (3–5). AID deaminates dCs on single stranded DNA to generate dU (6–11). Single stranded DNA is created in vivo by transcription (6, 10, 11) and WRCY (W = A or T, R = A or G, Y = C or T) hotspots are preferentially targeted for mutation (12). The resulting G–U mismatches may then be replicated to introduce G to A and C to T transitions or the dU is converted to an abasic site by uracil DNA glycosylase leading to both transition and transversion mutations (13, 14). Alternatively, G–U mismatches are recognized by the mismatch repair (MMR) system, excised, and replaced by error prone polymerases to produce flank-

The online version of this article contains supplemental material.

Address correspondence to Matthew D. Scharff, Dept. of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Channing 403, Bronx, NY 10461. Phone: (718) 430-2170; Fax: (718) 430-8574; email: Scharff@aecom.yu.edu

A. Martin's present address is Dept. of Immunology, University of Toronto, Medical Sciences Bldg., Toronto, Canada, M5S 1A8.

Abbreviations used in this paper: AID, activation-induced cytidine deaminase; CSR, class switch recombination; Exo1, exonuclease 1; MMR, mismatch repair; Msh, MutS homologues; SHM, somatic hypermutation; V, variable.

ing mutations primarily at A/T base pairs that are not in hot spots (15–17). The fact that AID is required for both SHM and CSR supports the idea that mutations are introduced before CSR (18).

Mice deficient in MutS homologue (Msh)2 or Msh6 have more mutations at G/C bases and more transition mutations at G/C basepairs and in hotspot motifs in SHM (for review see 19). Similar changes were also observed in mice with a mutation in the ATPase domain of Msh2 (20). In contrast, *Msh3*^{-/-} mice do not have a detectable change in the frequency or characteristics of variable (V) region mutation (21). These findings suggest that the Msh2–Msh6 heterodimer that is predominantly involved in the repair of base–base substitutions and single base insertion/deletions (22, 23), but not the Msh2–Msh3 heterodimer that is predominantly involved in the repair of single base insertion/deletions and larger insertion/deletion mismatches (23), generates mutations on A/T base pairs during SHM (for review see 19). Mice deficient or mutant in Msh2 have a decreased frequency of CSR (20, 24–26), suggesting that MMR plays a role in CSR and connects the processes of SHM and CSR (for review see 19). There has been controversy on whether MMR plays a direct role in SHM and CSR (19, 27). Recent studies have shown that exonuclease 1 (Exo1) (28, 29), which is a part of the MMR process, plays a role in SHM and CSR and that both Exo1 and Mlh1 are associated with the V region of cultured B cells only when they are undergoing SHM (30). Although this and other recent studies on MMR-deficient mice (31, 32) indicate a direct role for MMR in CSR and SHM, many additional proteins such as Ku, DNA-PK_{cs}, γ -H2AX, 53BP1, and ATM, which are required to repair double stranded DNA breaks, are involved in CSR but not in SHM (33–38). It is still unclear how these different factors are coordinated with the changes in the chromatin structure that make the switch regions accessible for sterile transcription, mutation, double stranded break formation, and processing and ligation of the DNA ends in CSR (2, 39–42).

When B cells from *Msh2*^{-/-} mice are stimulated to carry out CSR in short term culture in vitro, their $\Sigma\mu$ – $\Sigma\gamma$ 3 switch junctions have shorter microhomologies, more blunt junctions, and more insertions than cells from wild-type littermate mice (43). This has led to the suggestion that Msh2 recruits a complex(es) that converts the single stranded breaks created by AID in the switch regions (44) into double stranded breaks which are then processed and ligated (43). Even though Msh2 and Msh6 function together as a heterodimer, Msh2-deficient mice die of colon and other cancers, whereas Msh6-deficient mice predominantly die of B cell lymphomas (45). Since many B cell lymphomas arise in the germinal center cells, it is important to determine whether the role of Msh6 or Msh3 in CSR is the same as that of Msh2. In addition, it is important to determine whether the role of these proteins in the generation of switch region mutations in CSR is the same as in the generation of variable region mutations. To address these questions, we examined the efficiency of CSR, the

location and spectrum of switch region mutations, and the location and characteristics of switch junctions in *Msh3*^{-/-} and *Msh6*^{-/-} mice. We also examined the characteristics of V region mutation in *Msh3*^{-/-} and *Msh6*^{-/-} mice to compare the characteristics of the mutations in the V region to those in the switch junctions.

Our results indicate that, similar to *Msh2*^{-/-} mice, the frequency of CSR is lower in *Msh6*^{-/-} B cells than in wild-type B cells, whereas the frequency of CSR in *Msh3*^{-/-} B cells is the same as the littermate control B cells. These results are consistent with the idea that the Msh2–Msh6 complex, but not the Msh2–Msh3 complex, is involved in generating DNA breaks during CSR. Surprisingly, unlike the findings in *Msh2*^{-/-} mice (43) the $\Sigma\mu$ – $\Sigma\gamma$ 3 switch junctions of *Msh6*^{-/-} mice did not reveal any difference from those of littermate control mice. Even though neither the rate of CSR nor the length of microhomologies in the $\Sigma\mu$ – $\Sigma\gamma$ 3 switch junctions was affected by a deficiency in Msh3, there was a higher percentage of large inserts in the switch junctions in the *Msh3*^{-/-} mice than in their littermate control mice, consistent with the notion that Msh3 is involved, probably in conjunction with Msh2, in the processing of DNA ends before ligation. Comparison of the mutations in the switch regions to those in the JH2–JH4 segment of the V region revealed a similar but somewhat different role for Msh6 in SHM targeted to the V region and to the donor μ and recipient γ switch regions. These findings suggest that the MutS homologues Msh2, Msh3, and Msh6 play overlapping and distinct roles in both SHM and CSR.

Materials and Methods

Mice. The Msh3- and Msh6-deficient mice were reported previously (21, 46, 47). Both mouse lines were backcrossed to C57BL/6 mice for more than six generations and were housed in a pathogen-free facility. Homozygous mice were generated through heterozygous mating. In all experiments, homozygous-deficient mice were analyzed simultaneously with at least one littermate control mouse. For Msh6 switch junction analyses, mice that had been backcrossed separately by the Scharff and Edelman laboratories for >3 yr were compared and were indistinguishable. All mouse experiments were approved by the Albert Einstein Animal Use Committee.

In Vitro Switching Assay. 4–10-wk-old mice were used to determine the frequency of CSR. For Msh3, two *Msh3*^{+/+}, three *Msh3*^{+/-} littermate control mice, and five *Msh3*^{-/-} mice were analyzed as described previously (48). For Msh6, four wild-type, three heterozygous, and six homozygous *Msh6*^{-/-} mice were analyzed. B cells were obtained from the spleens of naive mice by RBC lysis and complement-mediated T cell depletion. Cells were then cultured in RPMI media containing 10% FCS and 50 μ g/ml LPS (Sigma-Aldrich) or 50 μ g/ml LPS and 50 ng/ml rIL-4 (R&D Systems) for 4 d. The stimulated cells were then harvested and stained with anti-IgM CY5 (Jackson Immunochemicals) and either anti-IgG1 or anti-IgG3 FITC (BD Biosciences). Stained cells were then washed with PBS, fixed with 1% paraformaldehyde in PBS, and analyzed with a FACSCalibur (Becton Dickinson). FACS[®] analysis was performed using the FlowJo software package (Treestar). Cells were gated for viability based on the forward- and side-scatter profiles. The IgG-positive popu-

lation was scored according to the gating profile as described previously (48).

Proliferation Assays. 2×10^5 total spleen cells in 200 μ l of the RPMI media were plated as 5-well replicates in a 96-well culture plate. On day three, the cells were pulsed with 1 μ Ci/well of 3 H-thymidine (NEN) for \sim 16 h before harvesting onto 96-well printed filtermats (Wallac) and measuring radioactive incorporation on a Wallac 1450 Microbeta LSC plate counter.

Mutation Analysis of the Upstream Region of the Core S μ . Genomic DNA was isolated using the DNAeasy kit (QIAGEN)

from cells stimulated with 50 μ g/ml LPS. DNA obtained from in vitro-stimulated B cells was used to amplify the upstream of the core S μ region using Pfu-turbo polymerase essentially as described (36), with the 5' primer 5'-AATGGATACCTCAGTG-GTTTTTAATGGTGGGTTTA-3' and the 3' primer 5'-GCCG-CGCCGGCTCATTCCAGTTCATTACAG-3'.

S μ -S γ 3 Switch Junction Analysis. DNA derived from four *Msh6*^{+/+}, two *Msh6*^{+/-}, and four *Msh6*^{-/-} mice was used to amplify the recombined sequences. For Msh3, two littermate controls and two *Msh3*^{-/-} mice were used. S μ -S γ 3 junctions were

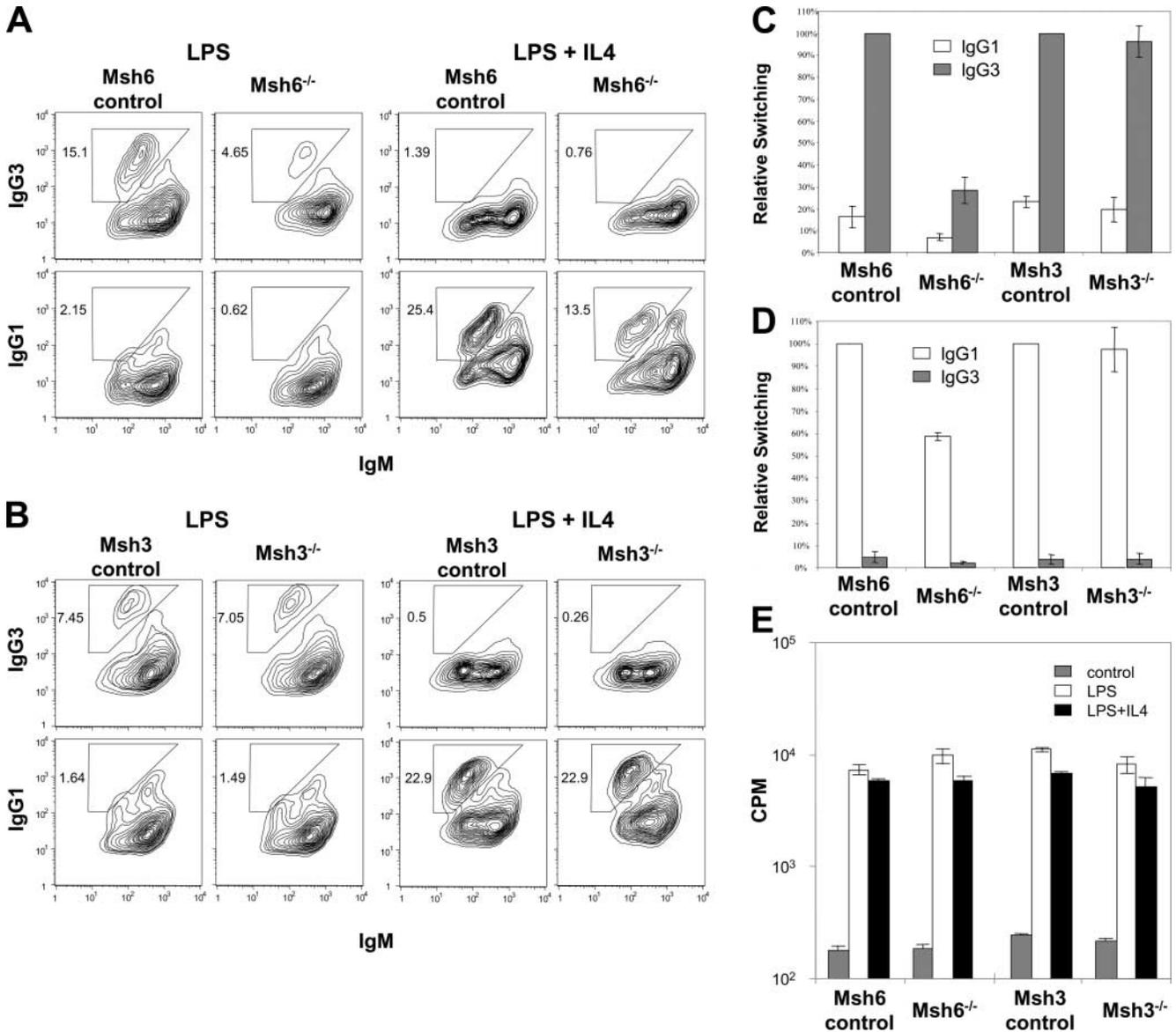


Figure 1. Effect of Msh6 deficiency and Msh3 deficiency on efficient CSR. LPS stimulates IgG3 switching, whereas LPS + IL4 stimulates IgG1 switching. (A) Representative FACS[®] profile showing the percentage of IgG1 and IgG3-positive cells from *Msh6* control and *Msh6*^{-/-} mice. (B) Representative FACS[®] profiles showing the percentage of IgG1 and IgG3-positive cells from *Msh3* control and *Msh3*^{-/-} mice. (C) Summary of multiple experiments in which IgG3 switching frequency from *Msh6*^{-/-} mice and *Msh3*^{-/-} mice is normalized to their respective controls as a percentage. The white bars represent the relative IgG1 switching in the presence of LPS compared with the respective wild-type IgG3 switching, which is defined as 100%, represented by the gray bars. (D) Summary of multiple experiments in which IgG1 switching frequency from *Msh6*^{-/-} mice and *Msh3*^{-/-} mice is normalized to their respective controls as a percentage. The gray bars represent the relative IgG3 switching, in the presence of LPS + IL4 compared with the respective wild-type IgG3 switching, which is defined as 100%, represented by the white bars. (E) B cell proliferation results showing *Msh3*^{-/-}, *Msh6*^{-/-}, and their respective controls.

Table I. Effect of *Msh6* Deficiency on the Characteristics of $S\mu$ - $S\gamma 3$ Switch Junctions from LPS-stimulated B Cells**A.** Microhomologies and Insertions and Blunt Junctions in the Switch Junctions from *Msh6*^{+/+}, *Msh6*^{+/-}, and *Msh6*^{-/-} Mice^a

	Blunt	Microhomologies (%)			Insertions (%)		Total
		1–2 nt ^b	3–4 nt	≥5 nt	1–4 nt	≥5 nt	
<i>Msh6</i> ^{+/+}	17 (17)	34 (34)	12 (12)	16 (16)	9 (9)	11 (11)	99 (100)
<i>Msh6</i> ^{+/-}	16 (19)	26 (31)	9 (11)	9 (11)	15 (18)	8 (10)	83 (100)
<i>Msh6</i> ^{-/-c}	9 (18)	13 (25)	5 (10)	12 (23)	6 (12)	6 (12)	51 (100)

B. GAGCT/GGGGT Consensus Sequence Targeting in the Switch Junctions for $S\mu$ and $S\gamma 3$ Segments from *Msh6*^{+/+}, *Msh6*^{+/-}, and *Msh6*^{-/-} Mice

	$S\mu$		$S\gamma 3$	
	Yes ^d (%)	No (%)	Yes ^d (%)	No (%)
<i>Msh6</i> ^{+/+}	66 (67)	33 (33)	19 (19)	80 (81)
<i>Msh6</i> ^{+/-}	54 (65)	29 (35)	23 (28)	60 (72)
<i>Msh6</i> ^{-/-e}	36 (71) ^e	15 (29) ^e	26 (51) ^f	25 (49) ^f

C. RGYW/WRCY Hotspot Targeting in the Switch Junctions for $S\mu$ and $S\gamma 3$ Segments from *Msh6*^{+/+}, *Msh6*^{+/-}, and *Msh6*^{-/-} Mice

	$S\mu$		$S\gamma 3$	
	Yes ^d (%)	No (%)	Yes ^d (%)	No (%)
<i>Msh6</i> ^{+/+}	77 (78)	22 (22)	64 (65)	35 (35)
<i>Msh6</i> ^{+/-}	55 (66)	28 (34)	60 (72)	23 (28)
<i>Msh6</i> ^{-/-e}	40 (78) ^e	11 (22) ^e	41 (80) ^g	10 (20) ^g

^aData presented in Table S1. Unique switch junctions compiled from four *Msh6*^{-/-}, two *Msh6*^{+/-}, and four *Msh6*^{+/+} littermate control mice.

^bnt, nucleotide.

^cNo statistically significant difference between *Msh6*^{-/-}, *Msh6*^{+/-}, and *Msh6*^{+/+} mice in any category.

^d“Yes” is defined as the junction is within or adjacent to the consensus or hotspot sequence.

^eNo statistically significant difference between *Msh6*^{-/-}, *Msh6*^{+/-}, and *Msh6*^{+/+} mice.

^fStatistically different between *Msh6*^{-/-} and *Msh6*^{+/+} mice (χ^2 test; $P = 0.0001$).

^gNo statistically significant difference between *Msh6*^{-/-} and *Msh6*^{+/+} mice (χ^2 test; $P = 0.0710$).

amplified by PCR using Expand Long Template Taq Polymerase (Roche) essentially as described (43). The primers were $\mu 3$ -H3: 5'-AACAAAGCTTGGCTTAACCGAGATGAGCC-3', and $\gamma 3$ -2: 5'-TACCCTGACCCAGGAGCTGCATAAC-3'. Amplified PCR products were cloned using the TA cloning kit (Invitrogen). Plasmid DNA was prepared using the Millipore 96-well miniprep kit. T7 primers (Invitrogen) were used to sequence the PCR inserts. Sequence alignments were constructed by comparing the PCR sequence with $S\mu$ germline sequence AF446347 and $S\gamma 3$ germline sequence Musighana using Blast2 alignment (NCBI). The selection of correct alignments was determined by the percentage of homology to the germline sequence, the length of homology, and the existence of the primer sequences used to amplify the junctions. Only unique junctions were tabulated for the analysis of the recombination sites.

Mutation Analysis of Switched DNA from $S\mu$ and $S\gamma 3$ Switch Regions. After the switch junctions were determined, mutations in the recombined DNA segments were scored by comparing them to the germline sequences of $S\mu$ or $S\gamma 3$. Only unique mutations were tabulated in the mutation spectrum analysis.

Somatic Hypermutation Analysis. To analyze V region SHM, Peyer's patch PNA high (PNA^{hi}) B cells of 9–15-mo-old mice were harvested, stained, and FACS[®] sorted. The JH2 to JH4 region was then amplified by using Pfu turbo (Stratagene) as described previously (20). The primers used to amplify JH2-JH4 region were 5' primer, 5'-GGCACCCTCTCACAGTCTCCTCAGG-3' and 3' primer, 5'-TGAGACCGAGGCTAGATGCC-3'. PCR products were cloned and sequenced. For *Msh3* mice, two 15-mo-old *Msh3* wild-type and two homozygous mice were analyzed. Additionally, 12 mutations from *Msh3* con-

trols and 17 mutations from *Msh3*^{-/-} mice reported previously (21) were included in the mutation spectra. For *Msh6*, one *Msh6*^{+/+}, one *Msh6*^{+/-}, and one *Msh6*^{-/-} mouse (~8-mo-old) were used. A pair of NP-immunized *Msh6*^{+/+} and *Msh6*^{-/-} mice (~2-mo-old) was also analyzed. Additionally, four mutations from *Msh6*^{+/+} and seven mutations from *Msh6*^{-/-} mice reported previously (21) were also used to obtain the mutation spectra. The spectra of mutation were not different when this old data was not pooled with the new data.

Statistical Analyses. For grouped or categorized microhomology, conserved repeat and hotspot data, the χ^2 test was used. If there were more than two categories, one was compared with the pooled data from the rest of the categories to evaluate the difference between two different genotypes within the same category. When, within any category, there was a number less than five, Fisher's exact test was used. $P < 0.05$ is considered statistically significant.

Online Supplemental Material. In Tables S1 and S2, $S\mu$ - $S\gamma 3$ junctions are shown from *Msh6* and *Msh3* mouse lines, respectively. Table S3 shows the consensus sequence targeting and hotspot targeting of $S\mu$ - $S\gamma 3$ junctions from *Msh3* mouse line. Table S4 shows the detailed mutation distribution in the recombined switch regions from *Msh6* and *Msh3* mouse lines. All supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20040355/DC1>.

Results

***Msh6* Deficiency Results in Decreased CSR but *Msh3* Deficiency Did Not.** To address whether *Msh3* or *Msh6* is involved in efficient CSR, splenic B cells from both mouse lines were stimulated with LPS or LPS plus IL-4 to induce switching to IgG3 or IgG1, respectively. In all CSR experiments, a homozygous mouse was always analyzed with a wild-type or heterozygous littermate mouse. The relative change in switching efficiency of a homozygous mouse was normalized to its wild-type or heterozygous littermate mouse. In both the *Msh6* and *Msh3* experiments, the relative switching efficiency of homozygous mice was comparable when normalized to either their wild-type or heterozygous littermate mice (Fig. 1, A–D). Therefore, different experiments were pooled and the relative switching efficiency was displayed as a percentage of the control littermates. Fig. 1, A and B, shows the representative FACS[®] data for *Msh6*^{-/-} and *Msh3*^{-/-} mice and their littermate controls, respectively. *Msh6*^{-/-} B cells treated with LPS had an approximately threefold reduction in switching to IgG3 compared with those from their littermate controls (Fig. 1, A and C). When B cells were stimulated with LPS and IL-4, *Msh6*^{-/-} B cells had an approximately twofold reduction in switching to IgG1 compared with the controls (Fig. 1, A and D). As shown in Fig. 1, B–D, *Msh3*^{-/-} mice did not exhibit any defect in switching to either IgG3 or IgG1. Neither *Msh3* nor *Msh6* deficiency affected B cell proliferation during cytokine stimulation (Fig. 1 E). The extent of the defect in CSR from *Msh6*-deficient mice is similar to that found in *Msh2*-deficient mice (20, 24, 26, 31), suggesting that the *Msh2*-*Msh6* heterodimer is involved in efficient CSR, as it is in SHM.

***Msh6* Deficiency Does Not Change the $S\mu$ - $S\gamma 3$ Junctions but Does Change the Targeting of Recombination Sites to the Consensus Repeat and Hotspots in the $S\gamma 3$ Region.** In addition to a decrease in the frequency of switching, *Msh2* deficiency is associated with increased frequencies of blunt junctions and shorter microhomologies (43). Since *Msh2* and *Msh6* both caused a decrease in the frequency of switching and function as a heterodimer in MMR (22), we examined the $S\mu$ - $S\gamma 3$ junctions from splenic B cells stimulated with LPS from *Msh6*^{-/-} mice and their littermate controls. 99 unique junctions were analyzed from four *Msh6*^{+/+} mice, 83 unique junctions from two *Msh6*^{+/-} mice, and 51 unique junctions from four *Msh6*^{-/-} mice. The alignments of these switch junctions are shown in Table S1, available at <http://www.jem.org/cgi/content/full/jem.20040355/DC1>. The data were categorized and summarized in Table I A. In contrast to *Msh2*^{-/-} mice, there was no increase in blunt junctions and shorter microhomologies in the switch junctions from *Msh6*-deficient mice compared with their littermate controls. However, *Msh6*^{+/+}, *Msh6*^{+/-}, and *Msh6*^{-/-} mice all had large inserts in their switch junctions (see Discussion).

Since there is an increased targeting of SHM to hotspots in the V region in *Msh6*^{-/-} mice (reference 21 and see Table IV), we analyzed the usage of the GAGCT/GGGGT consensus repeat sequence and RGYW/WRCY hotspots in the recombination junctions identified. In general, there was more targeting to the consensus repeats in $S\mu$ than in $S\gamma 3$ of the recombined switch regions (Table I B). This is probably due to the fact that there are more such consensus motifs in the $S\mu$ than in the $S\gamma 3$ region. However, in *Msh6*^{-/-} mice there was an increase in targeting to the consensus repeat (Table I B, right) in the $S\gamma 3$ region (51%) compared with *Msh6*^{+/+} mice (19%), whereas the *Msh6*^{+/-} mice showed an intermediate frequency (28%). This type of targeting change was not observed in the $S\mu$ region (Table I B, left). Similarly, an increased targeting to RGYW/WRCY hotspots was observed in the $S\gamma 3$ region from *Msh6*^{-/-} mice compared with *Msh6*^{+/+} mice (Table I C, right), although the p-value is 0.0710. Increased use of hotspots was not observed in $S\mu$ region (Table I C, left).

Table II. Effect of *Msh3* Deficiency on the Characteristics of $S\mu$ - $S\gamma 3$ Switch Junctions from LPS-stimulated B Cells^a

	Microhomologies (%)				Insertions (%)		Total
	Blunt	1–2 nt ^b	3–4 nt	≥5 nt	1–4 nt	≥5 nt ^c	
Controls	6 (14)	20 (46)	5 (12)	10 (23)	2 (5)	0 (0)	43 (100)
<i>Msh3</i> ^{-/-d}	5 (10)	19 (38)	6 (12)	9 (18)	5 (10)	6 (12)	50 (100)

^aData presented in Table S2. Unique switch junctions compiled from two *Msh3*^{-/-} mice and two littermate controls.

^bnt, nucleotide.

^{c,d}Insert lengths ≥5 nt from *Msh3*^{-/-} mice are statistically different compared to the controls ($P < 0.05$), whereas microhomology lengths are not.

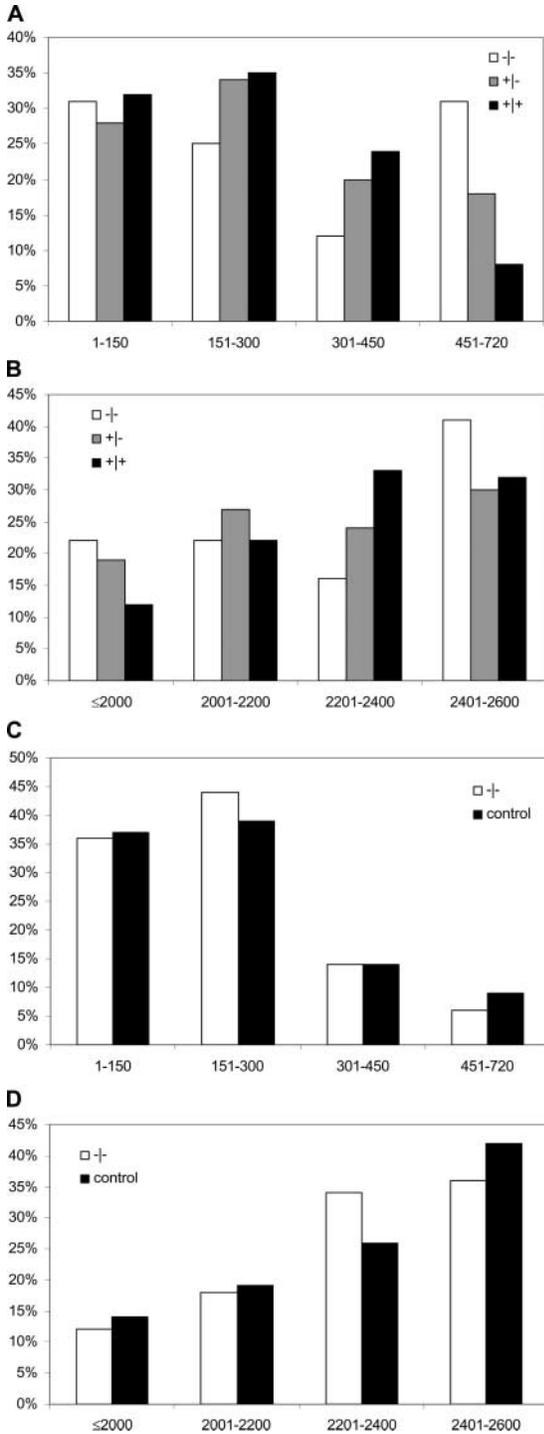


Figure 2. Effect of Msh6 deficiency and Msh3 deficiency on the positioning of junctions in $S\mu$ and $S\gamma 3$ regions in $S\mu$ - $S\gamma 3$ switch junctions. The core $S\mu$ (GenBank/EMBL/DDBJ accession no. AF446347.1) region was arbitrarily divided into four different segments: nucleotide 1–150, 151–300, 301–450, and 451–760. The $S\gamma 3$ region (GenBank/EMBL/DDBJ accession no. M12182) was arbitrarily divided into four different segments: nucleotide ≤ 2000 , 2001–2200, 2201–2400, and 2401–2600. Y axis stands for the percentage of junctions falling into different segments of switch regions. (A) Summary of junctions falling into different segments of switch regions from $Msh6^{+/+}$, $Msh6^{+/-}$, and $Msh6^{-/-}$ mice. (B) Summary of junctions falling into different $S\gamma 3$ segments from $Msh6^{+/+}$, $Msh6^{+/-}$, and $Msh6^{-/-}$ mice. (C) Summary of junctions falling into different $S\mu$

Msh3 Deficiency Does Not Change the Pattern of Microhomologies but Does Change the Pattern of Inserts in the $S\mu$ - $S\gamma 3$ Junctions. Studies in yeast have established that Msh2, Msh3, Rad1, and Rad10 are involved in removing the non-homologous DNA end sequences during double stranded break repair (49). Msh2 and Msh3 could function in a similar fashion in CSR. Although Msh3 deficiency has no effect on the frequency of switching, Msh3 might play a role in the late steps in CSR. Therefore, we examined the $S\mu$ - $S\gamma 3$ junctions from splenic B cells from Msh3-deficient mice stimulated with LPS. 43 unique junctions were obtained from the control mice, and 50 unique junctions were obtained from $Msh3^{-/-}$ mice. The complete alignments were shown in the Table S2, available at <http://www.jem.org/cgi/content/full/jem.20040355/DC1>. The data were categorized and are shown in Table II. There was no difference in the size distribution of the microhomologies in the junctions from the $Msh3^{-/-}$ and control mice. However, the Msh3-deficient mice had more large inserts (≥ 5 nt) than their littermate controls (Fisher's Exact test, $P < 0.05$). Therefore, Msh3 deficiency shares the increased occurrence of large inserts with Msh2-deficiency, even though $Msh3^{-/-}$ and $Msh2^{-/-}$ mice differ in the frequency of blunt junctions and in the size of the microhomologies in switch junctions. $Msh3^{-/-}$ mice and their littermate controls did not differ in the targeting of recombination to the GAGCT/GGGGT consensus repeats or hotspots in $S\mu$ or $S\gamma 3$ (Table S3, A and B, available at <http://www.jem.org/cgi/content/full/jem.20040355/DC1>). Together, these results suggest that Msh3 is neither involved in the generation of DNA breaks nor in the initial resolution of breaks, but it could be involved in processing the DNA ends before ligation occurs.

Msh6 Deficiency but not *Msh3* Deficiency Changes the Positioning of Junctions in the Switch Regions. Further analyses of switch junctions from the Msh3 and Msh6 mouse lines revealed that Msh6 deficiency was associated with a change of positioning of junctions in the switch regions. Fig. 2 A shows that Msh6 deficiency significantly increased the usage of 451–720-bp segment (χ^2 test, $P = 0.0006$) (the whole $S\mu$ core is 760 bp) but did not change the targeting of recombination to the $S\mu$ segments 1–150 bp or 151–300 bp. There also appears to be a decrease in the usage of segment 301–450 bp (χ^2 test, $P = 0.11$) (Fig. 2 A). In both 301–450-bp and 451–720-bp $S\mu$ segments, $Msh6^{+/-}$ mice showed an intermediate phenotype (Fig. 2 A). In the $S\gamma 3$ region, deficiency of Msh6 significantly decreased the usage of segment 2,201–2,400 bp (χ^2 test, $P = 0.0354$) (the whole $S\gamma 3$ is 2,705 bp), and there appeared to be an increase in the usage of segment $\leq 2,000$ bp (χ^2 test, $P = 0.2$) (Fig. 2 B). In parallel, we also analyzed the effect of Msh3 deficiency in the usage of different switch segments. Fig. 2 C shows the usage of different portions of $S\mu$ was not significantly different between the control and $Msh3^{-/-}$ mice in four different segments. Similar results were also observed in the $S\gamma 3$ region (Fig. 2 D).

segments from littermate control and $Msh3^{-/-}$ mice. (D) Summary of junctions falling into different $S\gamma 3$ segments from littermate control and $Msh3^{-/-}$ mice.

Table III. Effect of *Msh6* Deficiency and *Msh3* Deficiency on the Characteristics of Switch Region Mutations from LPS-stimulated B Cells

A. Sμ Region from <i>Msh6</i>^{+/+}, <i>Msh6</i>^{+/-}, and <i>Msh6</i>^{-/-} Mice			
	<i>Msh6</i> ^{+/+}	<i>Msh6</i> ^{+/-}	<i>Msh6</i> ^{-/-}
Mutation frequency ($\times 10^{-3}$)	12.9	7.1	11.3
GC mutations/total	184/278 (66%)	101/148 (68%)	161/211 (76%) ^a
Ts ^b mutations at GC/total	94/184 (51%)	54/101 (53%)	128/161 (80%) ^c
Hotspot mutations/total	92/278 (33%)	71/148 (48%) ^d	142/211 (67%) ^e
B. Sγ3 Region from <i>Msh6</i>^{+/+}, <i>Msh6</i>^{+/-}, and <i>Msh6</i>^{-/-} Mice			
	<i>Msh6</i> ^{+/+}	<i>Msh6</i> ^{+/-}	<i>Msh6</i> ^{-/-}
Mutation frequency ($\times 10^{-3}$)	5.8	4.3	5.7
GC mutations/total	96/161 (60%)	64/101 (63%)	64/87 (74%) ^f
Ts ^b mutations at GC/total	61/96 (64%)	40/64 (62%)	53/64 (83%) ^g
Hotspot mutations/total	29/161 (18%)	39/101 (39%) ^h	37/87 (42%) ^h
C. Sμ Region from Littermate Control and <i>Msh3</i>^{-/-} Mice			
	Control	<i>Msh3</i> ^{-/-i}	
Mutation frequency ($\times 10^{-3}$)	12.7	14.7	
GC mutations/total	88/127 (69%)	80/128 (63%)	
Ts ^b mutations at GC/total	48/88 (55%)	35/80 (44%)	
Hotspot mutations/total	49/127 (38%)	35/128 (27%)	
D. Sγ3 Region from Littermate Control and <i>Msh3</i>^{-/-} Mice			
	Control	<i>Msh3</i> ^{-/-j}	
Mutation frequency ($\times 10^{-3}$)	2.9	6.3	
GC mutations/total	19/37 (51%)	46/87 (53%)	
Ts ^b mutations at GC/total	15/19 (79%)	33/46 (72%)	
Hotspot mutations/total	9/37 (24%)	21/87 (24%)	

^aStatistically significant difference between *Msh6*^{-/-} or *Msh6*^{+/-} and *Msh6*^{+/+} mice (χ^2 test; ^aP = 0.0198).

^bTs, transition.

^{c,d,e,f,g,h}Statistically significant difference between *Msh6*^{-/-} or *Msh6*^{+/-} and *Msh6*^{+/+} mice (χ^2 test; ^cP < 0.0001, ^dP = 0.0037, ^eP < 0.0001, ^fP = 0.0404, ^gP = 0.0139, ^hP < 0.0001).

^{ij}No statistical difference between *Msh3*^{-/-} and control mice.

Neither Msh3 nor Msh6 Is Required to Generate Mutations in the Upstream of the Core S μ Region. To determine whether *Msh3* or *Msh6* is required for the generation of mutations in the switch regions, we first amplified and sequenced a 561-bp DNA fragment upstream of the core S μ from genomic DNA isolated from stimulated splenic B cells (36). The region sequenced corresponds to position 4600–5160 in the S μ germline sequence (GenBank/EMBL/DDBJ accession no. J0040). Of note, the mutations seen could be from both recombined and unrecombined

sequences. The frequency of mutations in *Msh6*^{+/+} and *Msh6*^{+/-} littermate mice was 2.8×10^{-4} and 2.9×10^{-4} , respectively, so the data was pooled. As shown in Fig. 3, the mutation frequency for the control (i.e., *Msh6*^{+/+} and *Msh6*^{+/-} mice) and *Msh6*^{-/-} mice was 2.9×10^{-4} and 3.4×10^{-4} , respectively. Although the frequency of mutation in the *Msh3*^{-/-} and control littermates was higher, the difference in the frequency of mutation between the *Msh6*- and *Msh3*-deficient mice and their respective controls was not statistically significant. These results indicate that both

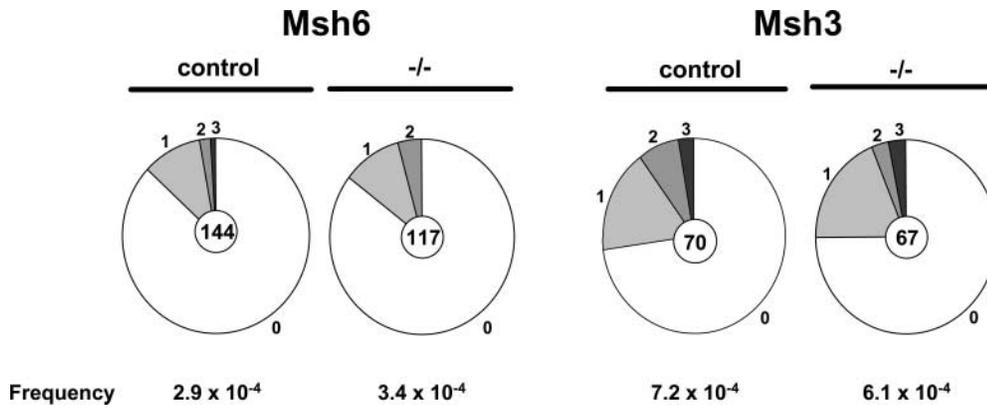


Figure 3. Effect of Msh6 deficiency and Msh3 deficiency on the mutation frequency of the upstream S μ region from LPS-stimulated B cells. *Msh3*^{-/-} mice and *Msh6*^{-/-} mice were compared with their own littermate controls. Pie charts were used to depict the distribution of mutation frequencies and the proportion of sequences with different number of mutations (numbers of mutations outside the pie slices).

Msh6 and Msh3 are dispensable for generating mutations upstream of μ switch region.

Msh6 Deficiency but not *Msh3* Deficiency Increases the Transition Mutations at G/C and Mutations at Hotspots in Switched DNA Sequences. To further characterize the mutations generated in switch regions, we analyzed mutations in rear-

ranged S μ and S γ 3 regions. As shown in Table III A, the mutation frequency in the S μ region of *Msh6*^{+/+} mice was similar to that of *Msh6*^{-/-} mice, whereas *Msh6*^{+/-} mice had a lower frequency of mutation than *Msh6*^{+/+} and *Msh6*^{-/-} mice (χ^2 test, $P < 0.0001$). The mutation frequency was lower in S γ 3 region than in S μ and *Msh6*^{+/-}

Table IV. Somatic Hypermutation of the JH2-JH4 Region from Peyer's Patch B Cells

A.	<i>Msh3</i> control					<i>Msh6</i> control				
	A	G	C	T	Total	A	G	C	T	Total
A	/	16	5	12	33	A	/	9	5	19
G	9	/	4	1	14	G	10	/	3	16
C	0	2	/	7	9	C	3	2	/	17
T	4	3	5	/	12	T	4	3	6	13
					68					65
	<i>Msh3</i> ^{-/-}					<i>Msh6</i> ^{-/-}				
	A	G	C	T	Total	A	G	C	T	Total
A	/	35	13	20	68	A	/	0	1	2
G	19	/	4	6	29	G	10	/	2	14
C	3	7	/	9	19	C	2	1	/	25
T	14	10	19	/	43	T	2	0	0	2
					159					43
B.	Controls		<i>Msh6</i> ^{-/-}		<i>Msh3</i> ^{-/-}					
GC mutations/total	56/133 (42%)		39/43 (91%) ^a		48/159 (30%) ^c					
Ts ^b mutations at GC/total	38/56 (68%)		32/39 (82%) ^d		28/48 (58%) ^e					
Hotspot mutations/total	25/133 (11%)		15/43 (35%) ^f		23/159 (14%) ^g					

^{a,f}Statistically significant difference between *Msh6*^{-/-} and control mice (χ^2 test; ^a $P < 0.0001$, ^f $P = 0.0478$).

^bTs, transition.

^cStatistically significant difference between *Msh3*^{-/-} and control mice (χ^2 test; ^c $P = 0.0218$).

^dNo statistically significant difference between *Msh6*^{-/-} and control mice.

^{e,g}No statistically significant difference between *Msh3*^{-/-} and control mice.

mice also had a lower frequency of mutation than *Msh6*^{+/+} and *Msh6*^{-/-} mice (Table III B). The characteristics of the individual base changes in recombined S μ and S γ 3 regions from *Msh6*^{+/+}, *Msh6*^{+/-}, and *Msh6*^{-/-} mice are tabulated in Table S4 A, available at <http://www.jem.org/cgi/content/full/jem.20040355/DC1>. To compare the mutations in the switch regions to those in the V region in the same mouse colonies at the same time, we analyzed the characteristics of SHM in the JH2-JH4 region from both *Msh3*- and *Msh6*-deficient mice.

Msh6-deficient mice had a statistically significant increase in the percentage of mutations at G/C basepairs in both rearranged S μ and S γ 3 regions compared with *Msh6*^{+/+} mice (χ^2 test, $P < 0.05$), whereas *Msh6*^{+/-} and *Msh6*^{+/+} mice did not differ (Table III, A and B). This small but statistically significant increase in mutations at G/C basepairs in the switch regions of *Msh6*^{-/-} mice is less dramatic than the increase in targeting to G/C basepairs seen in the V region previously (21) and in JH2-JH4 region in the *Msh6*^{-/-} mice used in this study (42% for the control versus 91% for *Msh6*^{-/-} mice; Table IV, A and B) (χ^2 test, $P < 0.0001$). *Msh6* deficiency also increased the percentage of transition mutations at G/C basepairs in both the S μ and S γ 3 switch regions (S μ region, 51% for *Msh6*^{+/+}, 55% for *Msh6*^{+/-}, and 80% for *Msh6*^{-/-} mice; S γ 3 region, 64% for *Msh6*^{+/+}, 62% for *Msh6*^{+/-}, and 83% for *Msh6*^{-/-} mice [Table III, A and B]). The extent of this increase in the S μ region is greater than in the V region (Table IV B), whereas it is similar in the S γ 3 region (Table IV B). In addition, *Msh6* deficiency increased the percentage of hotspot mutations in the S μ region (33% for *Msh6*^{+/+} and 67% for *Msh6*^{-/-} mice) (Table III A) and in the S γ 3 region (18% for *Msh6*^{+/+} and 42% for *Msh6*^{-/-} mice) (Table III B). This is consistent with the hotspot mutations in the JH2-JH4 region observed in *Msh6*^{-/-} mice (11% for the control versus 35% for *Msh6*^{-/-} mice) (Table IV B). Of note, *Msh6*^{+/-} mice also exhibited an increase in the percentage of hotspot mutations in the recombined S μ and S γ 3 regions compared with *Msh6*^{+/+} mice (Table III, A and B).

We performed similar analyses for *Msh3*^{-/-} mice and their littermate controls. The characteristics of the individual base changes in recombined S μ and S γ 3 regions from the control and *Msh3*^{-/-} mice are tabulated in Table S4 B. In the S μ region, the mutation frequency was 12.7×10^{-3} for the control mice and 14.7×10^{-3} for the *Msh3*^{-/-} mice (Table III C). In the S γ 3 region, the mutation frequency was 2.9×10^{-3} for the controls and 6.3×10^{-3} for the *Msh3*^{-/-} mice (Table III D). The lower rate of mutation in S γ 3 than in S μ is similar to findings in the *Msh6*^{-/-} mice. We had reported previously (21) that *Msh3* deficiency did not significantly change the frequency or the characteristics of the mutations in the V region. When we examined a larger number of mutations in this study, we found that there was a small but statistically significant decrease in mutation at G/C basepairs (30% compared with 42% [Table IV B]) (χ^2 test, $P < 0.05$). There appeared to be a decrease in transitions (58% compared with 68%) in

the JH2-JH4 regions of the *Msh3*^{-/-} mice (Table IV B). Similar small differences were observed in the characteristics of the mutations in the recombined S μ region of the *Msh3*^{-/-} mice when they were compared with their littermate controls. The transition mutation at G/C was 55% for the control mice compared with 44% of *Msh3*^{-/-} mice (Table III C). The percentage of hotspot mutations was 38% in the control mice compared with 27% in *Msh3*^{-/-} mice (Table III C). However, these differences were not observed in recombined S γ 3 region (Table III D).

Discussion

MMR plays an important role in maintaining the stability of the genome by correcting mismatched bases that arise during replication and recombination. In addition, when there is excessive DNA damage the MMR complex signals for the cell cycle arrest and apoptosis (22). MMR also prevents homeologous recombination in yeast between slightly divergent DNA sequences (50). The initial recognition of mismatched bases is performed by Msh2 in association with either Msh6 or Msh3 (45). The Msh2-Msh6 heterodimer recognizes single base mismatches and single-base insertions/deletions, whereas the Msh2-Msh3 heterodimer recognizes single-base insertions/deletions and larger mismatches (23, 45). Once a mismatch is identified, the Msh2-Msh6 or Msh2-Msh3 heterodimer recruits additional proteins in an ATP-dependent manner including the Mlh1-Pms2 heterodimer, Exo1, PCNA, and potentially other as yet unidentified factors to complete the process of mismatch repair (45). Since AID-induced mutations are required to trigger both SHM and CSR and result in G-U or G-abasic site mismatches, it is not surprising that Msh2, Msh6, Exo1, Mlh1, and Pms2 are involved in both of these processes. Deficiency in any of these proteins results in a reduced frequency of CSR. Interestingly, the switch junctions found in *Msh2*^{-/-} and those in *Mlh1*^{-/-} or *Pms2*^{-/-} mice differ (43, 51), suggesting that the function of Msh2 is different at certain stages of CSR from that of Mlh1 or Pms2. This is consistent with the fact that *Msh2*^{-/-} or *Msh6*^{-/-} mice and *Mlh1*^{-/-} or *Pms2*^{-/-} mice have different mutation spectra in SHM (for review see 19). It has been hypothesized that the Msh2-Msh6 complex affects CSR and SHM by participating in the repair of AID-generated mismatches (1, 19, 52). In addition, MMR proteins especially Msh3 could also play a role in CSR by processing the nonhomologous ends created by staggered DNA breaks (53) or other recombination structures that might form in the course of CSR. Since the switch regions contain many nonidentical repeats, large mismatches could be created during recombination that would require the participation of the Msh2-Msh3 heterodimer. Therefore, it is important to determine the precise role of Msh6 or Msh3 relative to Msh2 in CSR.

In this study, we have shown that *Msh6*^{-/-} mice exhibit a decrease in the frequency of CSR, whereas *Msh3*^{-/-} mice do not, suggesting that Msh6 is needed for efficient CSR,

whereas Msh3 is dispensable. It is notable that the extent of reduction in CSR observed in *Msh6*^{-/-} mice is similar to what has been reported in *Msh2*^{-/-} mice (20, 24, 26, 31), supporting the idea that Msh2 and Msh6 function together in CSR. Therefore, one might have expected that *Msh6*^{-/-} and *Msh2*^{-/-} mice would have had a similar pattern of switch junctions. However, after analyzing a large number of unique recombination sites from *Msh6*^{-/-}, *Msh6*^{+/-}, and *Msh6*^{+/+} mice, we were surprised to find that the frequency of blunt junctions and the distribution of various sizes of microhomologies and inserts observed from *Msh6*^{-/-} mice did not differ from littermate *Msh6*^{+/-} or *Msh6*^{+/+} mice. This is in contrast to the phenotype of *Msh2*^{-/-} mice, which have more blunt junctions, more inserts, and shorter microhomologies than wild-type mice in their switch junctions (43). The switch junction phenotype difference between *Msh6*^{-/-} mice and *Msh2*^{-/-} mice reveals that the loss of Msh2 and Msh6 has a different impact on CSR and suggests that they have different roles at certain stages of CSR. *Msh6*^{+/+} littermate mice did have some large inserts in their switch junctions which are not frequently found in wild-type littermate mice. We think that this is not due to genetic background because these mice are backcrossed to C57BL/6 for more than six generations. In addition, we do not believe that this is due to our analysis methods because the *Msh3*^{+/+} mice that were analyzed in parallel with *Msh6*^{+/+} mice do not show such inserts and resemble the wild-type littermate mice published previously (20, 30, 31, 38, 43). In addition, we also found these inserts in *Msh6*^{+/+} mice derived from a colony that was backcrossed to C57/B6 independently for >3 yr (see Materials and Methods). Although this could be due to the relatively large number of recombination sites examined in the study, the precise reasons for the large insertions in the control mice remain unexplained. This could be due to some unknown epigenetic effects, given that we used heterozygous Msh6 parents to generate the wild-type littermates.

Although the characteristics of the junctions in *Msh6*^{-/-} mice did not differ from those of littermate controls, deficiency in Msh6 did result in other differences in the recombination process. The loss of Msh6 was associated with an increase in the targeting of the recombination sites to GAGCT/GGGGT consensus repeats and to RGYW/WRCY hotspots in the Sy3 regions but not in the S μ regions. These results suggest that in the Sy3 region, but not in the S μ region, the generation and/or resolution of DNA breaks in CSR outside of these sequences is dependent on Msh6. Our data obtained from *Msh6*^{-/-} mice suggest that Msh2 and Msh6 can have different roles in targeting recombination to consensus sequences. Indeed, the increase targeting of the consensus repeats in S μ has been observed in *Msh2*^{-/-} mice (25). Moreover, mice deficient in both Msh2 and the consensus sequence in the S μ region showed a synergistic defect in CSR (26), suggesting that the generation or/and resolution of DNA breaks in CSR outside of consensus sequences is dependent on Msh2 in the S μ region (26). Interestingly, the difference observed in *Msh2*^{-/-}

and *Msh6*^{-/-} mice in the recombination sites is consistent with other functional differences between Msh2 and Msh6 in vivo. For example, *Msh2*^{-/-} mice show microsatellite instability and patients or mice with deficiency in Msh2 have a strong predisposition to early onset of colon cancer, whereas *Msh6*^{-/-} mice do not have microsatellite instability and mice with Msh6 deficiencies have a later onset of malignancies with a predominance of B cell lymphomas (45). In addition, Msh6 deficiency changed the positioning of the junction site in the S μ and Sy3 switch regions, suggesting that Msh6 plays a role in the selection of different S μ and Sy3 segments for recombination. This is likely to reflect inherent sequence or higher order DNA or chromatin structural differences in the switch region themselves. The shift in sites suggests that Msh6 either modifies initial lesions that are more common in one subregion or is required to resolve or stabilize the double stranded breaks that occur more frequently in one subregion than another.

In yeast, the Msh2-Msh3 heterodimer plays a role in removing nonhomologous ends from double stranded DNA breaks (53). In the studies reported here, Msh3 deficiency did not have any effect on the frequency of CSR, the targeting of mutation or recombination sites or on the size of microhomologies at the sites of recombination. However, there were more large inserts in the sites of recombination in the *Msh3*^{-/-} mice than in their littermate controls, a partial phenotype of Msh2 deficiency in the switch junctions. These results suggest that Msh3 is probably not needed to generate DNA breaks or to resolve the breaks in CSR. However, the Msh2-Msh3 heterodimer could play a similar role to that reported in yeast (53) in the processing of the double stranded breaks in CSR or Msh2 can function independently of Msh3 during the processing of DNA break ends.

We compared the characteristics of mutations in the recombined S μ and Sy3 switch regions from Msh6- and Msh3-deficient mice and their littermate controls. We analyzed ~1,000 base changes from various *Msh6* genotypes and 400 base changes from *Msh3* mice. All of the genotypes had many more mutations in S μ than in Sy3, consistent with the findings reported by others (32, 36, 54). Because there were more mutations in S μ than in Sy switch regions from cells that have been stimulated but have not undergone switch recombination, it has been suggested that AID-dependent mutations in the donor S μ initiate the process of CSR (32). There was no change in the frequency of mutations in either the donor S μ or recipient Sy3 switch regions in the *Msh6*^{-/-} mice compared with their *Msh6*^{+/+} littermates. This lack of effect on the frequency of mutations in the switch regions has also been observed for Msh2-deficient mice and suggests that even though MMR facilitates efficient CSR, it is not required for AID-dependent mutations in the switch region (32). There was a statistically significant lower frequency of mutation in the S μ region and Sy3 region in *Msh6*^{+/-} mice than in *Msh6*^{+/+} or *Msh6*^{-/-} mice. In addition, *Msh6*^{+/-}

mice showed an increased frequency of RGYW hotspot mutation targeting compared with *Msh6*^{+/+} mice. It is possible that haplo insufficiency of Msh6 affects the competition between MMR and other DNA repair pathways for the AID-generated mismatches, changes the balance between BER and MMR and outcome of the repair of G-U mismatches, and therefore, influences the outcome of the mutation frequency in recombined switch sequences.

We compared the characteristics of mutations in CSR and SHM from Msh6-deficient mice and their littermate controls. In *Msh6*^{-/-} mice, there was a slight increase of G/C mutations in the switch regions and a much higher increase of transition mutations at G/C and in hotspots in the recombined S μ and S γ 3 regions. When compared with the changes in the V region (JH2-JH4), the increase in targeting of G/C bases was more dramatic in the V regions, but targeting of transition mutation at G/C and hotspots was more dramatic in switch regions. These results suggest that, in CSR and SHM, Msh6 plays an overlapping and distinct role. Interestingly, the mutation spectra in the switch regions from *Msh6*^{-/-} mice are somewhat similar to those observed in SHM from *Ung*^{-/-} mice (15), suggesting that Msh6 and uracil DNA glycosylase are more closely connected in CSR than in SHM.

We had observed previously that a deficiency in Msh3 did not affect the frequency or characteristics of the AID-dependent mutations in the V region (21). When we analyzed many more base changes in V regions of *Msh3*^{-/-} mice and added them to the mutations we had examined previously (21), we noticed that there was a slight increase in the percentage of mutations at A/T bases. This is the opposite of what was seen in Msh2- and Msh6-deficient mice and could be due to the competition between Msh6 and Msh3 for Msh2, i.e., loss of Msh3 increases the chance for the formation of Msh2-Msh6 complex, which ultimately increases the percentage of A/T mutations. Interestingly, deficiency in ATM also results in an increase in the percent of A/T mutations in CSR (55). Error-prone polymerases may also participate in the mutational process in both the V and switch regions (1), and a deficiency in polymerase η results in fewer mutations at A/T in the V region and in the switch regions (16, 17, 56). Although pol iota has also been implicated in V region mutation in BL2 cells (57), this does not seem to be true in mice (32, 58). Together, all of these findings suggest that the competitive interactions of many enzymes determine the nature of the mutations that trigger CSR and the ultimate structure of the recombination sites.

In conclusion, we have shown that efficient CSR requires Msh6 but not Msh3, that *Msh3*^{-/-} mice and *Msh6*^{-/-} mice exhibit different switch junctions from those of *Msh2*^{-/-} mice, and that similar but different mutation spectra were observed between switch region and V region mutations from *Msh6*^{-/-} mice and *Msh3*^{-/-} mice. Our results support the idea that Msh2 and Msh6 function together at the initial step of generating breaks, Msh2 then functions together with Msh3 and/or Msh2 functions inde-

pendently in the processing of DNA break ends, whereas Msh6 is not involved in this process, and Msh6 may then function in the resolution of DNA breaks. Therefore, we favor the idea that the MutS homologues, Msh2, Msh3, and Msh6 have overlapping and distinct roles in the process of SHM and CSR.

We would like to thank M. Fan for technical help.

This work was supported by grants from the National Institutes of Health to W. Edelmann (CA76329 and CA93484) and to M.D. Scharff (CA 72649, CA102705, and AI 43937) who is also supported by the Harry Eagle Chair provided by the National Women's Division of the Albert Einstein College of Medicine. S.J. Scherer is supported by Fellowship of Deutsche Krebshilfe. D. Ronai is supported by Cancer Research Institute Postdoctoral Fellowship and Harry Eagle Fellowship. M.D. Iglesias-Ussel is a fellow of the Ministerio de Educacion, Cultura y Deporte (Spain). A. Martin is a special fellow of the Leukemia and Lymphoma Society. Z. Li is supported by Cancer Research Institute Postdoctoral Fellowship.

Submitted: 23 February 2004

Accepted: 17 May 2004

References

1. Martin, A., and M.D. Scharff. 2002. AID and mismatch repair in antibody diversification. *Nat. Rev. Immunol.* 2:605–614.
2. Stavnezer, J. 2000. Molecular processes that regulate class switching. *Curr. Top. Microbiol. Immunol.* 245:127–168.
3. Muramatsu, M., V.S. Sankaranand, S. Anant, M. Sugai, K. Kinoshita, N.O. Davidson, and T. Honjo. 1999. Specific expression of activation-induced cytidine deaminase (AID), a novel member of the RNA-editing deaminase family in germinal center B cells. *J. Biol. Chem.* 274:18470–18476.
4. 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.
5. Revy, P., T. Muto, Y. Levy, F. Geissmann, A. Plebani, O. Sanal, N. Catalan, M. Forveille, R. Dufourcq-Labelouse, A. Gennery, et al. 2000. Activation-induced cytidine deaminase (AID) deficiency causes the autosomal recessive form of the Hyper-IgM syndrome (HIGM2). *Cell.* 102:565–575.
6. Bransteitter, R., P. Pham, M.D. Scharff, and M.F. Goodman. 2003. Activation-induced cytidine deaminase deaminates deoxycytidine on single-stranded DNA but requires the action of RNase. *Proc. Natl. Acad. Sci. USA.* 100:4102–4107.
7. Chaudhuri, J., M. Tian, C. Khuong, K. Chua, E. Pinaud, and F.W. Alt. 2003. Transcription-targeted DNA deamination by the AID antibody diversification enzyme. *Nature.* 422:726–730.
8. Dickerson, S.K., E. Market, E. Besmer, and F.N. Papavasiliou. 2003. AID mediates hypermutation by deaminating single stranded DNA. *J. Exp. Med.* 197:1291–1296.
9. Sohail, A., J. Klapacz, M. Samaranyake, A. Ullah, and A.S. Bhagwat. 2003. Human activation-induced cytidine deaminase causes transcription-dependent, strand-biased C to U deaminations. *Nucleic Acids Res.* 31:2990–2994.
10. Pham, P., R. Bransteitter, J. Petruska, and M.F. Goodman. 2003. Processive AID-catalyzed cytosine deamination on single-stranded DNA simulates somatic hypermutation. *Nature.* 424:103–107.

11. Ramiro, A.R., P. Stavropoulos, M. Jankovic, and M.C. Nussenzweig. 2003. Transcription enhances AID-mediated cytidine deamination by exposing single-stranded DNA on the nontemplate strand. *Nat. Immunol.* 4:452–456.
12. Rogozin, I.B., and N.A. Kolchanov. 1992. Somatic hypermutagenesis in immunoglobulin genes. II. Influence of neighbouring base sequences on mutagenesis. *Biochim. Biophys. Acta.* 1171:11–18.
13. Di Noia, J., and M.S. Neuberger. 2002. Altering the pathway of immunoglobulin hypermutation by inhibiting uracil-DNA glycosylase. *Nature.* 419:43–48.
14. Petersen-Mahrt, S.K., R.S. Harris, and M.S. Neuberger. 2002. AID mutates *E. coli* suggesting a DNA deamination mechanism for antibody diversification. *Nature.* 418:99–103.
15. 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.
16. Zeng, X., D.B. Winter, C. Kasper, K.H. Kraemer, A.R. Lehmann, and P.J. Gearhart. 2001. DNA polymerase eta is an A-T mutator in somatic hypermutation of immunoglobulin variable genes. *Nat. Immunol.* 2:537–541.
17. Fails, A., S. Aoufouchi, S. Weller, F. Vuillier, A. Sary, A. Sarasin, C.A. Reynaud, and J.C. Weill. 2004. DNA polymerase {eta} is involved in hypermutation occurring during immunoglobulin class switch recombination. *J. Exp. Med.* 199:265–270.
18. Dunnick, W., M. Wilson, and J. Stavnezer. 1989. Mutations, duplication, and deletion of recombined switch regions suggest a role for DNA replication in the immunoglobulin heavy-chain switch. *Mol. Cell. Biol.* 9:1850–1856.
19. Li, Z., C.J. Woo, M.D. Iglesias-Ussel, D. Ronai, and M.D. Scharff. 2004. The generation of antibody diversity through somatic hypermutation and class switch recombination. *Genes Dev.* 18:1–11.
20. Martin, A., Z. Li, D. Lin, P.D. Bardwell, M.D. Iglesias-Ussel, W. Edelmann, and M.D. Scharff. 2003. Msh2 ATPase activity is essential for somatic hypermutation at A-T base-pairs and for efficient class switch recombination. *J. Exp. Med.* 198:1171–1178.
21. Wiesendanger, M., B. Kneitz, W. Edelmann, and M.D. Scharff. 2000. Somatic mutation in MSH3, MSH6, 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.
22. Kolodner, R. 1996. Biochemistry and genetics of eukaryotic mismatch repair. *Genes Dev.* 10:1433–1442.
23. Kolodner, R.D., and G.T. Marsischky. 1999. Eukaryotic DNA mismatch repair. *Curr. Opin. Genet. Dev.* 9:89–96.
24. Schrader, C.E., W. Edelmann, R. Kucherlapati, and J. Stavnezer. 1999. Reduced isotype switching in splenic B cells from mice deficient in mismatch repair enzymes. *J. Exp. Med.* 190:323–330.
25. Ehrenstein, M.R., and M.S. Neuberger. 1999. Deficiency in msh2 affects the efficiency and local sequence specificity of immunoglobulin class-switch recombination: parallels with somatic hypermutation. *EMBO J.* 18:3484–3490.
26. Min, I.M., C.E. Schrader, J. Vardo, T.M. Luby, N. D'Avirro, J. Stavnezer, and E. Selsing. 2003. The Smu tandem repeat region is critical for Ig isotype switching in the absence of Msh2. *Immunity.* 19:515–524.
27. Reynaud, C.A., B. Bertocci, S. Frey, F. Delbos, L. Quint, and J.C. Weill. 1999. Mismatch repair and immunoglobulin gene hypermutation: did we learn something? *Immunol. Today.* 20:522–527.
28. Szankasi, P., and G.R. Smith. 1995. A role for exonuclease I from *S. pombe* in mutation avoidance and mismatch correction. *Science.* 267:1166–1169.
29. Genschel, J., L.R. Bazemore, and P. Modrich. 2002. Human exonuclease I is required for 5' and 3' mismatch repair. *J. Biol. Chem.* 277:13302–13311.
30. Bardwell, P.D., C.J. Woo, K. Wei, W. Edelmann, and M.D. Scharff. 2004. The alteration of somatic hypermutation and immunoglobulin class switching in exonuclease 1 (Exo1) deficient mice. *Nat. Immunol.* 5:224–229.
31. Schrader, C.E., J. Vardo, and J. Stavnezer. 2003. Mlh1 can function in antibody class switch recombination independently of Msh2. *J. Exp. Med.* 197:1377–1383.
32. Schrader, C.E., S.P. Bradley, J. Vardo, S.N. Mochegova, E. Flanagan, and J. Stavnezer. 2003. Mutations occur in the Ig Smu region but rarely in Sgamma regions prior to class switch recombination. *EMBO J.* 22:5893–5903.
33. Manis, J.P., Y. Gu, R. Lansford, E. Sonoda, R. Ferrini, L. Davidson, K. Rajewsky, and F.W. Alt. 1998. Ku70 is required for late B cell development and immunoglobulin heavy chain class switching. *J. Exp. Med.* 187:2081–2089.
34. Casellas, R., A. Nussenzweig, R. Wuerffel, R. Pelanda, A. Reichlin, H. Suh, X.F. Qin, E. Besmer, A. Kenter, K. Rajewsky, and M.C. Nussenzweig. 1998. Ku80 is required for immunoglobulin isotype switching. *EMBO J.* 17:2404–2411.
35. Cook, A.J., L. Oganessian, P. Harumal, A. Basten, R. Brink, and C.J. Jolly. 2003. Reduced switching in SCID B cells is associated with altered somatic mutation of recombined S regions. *J. Immunol.* 171:6556–6564.
36. Reina-San-Martin, B., S. Difilippantonio, L. Hanitsch, R.F. Masilamani, A. Nussenzweig, and M.C. Nussenzweig. 2003. H2AX is required for recombination between immunoglobulin switch regions but not for intra-switch region recombination or somatic hypermutation. *J. Exp. Med.* 197:1767–1778.
37. Pan, Q., C. Petit-Frere, A. Lahdesmaki, H. Gregorek, K.H. Chrzanowska, and L. Hammarstrom. 2002. Alternative end joining during switch recombination in patients with ataxia-telangiectasia. *Eur. J. Immunol.* 32:1300–1308.
38. Manis, J.P., J.C. Morales, Z. Xia, J.L. Kutok, F.W. Alt, and P.B. Carpenter. 2004. 53BP1 links DNA damage-response pathways to immunoglobulin heavy chain class-switch recombination. *Nat. Immunol.* 5:481–487.
39. Mostoslavsky, R., F.W. Alt, and C.H. Bassing. 2003. Chromatin dynamics and locus accessibility in the immune system. *Nat. Immunol.* 4:603–606.
40. Nambu, Y., M. Sugai, H. Gonda, C.G. Lee, T. Katakai, Y. Agata, Y. Yokota, and A. Shimizu. 2003. Transcription-coupled events associating with immunoglobulin switch region chromatin. *Science.* 302:2137–2140.
41. Shinkura, R., M. Tian, M. Smith, K. Chua, Y. Fujiwara, and F.W. Alt. 2003. The influence of transcriptional orientation on endogenous switch region function. *Nat. Immunol.* 4:435–441.
42. Yu, K., F. Chedin, C.L. Hsieh, T.E. Wilson, and M.R. Lieber. 2003. R-loops at immunoglobulin class switch regions in the chromosomes of stimulated B cells. *Nat. Immunol.* 4:442–451.
43. Schrader, C.E., J. Vardo, and J. Stavnezer. 2002. Role for mismatch repair proteins Msh2, Mlh1, and Pms2 in immunoglobulin class switching shown by sequence analysis of recombination junctions. *J. Exp. Med.* 195:367–373.
44. Chen, X., K. Kinoshita, and T. Honjo. 2001. Variable deletion and duplication at recombination junction ends: implication for staggered double-strand cleavage in class-switch re-

- combination. *Proc. Natl. Acad. Sci. USA*. 98:13860–13865.
45. Wei, K., R. Kucherlapati, and W. Edelmann. 2002. Mouse models for human DNA mismatch-repair gene defects. *Trends Mol. Med.* 8:346–353.
 46. Edelmann, W., K. Yang, A. Umar, J. Heyer, K. Lau, K. Fan, W. Liedtke, P.E. Cohen, M.F. Kane, J.R. Lipford, et al. 1997. Mutation in the mismatch repair gene Msh6 causes cancer susceptibility. *Cell*. 91:467–477.
 47. Edelmann, W., A. Umar, K. Yang, J. Heyer, M. Kucherlapati, M. Lia, B. Kneitz, E. Avdievich, K. Fan, E. Wong, et al. 2000. The DNA mismatch repair genes Msh3 and Msh6 cooperate in intestinal tumor suppression. *Cancer Res.* 60:803–807.
 48. Bardwell, P.D., A. Martin, E. Wong, Z. Li, W. Edelmann, and M.D. Scharff. 2003. Cutting edge: The G-U mismatch glycosylase methyl-CpG binding domain 4 is dispensable for somatic hypermutation and class switch recombination. *J. Immunol.* 170:1620–1624.
 49. Evans, E., and E. Alani. 2000. Roles for mismatch repair factors in regulating genetic recombination. *Mol. Cell. Biol.* 20:7839–7844.
 50. Nicholson, A., M. Hendrix, S. Jinks-Robertson, and G.F. Crouse. 2000. Regulation of mitotic homeologous recombination in yeast. Functions of mismatch repair and nucleotide excision repair genes. *Genetics*. 154:133–146.
 51. Ehrenstein, M.R., C. Rada, A.M. Jones, C. Milstein, and M.S. Neuberger. 2001. Switch junction sequences in PMS2-deficient mice reveal a microhomology-mediated mechanism of Ig class switch recombination. *Proc. Natl. Acad. Sci. USA*. 98:14553–14558.
 52. Storb, U., and J. Stavnezer. 2002. Immunoglobulin genes: generating diversity with AID and UNG. *Curr. Biol.* 12:R725–R727.
 53. Sugawara, N., F. Paques, M. Colaiacovo, and J.E. Haber. 1997. Role of *Saccharomyces cerevisiae* Msh2 and Msh3 repair proteins in double-strand break-induced recombination. *Proc. Natl. Acad. Sci. USA*. 94:9214–9219.
 54. Nagaoka, H., M. Muramatsu, N. Yamamura, K. Kinoshita, and T. Honjo. 2002. Activation-induced deaminase (AID)-directed hypermutation in the immunoglobulin Smu region: implication of AID involvement in a common step of class switch recombination and somatic hypermutation. *J. Exp. Med.* 195:529–534.
 55. Pan-Hammarstrom, Q., S. Dai, Y. Zhao, I.F. van Dijk-Hard, R.A. Gatti, A.L. Borresen-Dale, and L. Hammarstrom. 2003. ATM is not required in somatic hypermutation of VH, but is involved in the introduction of mutations in the switch mu region. *J. Immunol.* 170:3707–3716.
 56. Zeng, X., G.A. Negrete, C. Kasmer, W.W. Yang, and P.J. Gearhart. 2004. Absence of DNA polymerase {eta} reveals targeting of C mutations on the nontranscribed strand in immunoglobulin switch regions. *J. Exp. Med.* 199:917–924.
 57. Faili, A., S. Aoufouchi, E. Flatter, Q. Gueranger, C.A. Reynaud, and J.C. Weill. 2002. Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase iota. *Nature*. 419:944–947.
 58. McDonald, J.P., E.G. Frank, B.S. Plosky, I.B. Rogozin, C. Masutani, F. Hanaoka, R. Woodgate, and P.J. Gearhart. 2003. 129-derived strains of mice are deficient in DNA polymerase iota and have normal immunoglobulin hypermutation. *J. Exp. Med.* 198:635–643.