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The Sμ Tandem Repeat Region Is Critical for Ig Isotype Switching in the Absence of Msh2

Irene M. Min,[1] Carol E. Schrader,[4] Joycelyn Vardo,[4] Thomas M. Luby,[2] Nicole D'Avirro,[1] Janet Stavnezer,[4] and Erik Selsing[1,2,3,*]
[1]Genetics Program,
[2]Immunology Program and
[3]Department of Pathology
Tufts University School of Medicine
Boston, Massachusetts 02111
[4]Program in Immunology and Virology and
Department of Molecular Genetics and Microbiology
University of Massachusetts Medical School
55 Lake Avenue North
Worcester, Massachusetts 01655

Summary

Deficiencies of the Msh2 protein or the Sμ tandem repeat (SμTR) sequences each reduce isotype switching in mice by about 2- to 3-fold. We find that switching in mice deficient for both Msh2 and SμTR is nearly ablated. We propose that the SμTR provides closely spaced cleavage sites that can undergo switch recombination independent of Msh2, whereas cleavages in sequences flanking the SμTR require Msh2 processing to allow recombinational joining. We also find that changes in Sμ sequences alter the focus of switch junctions within Sγ sequences, indicating that sequences of switch regions act together in the choice of switch recombination junctions. These findings help to explain the conservation of tandemly repeated switch regions associated with heavy chain constant genes in species capable of switching.

Introduction

B lymphocytes originate in the bone marrow, migrate into peripheral lymphoid tissues, and then mature into cells that express cell surface IgM and IgD antibodies. After encountering antigen, peripheral B cells proliferate and differentiate. Differentiating B cells can undergo isotype switching to express other antibody isotypes such as IgE, IgA, or one of several IgG subclasses (Gritzmacher, 1989; Manis et al., 2002b; Stavnezer, 1996). Isotype switching occurs via DNA recombination events that juxtapose H chain VDJ segments with different downstream CH gene segments (Iwasato et al., 1990; Matsuoka et al., 1990; von Schwedler et al., 1990). However, the mechanism of class switch recombination (CSR) and the targeting of the switch mechanism are not yet well understood.

Although there are no consensus DNA sequences found at individual switch recombination sites, switch junctions are generally found within tandemly repeated DNA regions that are located upstream of all CH gene segments that undergo isotype switching (Dunnick et al., 1993; Kataoka et al., 1981; Lee et al., 1998). The repeat units associated with the different CH genes vary. In mice, the switch (S) region associated with Cμ is composed of (GAGCT)n(GGGGT) where n is 3 on average but can range between 1 and 7. The pentamers GAGCT and GGGGT found in the Sμ region are a common feature of many, but not all, S regions in mice and humans. The unusual tandemly repeated structures of S regions have suggested they play a role in isotype switching. However, deletion of the Sμ tandem repeats (SμTR) only diminishes isotype switching by an average factor of 2- to 3-fold, indicating that DNA elements outside of the tandem repeats can be sufficient for targeting and activity of the switching mechanism (Luby et al., 2001). We will use Sμ to designate all intronic sequences that exhibit switch junction sites upstream of Cμ (essentially all sequences located between Iμ and Cμ) and SμTR to designate the subregion that is comprised of only the tandem repeats located within Sμ (Luby et al., 2001).

Although the mechanism of CSR is unknown, several proteins have been shown to have roles in CSR. Activation-induced cytidine deaminase (AID) has been found to be essential for the isotype-switching process (Muramatsu et al., 2000; Revy et al., 2000). The role of AID in the switching process is not known, although recent evidence suggests that AID deaminates cytidine residues in DNA, a modification that may initiate the DNA breaks required for switching (Di Noia and Neuberger, 2002; Petersen-Mahrt et al., 2002; Rada et al., 2002). In addition, studies have indicated that class switching involves nonhomologous end-joining (NHEJ) DNA repair activity because animals or cells that lack proteins such as Ku80, Ku70, or DNA-PKcs have major defects in isotype switching (Casellas et al., 1998; Manis et al., 1998, 2002a; Rolink et al., 1996). Presumably, NHEJ activity is involved in rejoining the DNA breaks that must accompany class switch DNA recombinations.

Disruptions of proteins involved in mismatch repair (MMR) also impair class-switching activity, although to a lesser extent (Ehrenstein and Neuberger, 1999; Schrader et al., 1999; Vora et al., 1999). Comparing switching efficiencies in wild-type mice and in mice deficient in MMR proteins (either Msh2, Pms2, or Mlh1) shows that B cells lacking MMR proteins exhibit 2- to 5-fold reductions in switch recombination (Ehrenstein and Neuberger, 1999; Ehrenstein et al., 2001; Schrader et al., 1999). Furthermore, the locations of switch region junction sites in mice that lack Msh2 show an increased focus on Sμ GAGCT and GGGGT pentamer sequences relative to the random locations of junction sites in wild-type mice (Ehrenstein and Neuberger, 1999).

The roles of MMR proteins in class switch recombination are not known. Various MMR proteins are classified as either MutS or MutL homologs. MutS homologs, Msh1 through Msh6, can recognize DNA disruptions. MutL homologs bind subsequently and recruit enzymes that excise a patch of single-strand DNA containing the mutation (Kolodner and Marsischky, 1999). In mammals, MutL homologs are Pms2, Mlh1, and Mlh3. MMR pro-

*Correspondence: erik.selsing@tufts.edu

teins Msh2, Msh3, Msh6, and Mlh1/Pms2 are involved in correcting DNA synthesis errors, but MMR proteins also appear to have other functions. MMR proteins inhibit recombination between sequences that are homologous but not identical (Datta et al., 1996; Matic et al., 1995); Msh2 and Msh6 can bind to Holliday junctions (Marsischky et al., 1999); Msh2 and Msh3 are required for removal of nonhomologous segments that neighbor DNA breaks during break repair in yeast (Sugawara et al., 1997); and Msh2, Msh6, Mlh1 and Pms2 appear to be involved in somatic hypermutation of antibody genes (Cascalho et al., 1998; Kim et al., 1999; Rada et al., 1998; Wiesendanger et al., 2000; Winter et al., 1998).

Because deletion of the SμTR reduces switching comparable to deficiencies in MMR proteins, and because Msh2 deficiency shifts the focus of switch recombination sites to the GAGCT and GGGGT sequences that are almost all eliminated by SμTR deletion, we decided to produce mice that lack both the SμTR and Msh2 to analyze the relationships between these two elements in the switching process. $S\mu^{-/-}$:*msh2*$^{-/-}$ mice show greatly reduced levels of isotype switching (5%–10% of wild-type mice). However, Msh2 deficiency does not significantly increase the focus of recombination to the few GAGCT/GGGGT pentamers remaining in the JH-Cμ region of $S\mu^{-/-}$ mice. Our results indicate that DNA cleavage and rejoining processes at sites within the SμTR are relatively Msh2 independent but highly Msh2 dependent at sites outside of the tandem repeats. We suggest that this could reflect a role for Msh2 in processing the broken DNA ends during the switching process coupled with differences in the types of ends generated within or outside of the SμTR region.

Results

Isotype Switching Is Substantially Reduced in $S\mu^{-/-}$:*msh2*$^{-/-}$ Mice

Isotype switching frequencies were assessed in B cells from wild-type, $S\mu^{-/-}$, *msh2*$^{-/-}$, and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice to determine how combined deficiencies of Msh2 and SμTR affected switching efficiency. Splenic B cells were isolated from unimmunized animals and stimulated in culture for 4 days with LPS together with particular cytokines that induce switching to various H chain isotypes. Previous studies have shown that such cell cultures from $S\mu^{-/-}$ and *msh2*$^{-/-}$ mice exhibit B cell proliferation and viabilities that are equivalent to cultures from wild-type mice (Luby et al., 2001; Schrader et al., 1999); the doubly mutant $S\mu^{-/-}$:*msh2*$^{-/-}$ mice, as expected, were also found to show comparable B cell proliferation and viabilities (data not shown).

The efficiency of switching to downstream isotypes was assessed by staining the B cells with isotype-specific antibody reagents and by analyzing the frequencies of cells expressing different isotypes by flow cytometry. A representative FACS analysis of B cells that were stimulated to switch to IgG1 with LPS and IL-4 is shown in Figure 1. The cells from $S\mu^{-/-}$ mice and *msh2*$^{-/-}$ mice exhibited, respectively, 2-fold and 2.7-fold decreases in switching to IgG1 relative to wild-type mice. These results are comparable to similar studies reported previously (Luby et al., 2001; Schrader et al., 1999). Strikingly, IgG1 switching was greatly reduced (about 33-fold relative to wild-type mice) in the cell cultures from $S\mu^{-/-}$:*msh2*$^{-/-}$ mice. Similar large reductions for isotype switching in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice were observed for all isotypes tested (Figure 2). The switching efficiency of $S\mu^{-/-}$:*msh2*$^{-/-}$ mice was generally only 5%–10% of the control wild-type mice. The extent of reduction appeared somewhat smaller for IgG3 and IgA switching; however, switching is less efficient for these isotypes in our in vitro cultures, and this impacts the decreases that can be detected.

Reductions in $S\mu^{-/-}$:*msh2*$^{-/-}$ Surface Ig Expression Reflect Decreases in Class Switch Recombination

To determine whether the low switching frequencies in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice are due to defects in class switch DNA recombination, we analyzed in vitro switched B cells at the molecular level by digestion-circularization (DC)-PCR. Using a primer upstream of Sμ together with a second primer downstream of either Sγ1 or Sγ3, the DC-PCR technique generates a PCR product of constant size that allows quantitation of class switch recombination events within polyclonal B cell populations (Chu et al., 1992). DC-PCR analyses of Sμ/Sγ1 and Sμ/Sγ3 switch recombination in B cell cultures from wild-type, $S\mu^{-/-}$, *msh2*$^{-/-}$, and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice are shown in Figure 3A. As reported previously, $S\mu^{-/-}$ and *msh2*$^{-/-}$ B cells showed about 2-fold reductions in γ1 and γ3 switch recombination relative to wild-type mice (Luby et al., 2001; Schrader et al., 1999). In contrast, Sμ/Sγ1 and Sμ/Sγ3 recombinations in cells from $S\mu^{-/-}$:*msh2*$^{-/-}$ cultures were barely detectable. Consistent with the FACS analyses, quantitative comparisons of the DC-PCR results indicated that the double-knockout cells have much greater reductions in class switch DNA recombinations (5%–10% of wild-type) than either *msh2*$^{-/-}$ or $S\mu^{-/-}$ B cells (Figure 3B).

We compared the data sets from the B cell cultures using one-way analyses of variance (ANOVA) together with Tukey tests; in these evaluations each FACS and DC-PCR analysis for both IgG1 and IgG3 was considered to be independent. Comparisons of IgG1 switch frequencies from wild-type mice versus each mutant line ($S\mu^{-/-}$, *msh2*$^{-/-}$, or $S\mu^{-/-}$:*msh2*$^{-/-}$) all showed significant reductions ($p < 0.001$). Furthermore, IgG1 switching levels in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice were also significantly reduced when compared either to $S\mu^{-/-}$ mice or to *msh2*$^{-/-}$ mice ($p < 0.001$). The differences in IgG3 switching levels between the different mouse groups were somewhat less dramatic. Differences in IgG3 switching were detectable between wild-type mice and *msh2*$^{-/-}$ mice ($p < 0.01$), but no significant differences were found between wild-type and $S\mu^{-/-}$ mice. The lack of either SμTR or Msh2 had highly significant impacts on IgG3 switching when the other factor was not present (*msh2*$^{-/-}$ versus $S\mu^{-/-}$:*msh2*$^{-/-}$, $p < 0.05$; $S\mu^{-/-}$ versus $S\mu^{-/-}$:*msh2*$^{-/-}$, $p < 0.001$). The clear differences in switching between the $S\mu^{-/-}$:*msh2*$^{-/-}$ mice when compared to either the $S\mu^{-/-}$ or the *msh2*$^{-/-}$ mice indicate that Msh2 and the SμTR element are likely to be involved in separate aspects of the switching process that, together, affect almost all isotype-switching events.

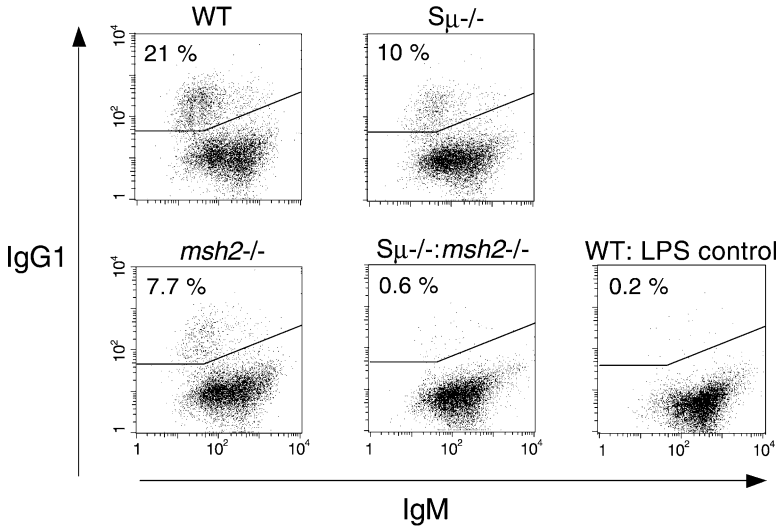

Figure 1. Isotype Switching to IgG1 Is Greatly Reduced in $S\mu^{-/-}$:*msh2*$^{-/-}$ Mice

Splenic B cells from age-matched mutant mice and control wild-type mice were cultured for 4 days with LPS and IL4 to stimulate IgG1 class switching. Flow cytometric analyses of IgG1-expressing B cells are shown; the percentages of IgG1-positive cells are indicated in each diagram. Note that wild-type cells treated with LPS alone did not switch to IgG1.

Msh2 Deficiency Does Not Focus Switch Recombination Sites to Pentamer Motifs When the Sμ Tandem Repeats Are Absent

Mice that lack Msh2 have been reported to exhibit switch recombination junction sites that focus on the pentamer sequences within the JH-Cμ intron; this contrasts with the relatively random location of switch junctions in wild-type mice (Ehrenstein and Neuberger, 1999; Dunnick et al., 1993). It has been proposed that Msh2 is involved in postcleavage DNA end-processing which would result in recombination junctions that are removed from the initial cleavage site. If Msh2 is absent, perhaps the final junction sites would be more indicative of the initial cleavage site (Ehrenstein and Neuberger, 1999; Ehrenstein et al., 2001; Schrader et al., 1999).

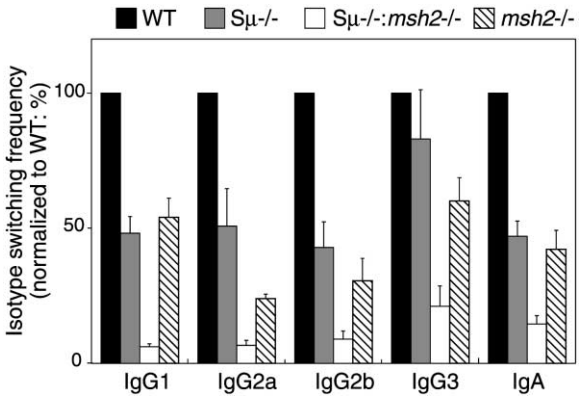

Figure 2. Reductions of Isotype Switching for All of the Major Isotypes in $S\mu^{-/-}$:*msh2*$^{-/-}$ Mice

As in Figure 1, flow cytometry was used to estimate the efficiency of isotype switching with in vitro stimulated B cells. The bar graphs represent the average switching frequencies and the corresponding standard error of means (SEM) in three to six independent experiments for the four mouse genotypes indicated. For each isotype, values for wild-type mice are set to 100%; values for each mutant genotype are then calculated relative to wild-type. For each isotype, the average frequencies of switched cells in the stimulated wild-type cultures were IgG1, 28%; IgG2a, 13.0%; IgG2b, 13.2%; IgG3, 6.3%; IgA, 4.0%.

Because the JH-Cμ region in $S\mu^{-/-}$ mice retains about 2% of the pentamers present in the wild-type JH-Cμ, we wished to determine whether the switch junction sites found in $S\mu^{-/-}$:*msh2*$^{-/-}$ B cells focused on these few remaining pentamers.

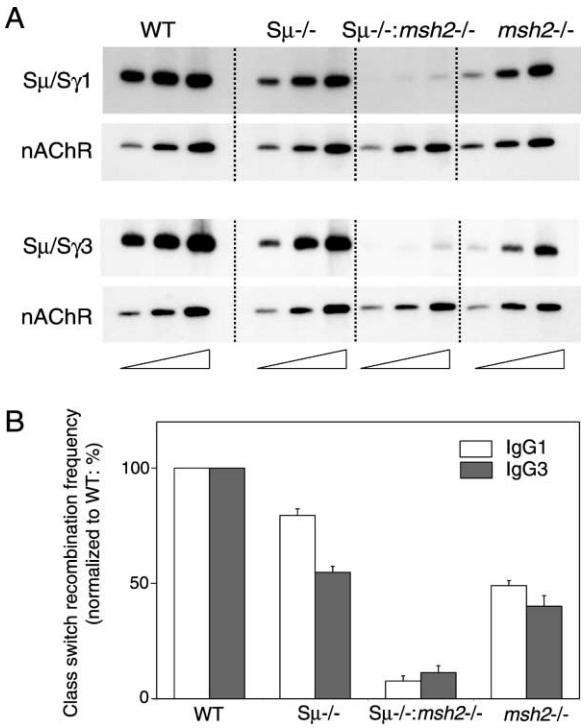

Figure 3. DC-PCR Analyses Show that Both IgG1 and IgG3 Class Switch DNA Recombinations Are Substantially Reduced in $S\mu^{-/-}$:*msh2*$^{-/-}$ Mice

(A) Genomic DNAs from IgG1 and IgG3 switched cells were prepared and 2-fold dilutions were subjected to Sμ/Sγ1 and Sμ/Sγ3 DC-PCR, respectively. Samples were normalized using control nicotinic acetylcholine receptor (nAChR) DC-PCR reactions.

(B) Average values for the levels of Sμ/Sγ1 and Sμ/Sγ3 switch recombinations in mutant mice relative to wild-type mice. Values for wild-type mice are set to 100%.

Table 1. Locations of Sμ/Sγ1 and Sμ/Sγ3 Junctions in $S\mu^{-/-}$ and $S\mu^{-/-}$:*msh2*$^{-/-}$ Mice

Sμ Germline Region[a] (4.5% pentamers; ~40% hotspots)		Junction Location Relative to Sμ GAGCT/GGGGT Pentamers[b]		Junction Location Relative to Sμ RGYW/WRCY Hotspots[b]	
		Inside	Outside	Inside	Outside
Sμ junctions with Sγ1	$S\mu^{-/-}$ mice	1	20	8	13
	$S\mu^{-/-}$:*msh2*$^{-/-}$ mice	4	15	11	8
Sμ junctions with Sγ3	$S\mu^{-/-}$ mice	0	16	7	9
	$S\mu^{-/-}$:*msh2*$^{-/-}$ mice	3	18	17[c]	4[c]
Sγ1 or Sγ3 Germline Regions[a]		**Junction Location Relative to Sγ1 or Sγ3 GAGCT/GGGGT Pentamers[b]**		**Junction Location Relative to Sγ1 or Sγ3 RGYW/WRCY Hotspots[b]**	
Sγ1 (3.8% pentamers; 39.5% hotspots) Sγ3 (~16% pentamers; ~49% hotspots)		Inside	Outside	Inside	Outside
Sγ1 junctions with Sμ	$S\mu^{-/-}$ mice	1	18	12	7
	$S\mu^{-/-}$:*msh2*$^{-/-}$ mice	2	16	10	8
Sγ3 junctions with Sμ	$S\mu^{-/-}$ mice	7	8	11	4
	$S\mu^{-/-}$:*msh2*$^{-/-}$ mice	10[d]	10[d]	15	5

[a] The percentage of nucleotides within or adjacent to both pentamer motifs (GAGCT and GGGGT) and somatic hypermutation hotspots (RGYW and WRCY) among 1600 nucleotides were analyzed for the Sμ, Sγ1, or Sγ3 regions. For each region, the 1600 bp analyzed were located contiguous to the primer that was used for corresponding switch junction analyses.

[b] The numbers of class switch recombination sites that occurred inside (within or adjacent to) or outside of pentamers and hotspots were counted. The frequencies of observed junction sites inside and outside of pentamer or hotspot motifs were not significantly different (by two-tailed χ^2 tests) from the frequencies expected assuming randomly located junction sites in the same DNA region, except for the cases noted in c and d below.

[c] The observed frequency of $S\mu^{-/-}$:*msh2*$^{-/-}$ junctions located inside Sμ RGYW/WRCY hotspots is significantly different from the frequency expected assuming randomly located junction sites in the same DNA region (χ^2 test, two-tailed, $p = 0.026$) and also significantly different from the frequency of $S\mu^{-/-}$ junctions located inside Sμ hotspots (Fisher's exact test, two-tailed, $p = 0.036$).

[d] The observed frequency of $S\mu^{-/-}$:*msh2*$^{-/-}$ switch junctions located inside pentamers is significantly different from the frequency expected assuming randomly located junction sites in the same DNA region (χ^2 test, two-tailed, $p = 0.043$).

We analyzed Sμ/Sγ1 and Sμ/Sγ3 switch sites in $S\mu^{-/-}$:*msh2*$^{-/-}$ B cells to determine whether $S\mu^{-/-}$:*msh2*$^{-/-}$ switch junctions occur at Sμ pentamers. $S\mu^{-/-}$ or $S\mu^{-/-}$:*msh2*$^{-/-}$ splenic B cells were cultured for 4 days either with LPS and IL-4 to induce IgG1 switching or with LPS alone to induce IgG3 switching. Genomic DNAs were extracted from the cultured B cells, and switch junctions were amplified using specific PCR reactions. PCR products were cloned and sequenced; 19 Sμ/Sγ1 junctions and 21 Sμ/Sγ3 junctions from $S\mu^{-/-}$:*msh2*$^{-/-}$ mice were analyzed. For comparison, similar numbers of Sμ/Sγ1 and Sμ/Sγ3 switch recombination sites from $S\mu^{-/-}$ mice were also analyzed. Sequences of the switch junctions are shown in Supplemental Figure S1 (at http://www.immunity.com/cgi/content/full/19/4/515/DC1), and positions of all switch junctions are located on a map of the JH-Cμ intron (Supplemental Figure S2). Analyses of switch junction structures included the data from previously reported Sμ/Sγ1 junctions in $S\mu^{-/-}$ mice (Luby et al., 2001).

As shown in Table 1, the majority of Sμ recombination sites for both Sμ/Sγ1 and Sμ/Sγ3 junctions in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice did not utilize the 13 pentamer sequences remaining in the $S\mu^{-/-}$ JH-Cμ region. Although there was a small increase in the number of switch junctions located within Sμ pentamers in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice when compared to $S\mu^{-/-}$ mice, this difference was not statistically significant for our data sets (Table 1). We also assessed the distance the junctions were located from the nearest Sμ pentamer and found that switch junctions in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice (relative to junctions in $S\mu^{-/-}$ mice) were located somewhat nearer to the Sμ pentamers (Supplemental Figure S3). However, it is again not clear whether this is statistically significant, given the limited data sets. These data suggest that previous findings showing that Sμ junctions in *msh2*$^{-/-}$ mice occur almost entirely within the tandem pentamer repeats (Ehrenstein and Neuberger, 1999) may be due to the repeated nature of the Sμ region rather than due to the pentamer sequences per se.

It is also possible that switch DNA cleavages might occur preferentially at RGYW and its complement WRCY sequences (R, purine; Y, pyrimidine; W, A/T); therefore, we also analyzed the usage of these sequence motifs at the switch junctions from $S\mu^{-/-}$and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice. RGYW/WRCY sequences are known to be "hotspots" for somatic hypermutation (Jolly et al., 1996) and have been suggested to be targets for in vivo AID activity in the hypermutational mechanism (Martin et al., 2002). Because AID is known to be required for both somatic hypermutation and isotype switching (Muramatsu et al., 2000; Revy et al., 2000), and because it has been hypothesized that the somatic hypermutation hotspots are AID targets, it has also been suggested that AID activity might be directed to these hotspot sequences during class switching (Di Noia and Neuberger, 2002; Storb and Stavnezer, 2002). The GAGCT pentamers contain the RGYW sequence and thus are a subset of these hotspots. As shown in Table 1, Msh2 deficiency significantly increased the focus of Sμ/Sγ3 junctions to Sμ hotspot sequences in cells that lacked SμTR. Although Msh2 deficiency also appeared to increase the Sμ hotspot focus for Sμ/Sγ1 junctions, the increase was not significant. There was no detectable effect on hotspot focus within either Sγ1 or Sγ3 sequences when the SμTR region was absent. Combining all of the results from $S\mu^{-/-}$ and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice, more junctions were found to occur at hotspots than not. This skewing could

be discerned even though a large proportion of S region sequences consist of hotspot motifs.

Sμ Sequences Affect Switch Junction Sites Used in Sγ

Comparisons of switch junction sites in wild-type, $S\mu^{-/-}$, and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice revealed that, although deletion of the SμTR did not affect the Sγ1 site used at the recombination junction, the Sγ3 sites used were altered. First, mice that lack the SμTR exhibited many Sγ3 switch junctions that occurred at GAGCT or GGGGT pentamer motifs within the Sγ3 region. This focus was not observed in wild-type Sγ3 junctions (Schrader et al., 2002) in which only 1 out of 32 junctions (3.1%) occurred at Sγ3 GAGCT/GGGGT sequences in contrast to 7 out of 15 (46.7%) and 10 out of 20 (50.0%) in $S\mu^{-/-}$ mice and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice, respectively (Table 1) (WT versus $S\mu^{-/-}$, two-tailed, $p = 0.001$; WT versus $S\mu^{-/-}$:*msh2*$^{-/-}$, two-tailed, $p < 0.0001$ by Fisher's exact test). Another study of Sγ3 recombinations in *msh2*$^{+/-}$ mice also showed a low frequency of junctions at Sγ3 pentamers (1 out of 29 Sγ3 junctions) (Ehrenstein and Neuberger, 1999). Furthermore, we found that about 25% of Sμ/Sγ3 recombination junctions in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice had unusually long stretches of microhomology at or near the junction site, and all of these unusual sites exhibited pentamer sequences at the Sμ side of the Sμ/Sγ3 junction (see Supplemental Figure S1 at http://www.immunity.com/cgi/content/full/19/4/515/DC1). Such features were not found among the Sμ/Sγ1 junctions that we have characterized from $S\mu^{-/-}$:*msh2*$^{-/-}$ mice. Taken together, these results suggest that, in mice, IgG3 and IgG1 isotype-switching recombinational pathways are somewhat different.

When Sμ Tandem Repeats Are Absent, Switch Recombination Sites Do Not Significantly Focus on Hairpin Structures in the JH-Cμ Intron

Several studies of switch junction sites have suggested that the initiation of switch recombination might involve the recognition of secondary structures within S sequences (Mussmann et al., 1997; Tashiro et al., 2001; Wuerffel et al., 1997). Switch junctions in several species have been found predominantly near the base or neck of hairpins predicted within the S region sequences. To determine whether switch junctions were similarly located in mice that lacked SμTR or both SμTR and Msh2, we analyzed the locations of both IgG1 and IgG3 switch junctions in $S\mu^{-/-}$ and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice. We used the same algorithm for predicting secondary structures (SantaLucia, 1998) and the same criteria as described previously in another study of switch regions (Tashiro et al., 2001) to determine whether individual nucleotides in the JH-Cμ region were located at the neck or base of hairpin structures. Switch recombination sites within the JH-Cμ region for both IgG1 and IgG3 switches were then located on the secondary structure map predicted for this region. The switch junction sites which are located immediately downstream of the deleted SμTR sequences are shown together with the predicted secondary structures in Figure 4; Table 2 summarizes the analyses for all of the junctions that we determined.

For the germline JH-Cμ DNA regions analyzed in $S\mu^{-/-}$ and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice, we found that 49% of the nucleotides were predicted to be at a neck or base of a stem-loop structure. As summarized in Table 2, Sμ/Sγ1 recombination sites from both $S\mu^{-/-}$ and $S\mu^{-/-}$:*msh2*$^{-/-}$ mice showed a slight bias toward the base or neck regions of hairpin structures. However, the significance of this bias was borderline when assessed using χ^2 test, and the Sμ/Sγ3 junctions showed no bias (Table 2). The lack of Msh2 did not appear to have any impact on switch junction locations relative to hairpin structures. When all switch recombination sites analyzed in this study were pooled, there was no detectable clustering of switch junctions at the base or neck regions of stem-loop structures. These results suggest that hairpin structures do not play a role in determining the location of switch recombination sites in the JH-Cμ region when the SμTR region is absent.

Discussion

Our studies show that mice lacking both Msh2 and SμTR exhibit large reductions in the efficiency of B cell class switch recombination. These results indicate that, in the absence of Msh2, the pentamer-rich SμTR region is very important for isotype switching.

Previous reports have shown that in *msh2*$^{-/-}$ mice, Sμ switch junctions strongly focus on GAGCT and GGGGT pentamers (Ehrenstein and Neuberger, 1999). These findings suggested that switching might be greatly reduced in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice because the vast majority of GAGCT and GGGGT sequences in the JH-Cμ region were removed. Furthermore, it might be expected that any switch recombinations remaining in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice would focus on the few JH-Cμ pentamers that were available.

Although our studies do show large reductions in isotype switching that are consistent with the hypothesized predictions, switch junctions in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice do not predominantly focus on the few JH-Cμ pentamers present. A shift of switch junctions to pentamer motifs is discernible, but the shift is not statistically significant. This result suggests that the marked reduction in switching for $S\mu^{-/-}$:*msh2*$^{-/-}$ mice is not caused by a lack of pentamers to provide cleavage sites for initiating the recombinational mechanism. Perhaps any possible preference for cleavage at pentamers is not great enough to observe when there are so few to compete with other sequences that might also serve as cleavage targets. It is also possible that cleavage sites do focus on pentamers but that the initial sites of DNA breaks are not necessarily identical to the final recombination sites. This latter possibility would seem less likely due to the data suggesting that Msh2 is involved in end-processing during the repair phase of CSR (Ehrenstein and Neuberger, 1999; Schrader et al., 2002).

A surprising finding from analyses of switch junctions in $S\mu^{-/-}$ mice is that deletion of the SμTR affects the spectrum of junction sites used within the Sγ3 switch region located about 60 kb downstream of Sμ. This finding is the first indication that the sequences present within Sμ can affect the usage of switch sites in downstream S regions. The role for the Sμ sequences could involve either altering the location of the cleavage sites

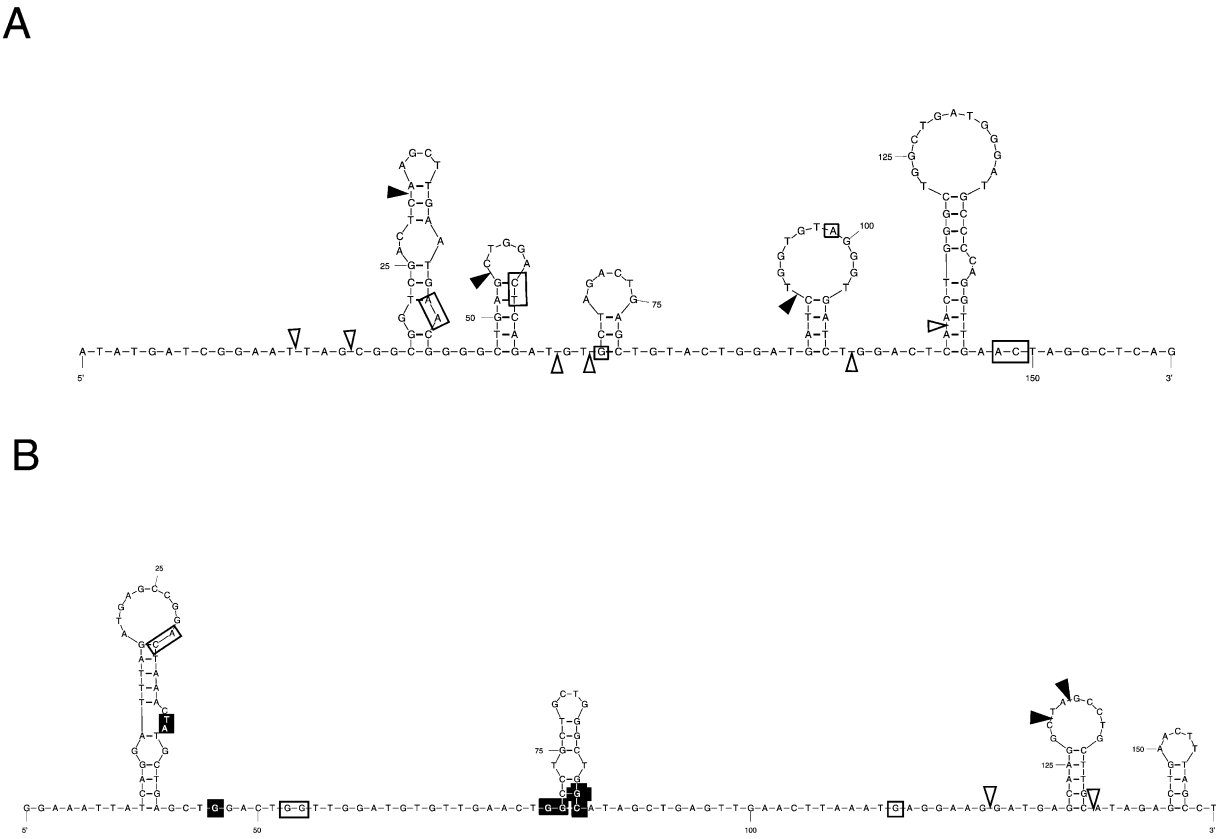

Figure 4. Switch Recombination Sites from Sμ$^{-/-}$ Mice Do Not Focus on Hairpin Structures in the JH-Cμ Intron

Two maps locating representative Sμ/Sγ1 and Sμ/Sγ3 junctions relative to predicted stem-loop structures of the single-stranded Sμ region are shown. Arrowheads indicate switch junction sites that have no shared microhomologies whereas junctions that exhibit shared microhomologies are marked as boxes. Switch sites that are derived from Sμ$^{-/-}$ mice are indicated by open arrowheads or open boxes. Switch sites from Sμ$^{-/-}$*:msh2*$^{-/-}$ mice are marked with solid arrowheads or solid boxes. The sequence in (A) spans the HindIII site located immediately downstream of the loxP site in the Sμ− allele (Luby et al., 2001) and this HindIII sequence corresponds to nucleotide 1207 in Sμ genomic sequence from GenBank accession number J00442. This HindIII site is present within the loop of the first hairpin on the left. The 5′ end of the sequence in (B) is located 314 bp downstream of the HindIII site in (A).

or structures in the Sγ3 region, or altering the selection of Sγ3 cleavage sites that are favored for religation. Similar alterations were not observed in our analyses of Sγ1 junction sites; this indicates that the nature of interactions between S regions depends on the sequences of the S regions involved and explains why this effect was not observed in a previous study of Sμ$^{-/-}$ mice in which only Sμ/Sγ1 junctions were examined

Table 2. Locations of Switch Recombination Sites Relative to Secondary Structures in the J_H-Cμ Intron

	Junctions Located 0 or 1 bp Distant from Neck or Stem			Junctions Located ≥2 bp Distant from Neck or Stem		
	Sμ/Sγ1	Sμ/Sγ3	Total	Sμ/Sγ1	Sμ/Sγ3	Total
Sμ$^{-/-}$ mice	13	7	20	6	6	12
Sμ$^{-/-}$*:msh2*$^{-/-}$ mice	13	9	22	5	9	14
Sums	26 (70%)[b]	16 (52%)	42 (62%)[a]	11 (30%)[b]	15 (48%)	26 (38%)[a]

Switch junctions located at or away from the necks of stem-loop structures in the J_H-Cμ intron region were counted. Numbers in parentheses indicate the percentage of switch junctions that were located either at or away from the necks of stem-loop structures. Those junctions exhibiting longer microhomologies were not included in this analysis when it was ambiguous whether the junction was either at or away from a neck. For the germline J_H-Cμ intron nucleotides analyzed 49% were 0 or 1 bp distant from the neck of a stem-loop structure, and the remaining 51% of the nucleotides were at least 2 bp away from necks.

[a] The observed number of switch junctions located at or away from necks were compared to the expected frequencies based on a random distribution of junctions. χ^2 test indicated that the association of switch junctions to the necks of stem-loop structures within the J_H-Cμ intron was not statistically significant (two-tailed, $p = 0.194$).

[b] Although the total number of Sμ/Sγ1 junction sites isolated from both Sμ$^{-/-}$ and Sμ$^{-/-}$*:msh2*$^{-/-}$ mice was slightly biased toward the necks of stem-loop structures, this association was not statistically significant when tested by χ^2 test (two-tailed, $p = 0.097$).

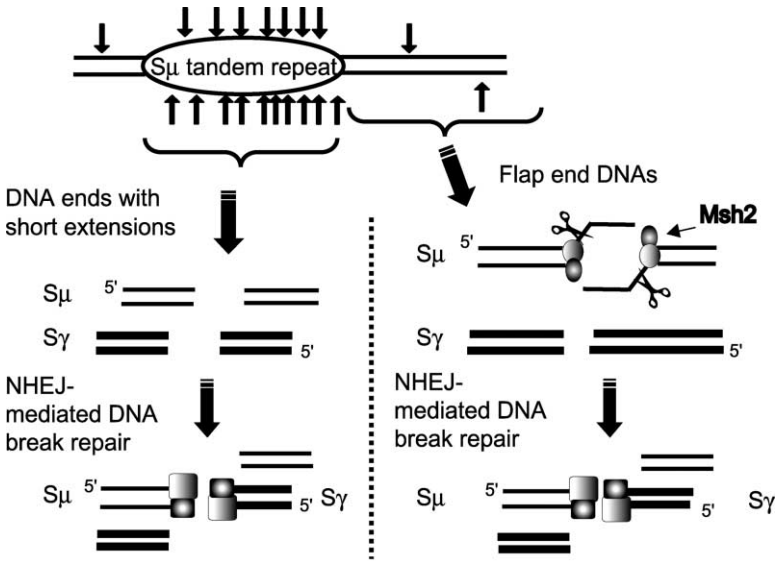

Figure 5. Model for Msh2 Action in SμTR Region and Sequences Surrounding the SμTR during Class Switch DNA Recombination

The top diagram shows the Sμ tandem repeat region together with DNA sequences flanking SμTR. Sites of single-stranded class switch DNA cleavages are indicated by arrows. DNA cleavages within SμTR are proposed to be closely spaced and, therefore, predominantly generate DNA ends that have short single-stranded extensions. These can be joined with downstream S region DNA ends by DNA-PK/Ku complex-mediated nonhomologous end-joining (NHEJ) pathway independent of Msh2 (left panel). In contrast, sequences outside of the SμTR region (right panel) have more widely spaced cleavage sites, which can lead to flap DNA ends with longer 5′ or 3′ extensions. Msh2 is suggested to recruit nucleases to end-process the 3′ flap DNA ends before they are recombined by the NHEJ mechanism. Rectangles and ovals represent the DNA-PK/Ku complex and Msh2 complex, respectively.

(Luby et al., 2001). Differences between IgG3 and IgG1 switching were also found in the use of Sμ hotspots at switch junction sites in $S\mu^{-/-}$:$msh2^{-/-}$ mice; recombinations in the Sμ region occur preferentially at RGYW/WRCY hotspots for Sμ/Sγ3 junctions but not for Sμ/Sγ1 junctions. Because hotspots have been suggested to represent preferential AID targets (Martin et al., 2002), the difference between hotspot usage that we observed in IgG1 and IgG3 switching might represent a post-AID mechanism. Recent in vitro data indicate that hotspots do represent AID targets (Pham et al., 2003), even though an earlier study did not find such targeting (Dickerson et al., 2003). It is intriguing to consider that the recently reported unique resistance of IgG1 switching to DNA-PKcs deficiency (Manis et al., 2002a) might be related to the differences observed between IgG1 and IgG3 switching in $S\mu^{-/-}$:$msh2^{-/-}$ mice, but how these features might be linked is not clear. It is also possible that postulated isotype-specific switching factors (Shanmugam et al., 2000; Ma et al., 2002) might be involved in the observed IgG1 and IgG3 junction site differences.

It is also noteworthy that deletion of most GAGCT and GGGGT pentamers within Sμ results in an increase in the use of such pentamers in Sγ3. Perhaps an initial cleavage is an important event during switching, and some pentamers represent favored sites for cleavage regardless of the S region location of the pentamer. For Sμ-Sγ3 switching in $S\mu^{-/-}$ mice, the numerous pentamers in Sγ3 might provide the predominant sites for this initial cleavage. However, for Sμ-Sγ1 switching, the pentamers in the large Sγ1 region may be too few to be detectably favored for cleavage relative to other sites that might be less favored but more numerous.

On the basis of the results obtained from $S\mu^{-/-}$:$msh2^{-/-}$ mice, we propose a model of class switch recombination to explain the critical role of the SμTR region for switching in mice that lack Msh2. There are likely to be other models that would be consistent with our data; the impetus for our model comes from the relative importance of Msh2 in class switching to sequences outside of the SμTR region and the relative Msh2 independence of class switching to sequences within the SμTR region. The model is based on the importance of the nonhomologous end-joining pathway of DNA repair in the switching mechanism and presumes that the cleaved DNA ends that are joined during switch recombination must be in the form of blunt ends or ends with short 5′ or 3′ extensions before the final joining step.

The nature of the DNA cleavages that initiate switching are not yet known; however, it is known that activation-induced deaminase is required for isotype switching and that AID appears to deaminate cytidine residues to introduce deoxyuracil (dU) residues. Removal of the dU residues is thought to involve base-excision repair and to result in a DNA break at the deaminated deoxycytidine residue (Di Noia and Neuberger, 2002; Rada et al., 2002; Stavnezer and Bradley, 2002; Storb and Stavnezer, 2002). It is not clear what sequence or structural features might determine which cytidine residues are suitable substrates for AID in vivo. As shown in Figure 5, our model proposes that the action of AID in cell populations that are undergoing switching yields cells with a variety of breaks in switch regions that include breaks with small 5′ and 3′ extensions and breaks with substantial 5′ or 3′ extensions. Breaks with small extensions can probably be repaired by the NHEJ mechanism; we suggest that one role for Msh2 in this switch model is to recruit nucleases (Paques and Haber, 1999; Sugawara et al., 1997) to process ends with substantial extensions and convert these into ends suitable for NHEJ.

To account for the substantial reductions in isotype switching that are observed in $S\mu^{-/-}$:$msh2^{-/-}$ mice relative to both $S\mu^{-/-}$ and $msh2^{-/-}$ mice, the proposed model includes the notion that the SμTR region has a much higher density of potential sites for switch DNA cleavages than are found in the DNA regions surrounding the SμTR. Because of this higher site density, SμTR sequences promote a greater chance of two DNA cleavages occurring near each other and thus generating a staggered end break with relatively short single-stranded extensions. This type of break could be joined by NHEJ with minimal end processing and would be

less dependent on Msh2. In contrast, in the DNA regions outside of the SμTR, most cleavages are more widely spaced, generating flap ends that require processing by Msh2 recruitment of nucleases (Paques and Haber, 1999; Sugawara et al., 1997) prior to joining by NHEJ. Therefore, class switching in $S\mu^{-/-}$ cells that lack SμTR would be more dependent on the presence of Msh2. Interestingly, although it is not known what sequences are required for switch cleavages, somatic hypermutation hotspots do represent about 50% of sequences within the SμTR region but only about 5%–10% of those sequences that flank the SμTR.

The proposed model suggests a preponderance of switch DNA breaks generated within the SμTR region that have only short extensions (Figure 5). This suggestion could be tied with the notion that the sequences of the Sμ tandem repeats (and other tandemly repeated switch regions) are particularly suited for the generation of closely spaced DNA cleavages. Although AID appears to be a cytidine deaminase, the efficiency of this activity on individual cytidine residues may well be affected by surrounding sequences or by other proteins that may target AID activity (Dickerson et al., 2003; Sohail et al., 2003). Indeed, some evidence for AID hotspots has already been found (Petersen-Mahrt et al., 2002). The Sμ GAGCT sequence is a known hotspot for somatic hypermutation, and perhaps it is also a hotspot for AID activity during class switching. Possibly the tandem repeat structure is important in presenting favorable AID-sensitive sites; this suggestion might explain the presence of tandem repeats associated with all CH gene segments capable of switching, even when these repeats are not rich with the cytidine residues that appear to be the target for AID (Gritzmacher, 1989; Mussmann et al., 1997). It is noteworthy that, of the two characterized DNA breaks within the Sγ3 tandem repeat region that have been detected in cells undergoing class switch recombination, one is located at a site bordering tandem pentamer sequences (Wuerffel et al., 1997). Analyses of the locations and structures of DNA ends generated by cleavage within or outside of the Sμ repeat region could provide data to test the model that we propose.

Experimental Procedures

Mice

$S\mu^{-/-}$ (previously ΔSμ) mice and *msh2*$^{-/-}$ mice have been described (Luby et al., 2001; Reitmair et al., 1995). $S\mu^{-/-}$ and *msh2*$^{-/-}$ mice were crossed to generate $S\mu^{-/-}$:*msh2*$^{-/-}$ mice; offspring were genotyped using PCR analyses of tail DNAs. A 350 bp fragment that identified Sμ− alleles was PCR amplified by using a primer downstream of Eμ, 3EμFOR (5′-ACTCTCCAGCCACAGTAATGACCC-3′) and a primer upstream of Cμ, 5CμBK (5′-TCATGGTCCCAGTC CATCTTTTCC-3′). Wild-type Sμ alleles were identified using the 5CμBK primer together with a primer located within the Sμ region, 3SμFOR (5′-GGCCAGAACCAGAATCAATTAGGC-3′) to amplify a 350 bp fragment. Samples were amplified for 30 cycles comprising 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s, together with a 7 min extension at 72°C. The genotyping PCR primers and procedures for *msh2*$^{-/-}$ mice have been described previously (Jenab-Wolcott et al., 2000).

In Vitro B Cell Cultures and Flow Cytometry

T cell-depleted splenic B cells were cultured at 2×10^5 cells/ml in RPMI-1640/10% FCS. Cultures were supplemented with LPS (50 μg/ml; Sigma Chemical Co.) and anti-δ-dextran (0.3 ng/ml; gift of C. Snapper, Uniformed Services University of the Health Sciences, Bethesda, MD) to induce IgG3 switching, or with LPS and cytokines to induce isotype switching to IgG1 (IL-4, 800 U/ml; gift of W. Paul, National Institutes of Health, Bethesda, MD), IgG2a (IFNγ, 10 U/ml; gift of American Cancer Society, New York, NY), IgG2b (dextran sulfate, 30 μg/ml; Amersham Pharmacia Biotech), or IgA (IL-4, 800 U/ml; IL-5, 150 U/ml; TGF-β1 [R&D Systems], 2 ng/ml; anti-δ-dextran, 0.3 ng/ml), as previously described (Luby et al., 2001). B cells from day 4 cultures were stained with FITC-conjugated goat anti-IgM antibody and PE-goat F(ab′)2 anti-mouse IgG1, IgG2a, IgG2b, IgG3, or IgA antibodies (Southern Biotechnology Associates, Inc.). Cells were analyzed using FACScan (Becton Dickinson) and Flowjo (Tree Star Inc.) software. Live lymphocytes that were gated based on forward and side scatter profiles were analyzed.

Digestion-Circularization-PCR

DC-PCR was carried out basically as described previously (Chu et al., 1992) with some modifications. Genomic DNAs (2 μg/100 μl) from cultured B cells were digested with EcoRI, diluted to 1440 ng/100 μl for circularization with T4 DNA ligase, and then dialyzed using ddH2O. Two-fold serial dilutions of ligated DNA were subjected to PCR. The PCR primer pairs and reaction conditions for both Sμ/Sγ1 DC-PCR and nicotinic acetylcholine receptor (nAChR) DC-PCR have been published previously (Chu et al., 1992). Sμ/Sγ3 DC-PCR products were amplified using published primer pairs, DCM1 (5′-GAAGCCCTTCACGCCACTGACTGACTG-3′) and DCG31 (5′-TTG ATCTTACAGCACAAAGGCCACG-3′) for 32 cycles (94°C for 1 min, 65°C for 1 min, and 72°C for 1 min) (Wuerffel et al., 2001). For quantitation, both [α-^{32}P]-dCTP and [α-^{32}P]-dATP were added to reaction mixtures to detect DC-PCR signals in samples from the mutant mouse lines. Radiolabeled PCR samples were separated on a polyacrylamide gel, dried, and exposed to X-ray film. DC-PCR bands were quantitated using Scion imaging software (Scion Corp.). For Sμ/Sγ3 DC-PCR, B cell cultures from two different mice were used to prepare circularized DNA samples, and these DNAs were amplified in duplicate PCR reactions. PCR products were quantitated by densitometry of several autoradiographs that differed by 2-fold exposure times, providing 12 sets of data that were averaged. For Sμ/Sγ1, duplicate PCR reactions from one mouse were analyzed and provided six sets of data that were averaged. Intensity values for both Sμ/Sγ3 and Sμ/Sγ1 DC-PCR products were normalized using nAChR DC-PCR products as controls.

Sμ/Sγ1 and Sμ/Sγ3 Recombination Junction Analyses

Two rounds of PCR using nested primer sets were used for Sμ/Sγ1 junction amplification. The outside primers for the first round were Sμ1.2In (5′-CAGGCTAAGAAGGCAATCCTGG-3′) and G1OUT (5′-TTGACCTGTAACCTACCCAGGAGAC-3′); 36 cycles of PCR were performed (94°C for 30 s, 62°C for 30 s, 68°C for 8 min). The inside primers for the second round PCR were SμOUT (5′-GATCCAAGGT GAGTGTGAGAGGACA-3′) and G1IN (5′-CATCCTGTCACCTATA CAGCTAAGCTG-3′); PCR reactions and cycle numbers were the same as above except that 64°C was used during the annealing step. Sμ/Sγ3 junctions in $S\mu^{-/-}$ mice were amplified by one round of PCR. Primers SμOUT (above) and Sγ3BK (5′-TACCCTGACCCAG GAGCTGCATAAC-3′) were used in PCR reactions for 36 cycles of amplification (94°C for 30 s, 62°C for 30 s, 68°C for 4 min). For isolating Sμ/Sγ3 junction sites in $S\mu^{-/-}$:*msh2*$^{-/-}$ mice, two rounds of PCR were carried out. The outside primers were Sμ1.1O (5′-TAGAATCAGTAAGGAGGGACC-3′) and Sγ3.1O (5′-CTACTGAG TTCCTGTGCTTG-3′); 36 cycles of PCR were performed (94°C for 30 s, 57°C for 30 s, 68°C for 4 min). The second round was identical except that primers Sμ1.2In (above) and Sγ3.2In (5′-TTGACCTGG TACCCTAGCACTC-3′) were used and the annealing temperature was increased to 61°C. All switch recombination sites were amplified using the Expand Long Template Taq and Pfu polymerase mix (Roche) to promote the production of longer PCR products and to decrease the occurrence of mutations during amplification. PCR products were cloned into pGEM-T Easy vector (Promega). Clones containing switch recombination sites were identified either by colony hybridization assays using the SμASO oligonucleotide probe (5′-GAATTGAGAAAGAATAGAGACCTGCAGTTG-3′) located near the upstream Sμ primer or by restriction enzyme digestion patterns.

Cloned PCR products were randomly selected for sequencing. Switch junction sequences were aligned with corresponding germline switch sequences (derived from Balb/c strain) using GAP (Accelrys) or BLAST2 (NCBI) programs. Mutations in individual switch sequences for $S\mu^{-/-}$ and $S\mu^{-/-}$:*msh2*$^{-/-}$ were identified by comparisons with compilations of switch region sequences that we have accumulated in both this and a previous study (Luby et al., 2001). The locations of switch recombination sites were assigned to minimize the number of mutations in the junction sequences when compared to germline switch sequences.

Secondary Structure Analyses of Switch Sequences

The procedures used to analyze the secondary structures of switch sequences closely followed the approaches that have previously been described (Tashiro et al., 2001). Structures of single-stranded switch sequences were predicted using a DNA folding program designed by Michael Zuker (Rensselaer Polytechnic Institute; http://www.bioinfo.rpi.edu/applications/mfold/old/dna); the folding conditions were 37°C with [Na^+] and [Mg^{2+}] concentrations of 150 and 0.5 mM, respectively. Structures were predicted for ~100–200 bp regions surrounding all the sequenced switch recombination sites; for each region only the most stable secondary structure was used for further analysis.

Statistics

Fisher's exact probability tests were used to assess the significance of differences between the numbers of class switch recombination sites located at pentamer sequences or somatic hypermutation hotspot motifs in switch regions within $S\mu^{-/-}$:*msh2*$^{-/-}$ mice relative to $S\mu^{-/-}$ mice. χ-squared tests were used to analyze whether the associations of switch junction sites to either pentamers or hotspots were significantly different from the germline frequency of pentamers or hotspots in switch regions and to assess the clustering of switch junctions to base and neck regions of stem-loop structures in the Sμ region. One-way analysis of variance followed by a Tukey test was used to compare differences in switching among multiple experimental mouse groups. Statistic tests were done with Graphpad Instat and Prism; p values of less than 0.05 were considered to be statistically significant.

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