Brief Definitive Report

Role for Mismatch Repair Proteins Msh2, Mlh1, and Pms2 in Immunoglobulin Class Switching Shown by Sequence Analysis of Recombination Junctions

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Abstract

B cells from mice deficient in mismatch repair (MMR) proteins show decreased ability to undergo class switch recombination in vitro and in vivo. The deficit is not accompanied by any reduction in cell viability or alterations in the cell cycle in B cells cultured in vitro. To assess the role of MMR in switching we examined the nucleotide sequences of Sμ–Sγ3 recombination junctions in splenic B cells induced in culture to switch to IgG3. The data demonstrate clear differences in the sequences of switch junctions in wild-type B cells in comparison with Msh2-, Mlh1-, and Pms2-deficient B cells. Sequences of switch junctions from Msh2-deficient cells showed decreased lengths of microhomology between Sμ and Sγ3 relative to junctions from wild-type cells and an increase in insertions, i.e., nucleotides which do not appear to be derived from either the Sμ or Sγ3 parental sequence. By contrast, 23% of junctions from Mlh1- and Pms2-deficient cells occurred at unusually long stretches of microhomology. The data indicate that MMR proteins are directly involved in class switching and that the role of Msh2 differs from that of Mlh1 and Pms2.

Key words: splenic B cells • DNA recombination • DNA repair • antibody heavy chain isotypes • mismatch repair

Introduction

Upon activation, B cells expressing IgM and IgD undergo Ig isotype (class) switching to express IgG, IgE, or IgA. Class switching occurs by a DNA recombination event that results in exchanging the constant region of the Ig heavy chain, without changing the antibody variable region. This process changes the effector functions of the antibody but does not affect antigen-binding specificity. Class switch recombination (CSR) occurs by an intrachromosomal deletional recombination between switch (S) sequences located upstream of the constant region genes (for a review, see reference 1).

S sequences consist of tandem repeats of short (20–80 bp) consensus elements, extending from 1 to 10 kb in length, and CSR can occur at any site within the S regions (2). Although the S regions have short elements in common, e.g., GGGGT or GAGCT, the S regions of different heavy chain genes (isotypes) differ too much to undergo homologous recombination. Instead, CSR is thought to occur by a type of nonhomologous end joining (NHEJ, reference 3). Ku70, Ku80, and DNA-PK, proteins known to be important for NHEJ, are essential for normal CSR (4–6). This hypothesis is supported by the fact that one often observes short bits of microhomology at the S-S junctions, which is typical of NHEJ. However, whether these microhomologies play a role in the recombination is unknown as their presence may simply be due to shared sequence elements among S regions (2).

An interesting feature of S recombination junctions is the presence of nucleotide substitutions, deletions and insertions, which has led to the proposal that CSR occurs by a process involving error-prone DNA synthesis (2, 7). The mutations appear quite similar to those due to somatic hypermutation of antibody variable regions, and these two processes have many other similarities. Both CSR and somatic hypermutation occur during antigen activation of B cells and require transcription; both appear to be initiated by double-strand breaks (8–10), and both require activation-induced deaminase (11).

Recently, mismatch repair (MMR) proteins have been shown to be involved in both CSR and somatic hypermutation. MMR proteins in eukaryotes fall into two classes: (i)

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the MutS homologs (Msh1–6) which recognize DNA mismatches, loops, and other distortions, and (ii) the MutL homologs (Pms1, Pms2, and Mlh1 in mammals) which bind to MutS homologs bound to DNA (for a review, see reference 12). It is well established that MMR proteins have additional roles besides the correction of nucleotide substitutions and small insertions or deletions created by DNA synthesis errors (12). Msh2, Msh6, Mlh1, and Pms2 are involved in, but not required for, somatic hypermutation (13–16). In the absence of these proteins, the frequency of somatic hypermutation is decreased. In addition, some MMR proteins have roles in homologous DNA recombination. MMR proteins have been shown to prevent recombination between homeologous sequences (sequences that are homologous, but not identical) (for a review, see reference 17). Msh 2 and 6 have been shown to bind to Holliday junctions (18) and Mlh1 and Pms2 are found bound to chromosomes undergoing meiosis in spermatogonia. In addition, Mlh1 mice and male Pms2 mice are sterile (19, 20). DSB repair in yeast requires removal of nonhomologous DNA segments adjacent to the break before the break can be repaired. Msh2 and Msh3 are required for this end-processing if 30 nts or more of such heterologous sequences are present, and their role is to recruit an endonuclease complex (Rad1/XPF and Rad 10/ERCC1) to excise the heterologous 3′ single-strand tail (21, 22).

By testing the ability of splenic B cells from mice deficient in three MMR proteins, Msh2, Mlh1 and Pms2, to undergo CSR in culture, we have previously shown that MMR proteins are required for optimal switching in these cultures, although they are not essential (23). Depending on the particular isotype, switch recombination is reduced by two to fourfold. MMR-deficient B cells proliferate as well as wild-type B cells and are no more susceptible to apoptosis than wild-type B cells in these cultures. Experiments in which the effect of Msh2 deficiency was examined during in vivo immune responses also showed a deficit in class switching, and the deficit was somewhat greater using this approach (24, 25).

To begin to determine the role of MMR in switch recombination, we examined the Sμ-Sγ3 junctions in B cells induced to switch to IgG3 in culture. We have compared the junctions obtained from Msh2-, Mlh1- and Pms2-deficient mice with junctions obtained from wild-type littermates. Our results demonstrate that all three MMR proteins are involved in CSR, but that Msh2 appears to be involved at a different step from Mlh1 and Pms2. Msh2, but not Mlh1 and Pms2, may be involved in processing the ends after DSB formation, while Mlh1 and Pms2 may be involved in stabilizing the recombination complex before DNA ligation.

Materials and Methods

Mice. Mice made deficient in Pms2 or Mlh1 by gene targeting were obtained from R.M. Liskay, Oregon Health Sciences University, Portland, OR (19, 20). Msh2-deficient mice were obtained from W. Edelmann and R. Kucherlapati, Albert Einstein College of Medicine, Bronx, NY (26). Mouse strains were carried as heterozygotes and wild-type littermates were used as controls. The background strains are 129 and C57Bl/6.

B Cell Isolation and Cultures. B cells were isolated from spleens by depletion of RBCs by lysis in Gey's solution for 5 min on ice and by depletion of T cells with a cocktail of anti-T cell reagents, anti-CD4 (GK1.5), anti-CD8 (3.168), and anti-Thy1 (HO13.4 and J1J10), followed by anti-rat κ-chain mAb (MAR18.5) and guinea pig complement (Pelfreeze Biochem). Viable cells were isolated by flotation on Ficoll/Hypaque gradients ($\delta = 1.09$). 10^6 B cells were cultured at 2×10^5 cells per milliliter in 6-well plates for 4 d in RPMI 1640 (BioWhittaker), with 10% FCS (Hyclone), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO BRL), and 1X MEM nonessential amino acid solution, 1 mM sodium pyruvate, and 5×10^{-5} M 2-mercaptoethanol (all from Sigma-Aldrich). LPS (50 μg/ml, *Escherichia coli* 055:B5; Sigma-Aldrich) was added at the initiation of culture.

PCR Amplification of Sμ-Sγ3 Junctions and Germline Sμ and Sγ3 Segments. Genomic DNA was isolated from B cells, either resting or cultured with LPS for 4 d. Cell pellets were incubated with proteinase K (0.5 mg/ml), RNaseA (100 μg/ml), and SDS (0.5%) in STE (0.1 M NaCl, 20 mM Tris, 1 mM EDTA) for 2 h at 37°C, followed by 3–4 extractions with phenol/chloroform (1:1) and precipitation with 0.3 M sodium acetate, pH 7, and ethanol. DNA was wound out on glass rods and resuspended in TE, pH 8. The germline Sγ3 segment was amplified by PCR from resting purified B cells from WT(129 × B6) mice for comparison to Sμ-Sγ3 junctions from cells induced to switch to IgG3. Expand HiFidelity Taq polymerase (Roche Laboratories) was used with the following primers: g3–1 (5′CAGGCTAAGATGGATGCTACAGGG-3′) (MUSIGHANA 404–427) and g3–2 (5′TACCCTGACCCAGGAGCTGCATAAC-3′) (MUSIGHANA 2603–2628) to amplify the 2.22-kb fragment of germline Sγ3. Sμ-Sγ3 junctions were amplified by PCR using Expand Long Template Taq polymerase (Roche Laboratories) and the primers μ3-H3 (5′AACAAGCTTGGCTTAACCGAGATGAGCC-3′) and g3–2 (above). The germline Sμ sequence was deduced by comparing the sequences of a large number of Sμ-Sγ3 junctions from wild-type mice. For the sequence analyses, the wild-type sequences from the corresponding littermates were used.

Cloning, Identification, and Sequence Analysis of PCR Products. PCR products were cloned into the vector pGEM®-T Easy (Promega) using blue/white screening for inserts. DNA was isolated from white colonies using QIAprep spin miniprep kit (QIAGEN). Inserts of the proper size for germline Sμ and Sγ3 segments were sequenced and compared with the corresponding germline 129 × B6 or BALB/c sequences. Clones containing Sμ-Sγ3 junction inserts of varying sizes were chosen to optimize the identification of unique junctions. For Msh2 clones, colonies containing inserts were identified by colony hybridization using the germline BALB/c Sμ 1.8-kb HindIII fragment. Sequence analysis of the cloned inserts was performed, using standard T7 and SP6 primers, by the UMass Nucleic Acid Facility using an ABI 377 DNA sequencer and Big Dyes. Sequences were aligned using the Clustal program of MacVector 6.5.3. Alignments were generally obvious, although in a few cases more than one alignment was possible due to the repetitious nature of the repeats and occasional internal deletions. In these cases, alignments were optimized to reduce nucleotide differences between the germline and recombinant sequences. The sequences which had to be aligned by minimiz-

ing mutations are WT28 (Sγ3), WT44 (Sμ), Msh195 (Sγ3), and Mlh124 (Sμ).

Results

To obtain Sμ-Sγ3 junctions for nucleotide sequence analysis, splenic B cells from wild-type or MMR-deficient mice were cultured with LPS to induce switching to IgG3 (23). On day 4, genomic DNA was isolated and Sμ-Sγ3 junctions were amplified by PCR using a primer located at the 5′ end of Sμ and a primer located at the 3′ end of Sγ3. PCR products were cloned and plasmids containing inserts of various sizes were chosen for sequence analysis. The nucleotide sequences of the junctions obtained from wild-type littermates of Mlh1- and Pms2-deficient mice are shown in Fig. 1. The upper sequence in each set is the corresponding unrearranged, or germline, Sμ sequence (labeled 129 × B6 Sm), or if not available for the particular junction shown, then from the BALB/c Sμ sequence (GenBank locus MUSIGCD09). The third sequence of each set is the unrearranged Sγ3 sequence from IgM$^+$ cells of wild-type littermates (labeled 129 × B6 Sg3), or if not available, the BALB/c Sγ3 sequence (GenBank locus MUSIGHANA). The middle sequence shows the segment surrounding the Sμ-Sγ3 junction, with the junction either marked as a vertical line (if there is no microhomology at the junction) or enclosed with a box to indicate nucleotides that may have been derived from either the Sμ or Sγ3 segments, i.e., the microhomology at the junction. One junction appears to have a short insert that does not correspond to either parental sequence (WT3–16), although alternatively, it could have two mutated nucleotides adjacent to a junction with 0 nucleotides of microhomology. As shown in Fig. 1, the sequences from wild-type B cells generally show 0 to 4 nucleotides of identity at the Sμ-Sγ3 junctions, although one sequence (WT1–87) has 7 nucleotides of identity.

Although the recombinant S junctions have nucleotide substitutions and small deletions or insertions typical of switch recombination junctions, these clones are PCR prod-

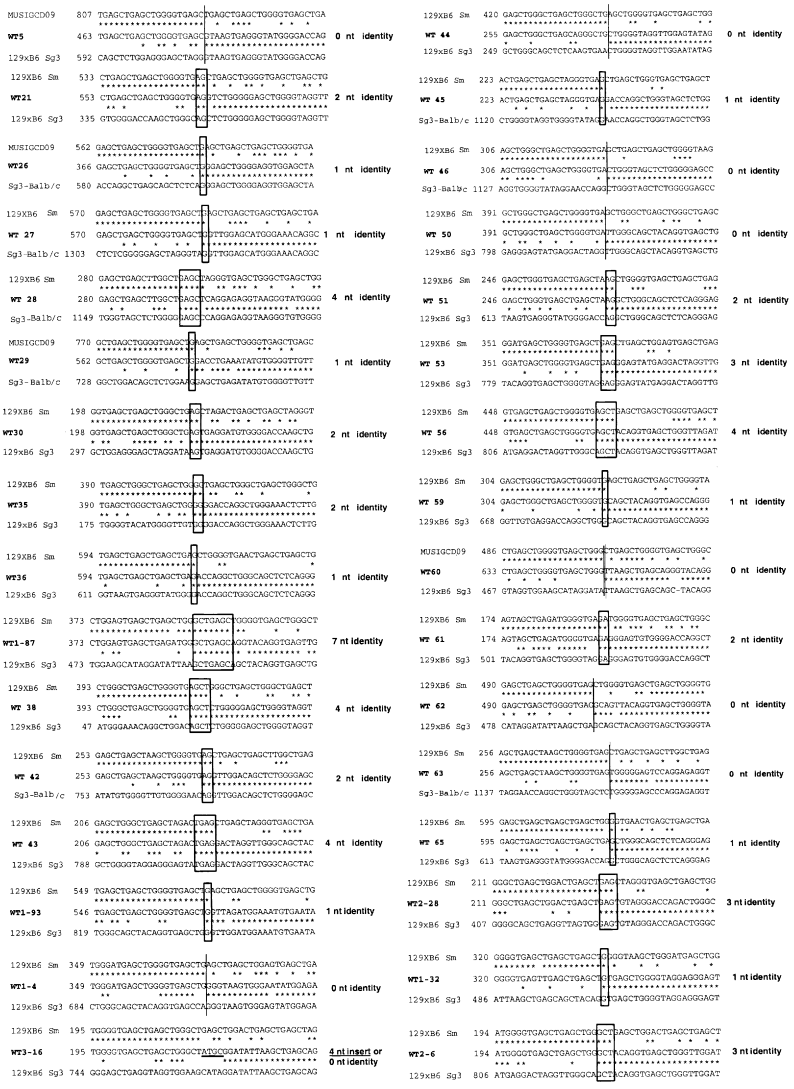

Figure 1. Nucleotide sequences surrounding Sμ-Sγ3 junctions from wild-type mice. PCR products obtained from 12 individual cultures from two mice were cloned and sequenced. DNA from each culture was amplified individually. The sequences are aligned with the Sμ sequence cloned from these mice, numbered from the first nucleotide of the segments cloned in pGEM®-Teasy (GenBank/EMBL/DDBJ AF446347; nt 1 = nt 5206 in MUSIGCD07) and with the sequenced region of the 129 × B6 Sγ3 segment. If the 129 × B6 sequence has not been determined in the region at the junction, the sequence is aligned with the BALB/c Sμ sequence MUSIGCD09 (numbering from nt 1), which is located 3′ to MUSIGCD07, or the Sγ3 sequence, MUSIGHANA (numbering from nt 404 in MUSIGHANA). The Sγ3 consensus tandem repeats begin at nt 195 and terminate at nt 2203 (repeat 42) in the BALB/c Sγ3 sequence. The BALB/c MUSIGCD09 and 129 × B6 Sμ sequenced region (760 nts) do not overlap, whereas nucleotide position 1245 in the BALB/c 2.2-kb Sγ3 sequence corresponds to nt position 1 in the 129 × B6 sequence. There are a few nt differences between the 129 × B6 and BALB/c sequences. To the right of each sequence is indicated the number of nucleotides (nts) of this identity at the junction. Underlined nts at the junction do not appear to be derived from either Sμ or Sγ3 and may represent nts inserted during recombination. Differences between the recombinant sequence and the parental sequence may be due to mutations introduced during CSR (2, 7) or to PCR errors.

ucts so it is not clear if all of the mutations were introduced during switching. Furthermore, we did not observe any clear differences in mutation frequency among the sequences obtained from WT or the three MMR-deficient B cells.

Sμ-Sγ3 junctions obtained from Msh2-deficient B cells are shown in Fig. 2. These sequences show shorter elements of microhomology at the junctions than WT ($P = 0.004$), usually 1 or 2 nts of identity, ranging up to 3 nts at most. In addition, 19% (6 of 32) have short inserts or nucleotide mutations at the junctions. Although WT junctions also have inserts (Fig. 1, and reference 2), the frequency of inserts in Msh2$^{-/-}$ junctions was significantly higher than the WT frequency ($P = 0.002$).

The Sμ-Sγ3 junctions obtained from Mlh1-deficient B cells, shown in Fig. 3, differ from the sequences of both Msh2$^{-/-}$ and of wild-type B cells. Although 78% of these sequences have junctional microhomologies of 4 nts or less, similar to wild-type junctions, 22% of them show ≥5 nts of microhomology at the S junctions, extending up to 14 nts of identity. The junctions from Pms2-deficient B cells have the same feature, with 24% showing junctional microhomology of 5 or more nts, extending up to 11 nts (Fig. 4). Table I presents a summary of the microhomology analyses. These data suggest that Mlh1 and Pms2 are also involved in CSR, but that their role differs from the role of Msh2.

Discussion

The finding that the sequences of Sμ-Sγ3 junctions differ between Msh2-deficient and wild-type B cells suggests that Msh2 is involved in the recombination process itself. One attractive possibility is that Msh2 is involved in DNA end-processing, similar to its role in DSB repair in yeast. In this model, single-strand DNA ends produced after DSB formation and during the alignment of the donor and acceptor S regions would be clipped off by an endonuclease recruited by Msh2 (presumably as a heterodimer with either Msh3 or Msh6). In yeast DSB repair, the recruited endonuclease is a complex of Rad 1 and 10 (homologs of mammalian XPF and ERCC1). We have previously described this model (Fig. 5 in reference 23). In the absence of Msh2, lack of this type of end-processing might

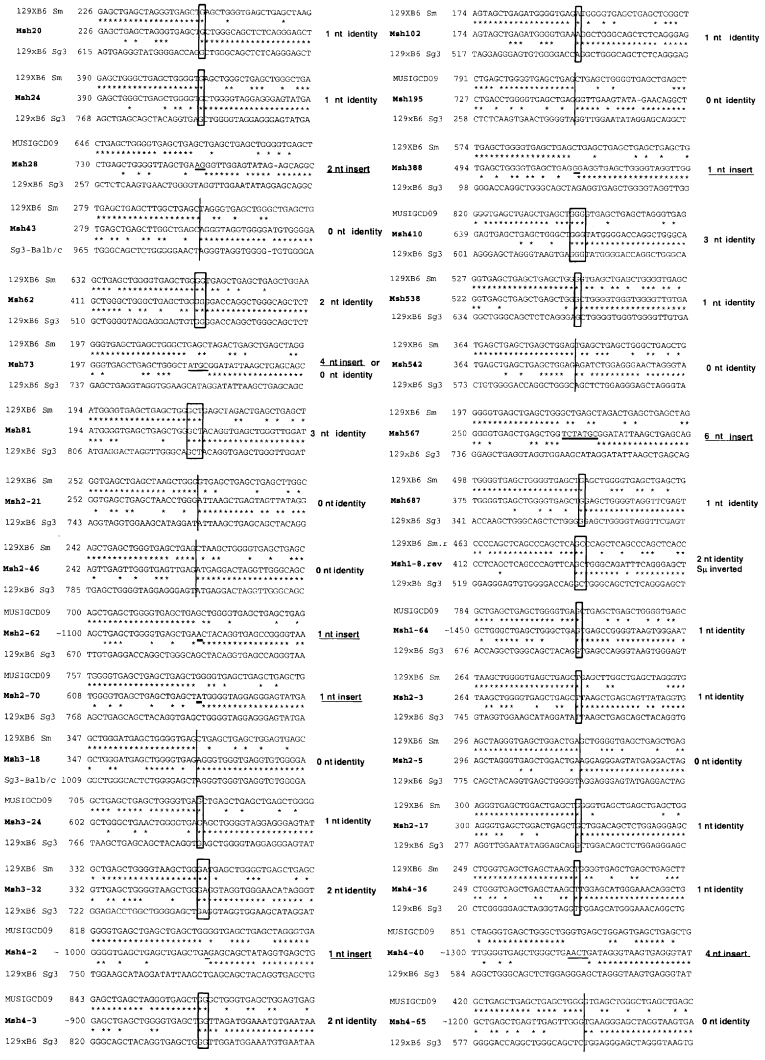

Figure 2. Nucleotide sequences surrounding Sμ-Sγ3 junctions from Msh2-deficient mice. Methods and notation identical to Fig.1. Sequences were obtained from 21 cultures of B cells from 5 mice.

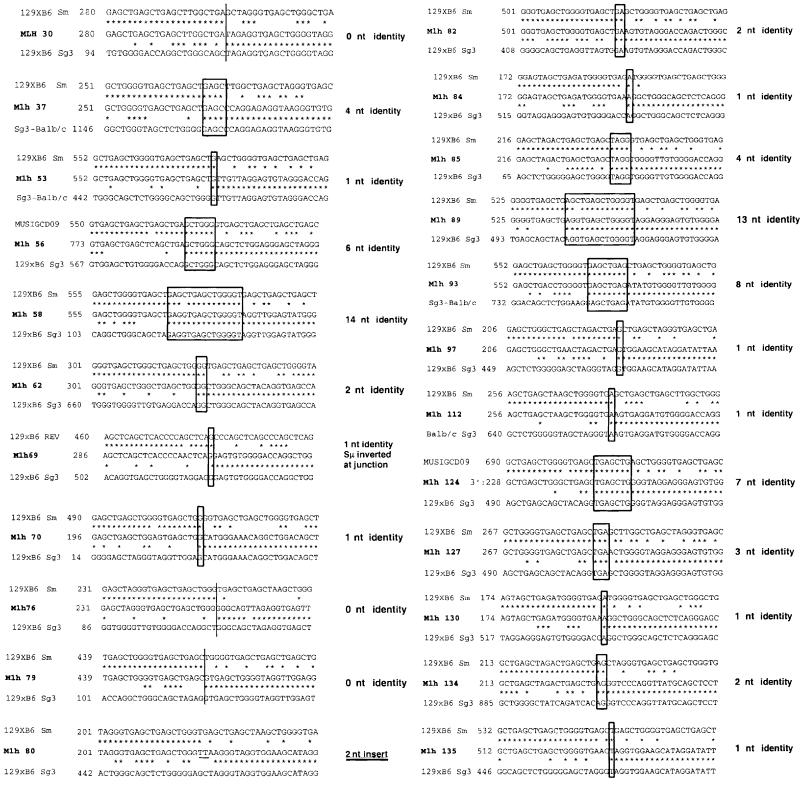

Figure 3. Sμ-Sγ3 junctions from Mlh1-deficient B cells. Note that the boxes include single nts that are not identical with both the Sμ and Sγ3 sequences which are included only if they are preceded by 2 or more identical nts. The numbers of identical nts indicated to the right of each sequence do not count these non-identical nts within the boxed regions. In the Mlh 69 sequence, the Sμ segment adjacent to Sγ3 had undergone inversion. This has been observed previously (reference 2). Sequences were obtained from 5 cultures from 2 mice.

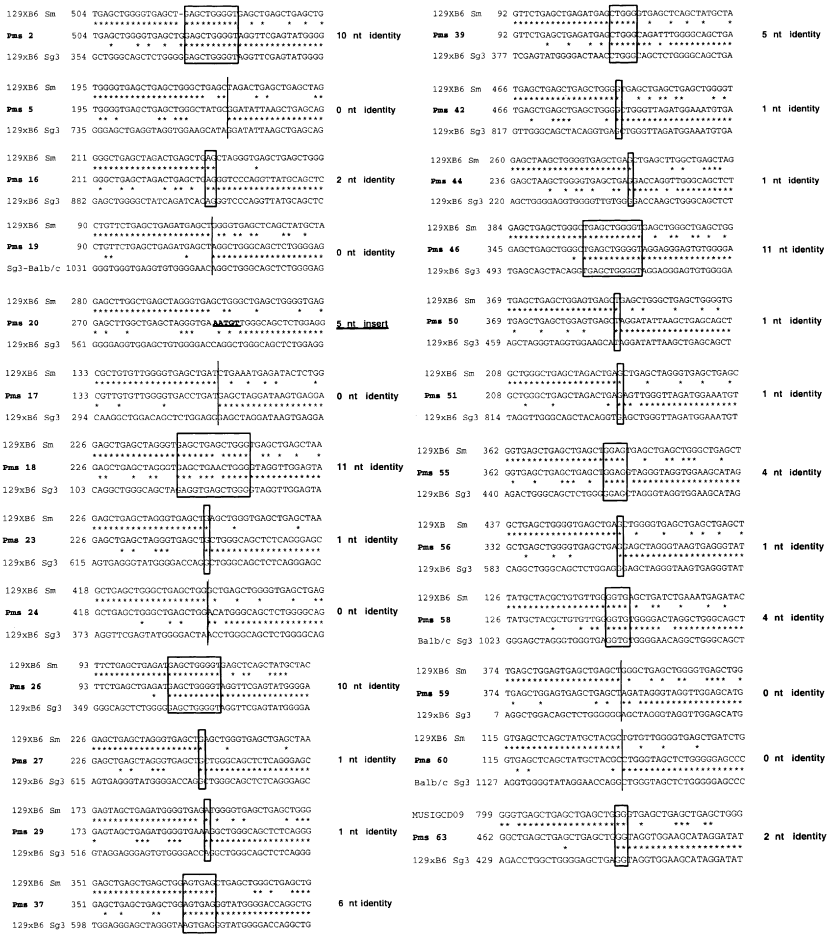

Figure 4. Sμ-Sγ3 junctions from Pms2-deficient B cells. Sequences were obtained from 4 cultures from 2 mice.

Table I. *Lengths of Microhomologies at Sμ-Sγ3 Junctions in MMR-deficient Cells Differ from Junctions in Wild-Type Cells*

	Percentage of junctions with indicated length of microhomology					
Mouse	≥2 bp	≥5 bp	≥8 bp	≥10 bp	*P* value[a]	Nbr of sequences
	%					
Wild-type	44	3	0	0		32
Mlh1$^{-/-}$	41	22	13	9	0.035	23
Pms2$^{-/-}$	40	24	16	16	0.047	25
Msh$^{-/-}$	19	0	0	0	0.004	32

[a]Significance of difference in length of microhomology from WT, using Student's one-tailed *t* test.

lead to reduced microhomology at the junctions. We found a decrease in microhomology at the junctions from Msh2-deficient cells compared with wild-type: twofold fewer junctions with 2 or more nts of identity (19 vs. 44%, Table I) and an increase in frequency of insertions at the junctions (19 vs. 3%, Figs. 1 and 2). Perhaps the insertions and mutations observed at the Sμ-Sγ3 junctions in the absence of Msh2 are due to a lack of normal processing of ends. Inability to properly process DNA ends could reduce the efficiency of switching, and this reduction could vary depending on the sequence of the particular downstream S region.

The sequences of the Sμ-Sγ3 junctions from Mlh1- and Pms2-deficient mice are similar to each other. About one-fourth of the junctions from these B cells show unusually long microhomologies, which suggests that these proteins are not performing the same function as Msh2. Mlh1 and Pms2 form a heterodimer and therefore it is reasonable that a deficiency in either of these proteins has the same phenotype. The Mlh1-Pms2 heterodimer is known to bind to Msh2-Msh6 and to Msh2-Msh3 heterodimers bound to DNA mismatches. The Mlh1-Pms2 heterodimer has been shown to greatly increase the affinity and thereby stabilize the binding of Msh2-Msh3 when bound to mismatches (27). In addition, it has been recently shown that the yeast Mlh1-Pms1 heterodimer (yeast Pms1 is equivalent to mammalian Pms2) can directly bind DNA in the absence of the Msh2 heterodimer. Interestingly, the heterodimer has two DNA binding sites and thus can bind to two different DNA molecules simultaneously (28). Consistent with these data, our sequencing results suggest that Mlh1-Pms2 might stabilize a recombination intermediate and that in the absence of this heterodimer, increased stability might be provided by increased lengths of microhomology. The increased lengths of microhomology also suggest that Mlh1 and Pms2 are probably not involved in processing the single-strand ends. If they were, one might predict that in their absence the lengths of microhomologies might decrease. This conclusion is in agreement with the lack of requirement for yeast Mlh1 and Pms1 (equivalent of mammalian Pms2) in DNA end-processing in DSB repair (21).

It was previously reported that Sμ-Sγ3 and Sμ-Sα junctions in Msh2-deficient B cells occur more frequently at the consensus elements GAGCT and GGGGT than do junctions from wild-type cells (24). We could not examine this in our data set, because nearly all of the wild-type and Msh2$^{-/-}$ switch junctions we obtained occurred in or near the Sμ tandem repeats, presumably because our 5′ Sμ primer is located near the beginning of the tandem repeats. We also examined the location of junctions within the Sγ3 consensus repeats and found no difference in the frequency of recombination within the Sγ3 SNIP and SNAP elements (8) in wild-type and MMR-deficient mice (data not shown).

In conclusion, the sequences of the Sμ-Sγ3 junctions from MMR-deficient mice indicate that these proteins are involved in the recombination process itself and that Msh2 has a different role from Mlh1 and Pms2. It is possible that Msh2 is present at the stage of alignment of the two S regions, perhaps recruiting an endonuclease to process the DNA ends and also attracting Mlh1-Pms2 to stabilize the recombination intermediate. In the absence of Mlh1 or Pms2, we hypothesize that Msh2 could still recruit endonuclease for end-processing, but the recombination complex would be less stable, with the result that recombination intermediates with longer stretches of microhomology would be favored.

We thank Drs. Rachel Gerstein and Erik Selsing for helpful comments on the manuscript. We thank Drs. Kenan Murphy, Michael Ehrenstein, and Claude-Agnes Reynaud for helpful discussions. We thank Dr. R.M. Liskay for mice deficient in Mlh1 and Pms2 and Drs. W. Edelmann and R. Kucherlapti for mice deficient in Msh2.

The research was supported by a grant from the National Institutes of Health RO1 AI23283.

Submitted: 12 November 2001
Accepted: 11 December 2001

Note Added in Proof: Similar data showing an increase in microhomology length at switch junctions in Pms2-deficient mice have recently been reported by Ehrenstein, M.R., C. Rita, A.-M. Jones, C. Milstein, and M.S. Neuberger. 2001. *Proc. Natl. Acad. Sci. USA.* 98:14553–14558.

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