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Increased Hypermutation at G and C Nucleotides in Immunoglobulin Variable Genes from Mice Deficient in the MSH2 Mismatch Repair Protein

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Summary

Rearranged immunoglobulin variable genes are extensively mutated after stimulation of B lymphocytes by antigen. Mutations are likely generated by an error-prone DNA polymerase, and the mismatch repair pathway may process the mispairs. To examine the role of the MSH2 mismatch repair protein in hypermutation, *Msh2*$^{-/-}$ mice were immunized with oxazolone, and B cells were analyzed for mutation in their V_κOx1 light chain genes. The frequency of mutation in the repair-deficient mice was similar to that in *Msh2*$^{+/+}$ mice, showing that MSH2-dependent mismatch repair does not cause hypermutation. However, there was a striking bias for mutations to occur at germline G and C nucleotides. The results suggest that the hypermutation pathway frequently mutates G·C pairs, and a MSH2-dependent pathway preferentially corrects mismatches at G and C.

Key words: biological sciences • genetics • genes, immunoglobulin • mutation • DNA repair

Hypermutation of immunoglobulin variable (V) genes occurs in B lymphocytes after antigen stimulation. Mutations are generated 1,000,000 times more frequently in V genes than in other genes, which implies that the mechanism that causes hypermutation is different than those that generate spontaneous mutations. Furthermore, the hypermutation mechanism is unique because it introduces mutations into a small area on three chromosomes that contain rearranged V, diversity, and joining (J) gene segments for heavy, κ, and λ immunoglobulin chains (for review see reference 1). A survey of the substitutions shows that transitions are found twice as frequently as transversions; germline G, A, and C nucleotides on the coding strand have an equal frequency of undergoing mutation, and T is mutated less frequently (2). Once mutations occur on the DNA strands, they would typically be substrates for the mismatch repair pathway.

During semiconservative replication, mismatched base pairs are recognized and corrected by several repair proteins. These proteins, MutS homologue 2 (MSH2)[1], MSH3, MSH6, post-meiotic segregation 2 (PMS2), and Mut L homolog 1 (MLH1), form a multiprotein complex that recognizes and excises mismatch errors (for review see reference 3). Once excised, DNA polymerases δ or ϵ resynthesize the repair gap. There are several ways in which the mismatch repair pathway could be involved in somatic hypermutation: (*a*) mismatch repair may actually cause the mutations by processing heteroduplex secondary structures that could form in the V region (4), (*b*) during occasional repair of DNA damage, an error-prone DNA polymerase might introduce mismatches in the repair gap (5, 6); or (*c*) mismatch repair might be actively suppressed in the V region, allowing normal replicative mismatch errors to accumulate. In any of these cases, mismatch repair may exhibit a preference for removing particular mismatches.

We analyzed the frequency and pattern of mutation in V genes from mice deficient for the MSH2 protein because this protein is a critical component of repair complexes that initially bind DNA mismatches. MSH2-deficient mice demonstrate a lack of mismatch repair in that they have unstable microsatellite repetitive sequences in transformed cells, and they have a high incidence of lymphoid tumors (7–9). MSH2-deficient cells from mice or humans are also defective in repairing single-base mismatches (10) and have

[1]*Abbreviations used in this paper:* MLH, Mut L homologue; MSH, Mut S homologue; *Pfu*, Pyrococcus furiosus; PMS, post-meiotic segregation; VκOx1, V gene for the κ chain that binds to oxazolone.

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elevated mutation rates (11–13). Overall, the immune system of *Msh2*$^{-/-}$ mice appears to be relatively normal in that the composition of T and B cells is similar to *Msh2*$^{+/+}$ mice (8). Thus, these mice should respond to antigen, and it will be possible to see what the pattern of hypermutation looks like before MSH2-dependent repair processes the mutations.

Materials and Methods

Mice. MSH2-deficient mice were generated by an insertion of a neomycin-resistance (*neo*) gene into one of the exons encoding MSH2 (8). Mice heterozygous for the *Msh2* gene on a mixed C57BL/6 and 129/Ola background were mated to produce F1 progeny. The mice were genotyped using a PCR-specific assay on DNA from ear notches. Two 8-wk-old *Msh2*$^{-/-}$ mice were immunized intraperitoneally with 100 μg of phenyl-oxazolone coupled to chicken serum albumin (provided by M. Neuberger, Cambridge, UK) in adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT). 4 wk later, the mice were given a secondary injection of 40 μg of antigen in adjuvant. 4 d later, the mice were killed and the spleens removed. Some 5% of splenic B cells that bound the B220 surface marker and peanut agglutinin (PNA) were sorted by flow cytometry using a FACStar Plus® (Becton Dickinson, San Jose, CA). Approximately 100 ng of DNA was isolated from about 50,000 cells after proteinase K digestion and phenol/chloroform extraction.

Detection of Neo Insert. To confirm that the mice used in this study contained the *neo* insert on both alleles, splenic DNA from the same B220^{+}PNA^{+} B cells that were analyzed for mutation was amplified by PCR using primer sequences obtained from Tak Mak (Amgen Institute, Toronto, Canada). To detect the wild-type exon, primers specific for the 5′ intron and *Msh2* exon were used, which would generate a 174-bp fragment: 5′ primer U771, 5′GCTCACTTAGACGCCATTGT3′, and 3′ primer L926, 5′AAAGTGCACGTCATTTGGA3′. To detect the *neo* insert, primers specific for the 5′ intron and the *neo* gene were used, which would generate a 460-bp product: 5′ primer U771 as above, and 3′ primer L1211, 5′GCCTTCTTGACGAGTTCTTC3′. A heterozygous mouse would produce both fragments. 10 ng of DNA from B220^{+}PNA^{+} spleen cells from C57BL/6 mice or *Msh2*$^{-/-}$ mice was amplified for 40 cycles and the products were detected by gel electrophoresis. As shown in Fig. 1, C57Bl/6 DNA contained the wild-type *Msh2* gene, and the *Msh2*$^{-/-}$ DNA had the correct size for a *neo* insertion into the *Msh2* gene.

V Gene Cloning and Sequencing. The rearranged V gene for the κ light chain that binds to oxazolone (VκOx1) gene was amplified from 20 ng of DNA by 30 rounds of PCR with *Pyrococcus furiosus* (*Pfu*) polymerase (Stratagene, La Jolla, CA) using a primer specific for the leader sequence on the 5′ side of the gene and a primer specific for the $J_κ5$ gene segment on the 3′ side. 1/25 of the reaction was then amplified for another 30 rounds using nested primers containing restriction sites. The 487-bp product, which included 5′ intron and V-J exon sequences, was cloned into M13 bacteriophage, and DNA from plaques was sequenced. The error-rate for *Pfu* polymerase under these conditions is 8×10^{-7} mutations/bp/duplication (14; Gearhart, P.J., data not shown); after 60 rounds of amplification of a 487-bp fragment, the cumulative error rate is 200-fold less than the number of mutations detected in the $V_κ$Ox1 gene.

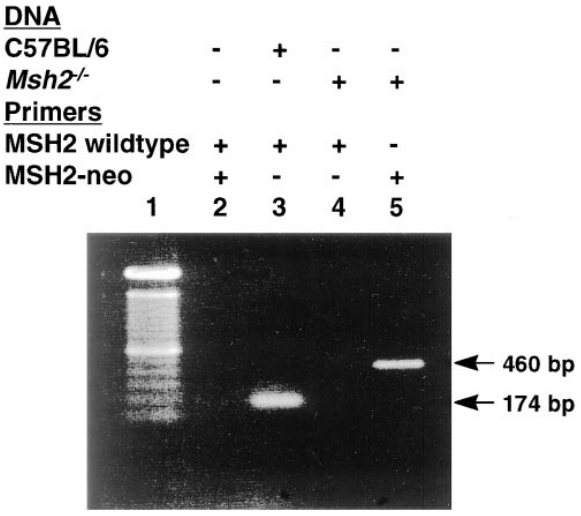

Figure 1. Detection of a *neo* insert in the *Msh2* gene in B220^{+}PNA^{+} splenic B cells from *Msh2*$^{-/-}$ mice. PCR products were electrophoresed through an agarose gel and stained with ethidium bromide. Lane *1*, 100-bp ladder; lanes *2–5*, amplification of DNA with primers as noted.

Statistical Analysis. A statistical test of whether the mutation frequency in A·T pairs is equal to the mutation frequency in G·C pairs was based on the ratio of the number of mutations in A·T pairs to the number of mutations in G·C pairs. The level of significance was determined using exact Poisson calculations (15), with correction for the unequal base composition in the region studied, where there are 263 A·T pairs and 203 G·C pairs.

Results

MSH2-Deficient Mice Have Hypermutated Antibodies. We studied mutation in the rearranged $V_κ$Ox1 gene segment because immunization of mice with oxazolone elicits a well-characterized antibody response (16). Some 38 clones were sequenced over a length of 487 bp, which included 190 nucleotides of 5′ intron sequence between the leader and V gene segment, and 297 nucleotides of the V-J gene. Sequences at the V-J junction were used to establish clonal identity, but were not included in the mutational analysis because variant nucleotides at the site of joining may be introduced by the recombination mechanism rather than by the hypermutation pathway. Thus, mutations were recorded for 466 nucleotides starting downstream of the leader sequence in the 5′ intron and ending in the V gene before the J gene segment. 44 out of 60 *Msh2*$^{-/-}$ clones had mutation, or 73%, compared to 49 out of 82 *Msh2*$^{+/+}$ clones with mutation, or 60%. To calculate the frequency of mutation, we only considered the mutated clones. This ensures that the analysis is performed on clones from B cells that have been activated to mutate, rather than including nonmutated clones from B cells that may not have been stimulated. 22 of the mutated *Msh2*$^{-/-}$ clones were unique in that they either had different sequences at the V-J junction, indicating they came from independent precursor B cells, or they had unique single substitutions that were not shared by other clones. As listed in Table 1, the number of

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Table 1. *Mutations in $V_\kappa Ox1$ Genes from MSH2-deficient Mice*

Clone	Number	Position*
M42	1	C30T
M70	1	G160A
M5	2	G37T, C97A
M53	4	G25C, C97A, A104T, G227A
M128	5	G37A, C78T, C97A, A104T, G227A
M89	5	G25A, C78T, C97A, A104T, G227A
M34	5	G77A, A87T, G89C, C97A, C261T
M71	5	G25A, G96A, C99G, A104T, G119C
M95	6	G227A, G235A, G264A, G267A, G269A, G270T
M106	6	T-129A, G166A, C176T, G195A, A226C, C228A
M85	7	G71C, G73C, C78T, G96A, C99G, A104T, G227A
M11	7	C20A, G71T, C99G, C122T, C126A, G175A, G227A
M23	7	G25A, G96A, C99G, A104T, G166A, C176T, G227C
M10	7	G-79T, C78G, C99G, A104T, G119C, C225T, G227A
M36	7	G-125A, C-122T, G25A, G96A, C99G, A104T, G119C
M115	7	C41T, C43T, C69T, C97A, G166A, C176T, G227C
M117	7	C60A, C66T, T72G, G96A, T115C, C120A, A121T
M120	7	G71A, G73A, G77A, G83A, G89A, C105T, C204T
M101	8	C41T, C43T, G96A, C99A, C105T, G166A, G224A, C228T
M119	8	G68A, G77C, C99G, A104T, G108A, A207T, G224A, G207A
M64	9	C78T, G83C, G96A, C120T, C123T, C125T, G175A, G224A, G227A
M82	14	G71C, G73C, C78T, C80T, G96A, C99G, A104T, C123T, C126T, A133T, G134T, A217T, G224A, G227A

Total = 22 clones (10,252 bp); 135 mutations; 1.3% mutations/bp.
*Mutations are listed with the first letter representing the germline nucleotide, the number depicting the position of the mutation as shown in Fig. 2, and the second letter signifying the mutant nucleotide. Negative numbers correspond to mutations in the 5′ intron (17). Tandem mutations are underlined.

mutations per *Msh2*$^{-/-}$ clone ranged from 1 to 14, with an average frequency of 1.3% mutations/bp. This frequency is very similar to that found in *Msh2*$^{+/+}$ C57BL/6 clones for the V_κOx1 gene, where the number of mutations per clone ranged from 1 to 17, with an average frequency of 1.4% mutations/bp (18). All 135 mutations in Table 1 were nucleotide substitutions, and only three were located in the 5′ intron. Three pairs of tandem mutations, or two substitutions in a row, were observed.

To determine if the pattern of mutation differs between *Msh2*$^{-/-}$ clones and *Msh2*$^{+/+}$ C57BL/6 clones, the position and types of mutations were identified. As shown in Fig. 2, many of the mutations in both strains of mice were located in the first complementarity-determining region at amino acid codons 34 and 36 (nucleotides [nt] 97–99 and 103–105). Mutations at these residues have been shown to increase the affinity of antibodies by 10-fold (20) because these sites directly interact with the oxazolone molecule (21). Thus, B cells expressing antibodies with mutations in codons 34 and 36 are highly selected based on their greater affinity for antigen than nonmutated antibodies. Comparing the patterns, several hypermutable sites were observed in *Msh2*$^{-/-}$ clones but not in *Msh2*$^{+/+}$,C57BL/6 clones: nt 96 (codon 33), which is not located in the RGYW motif that is suggested to be a preferential sequence for mutation (22), and nt 227 (codon 77), which is in an RGYW sequence.

Msh2$^{-/-}$ *Clones Have Predominantly Mutations at G and C Nucleotides.* The data in Fig. 2 demonstrate that most of the mutations in the *Msh2*$^{-/-}$ clones are substitutions for germline G or C. An analysis of the nucleotide changes for each base is summarized in Table 2. For comparison, data from identical mutational analyses of V_κOx1 genes (18) from another mismatch repair–deficient strain, *Pms2*$^{-/-}$, a nucleotide excision repair–deficient strain, xeroderma pigmentosum group A (*Xpa*$^{-/-}$), and the C57BL/6 strain are also included. In the *Msh2*$^{-/-}$ sequences, very few mutations at germline A and T and a much greater proportion of mutations at germline G and C were observed in comparison to the other three strains. *Msh2*$^{-/-}$ clones exhibited only nine mutations at A and T versus 91 mutations at G and C, whereas clones derived from the other strains had

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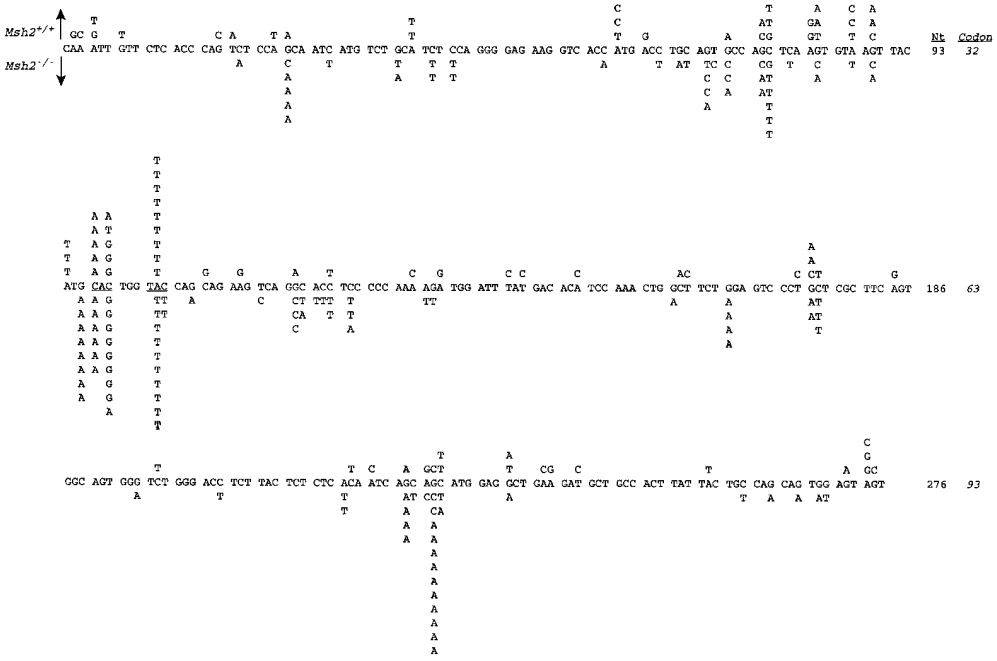

Figure 2. Location of mutations in the V_κOx1 gene from *Msh2*$^{+/+}$ C57BL/6 and *Msh2*$^{-/-}$ mice. The coding region of the V_κOx1 gene segment is shown; nucleotide and codon numbering is according to Kabat et al. (19). The 276-bp coding region contained 90% of all mutations occurring in the entire 466-bp sequence. *Msh2*$^{+/+}$ C57BL/6 substitutions (18) are displayed above the coding sequence and *Msh2*$^{-/-}$ substitutions are shown below. Codons 34 (nt 97–99) and 36 (nt 103–105) are underlined.

approximately the same number of mutations at A·T and G·C pairs. When corrected for nucleotide composition, a ratio of 1.3:1 mutations at A·T to G·C pairs would be expected if the mutation rates at both pairs were equal. As shown in Fig. 3, *P* values for whether the ratio was 1.3:1 were $<10^{-6}$ for *Msh2*$^{-/-}$ clones, 0.21 for *Pms2*$^{-/-}$ clones, 0.20 for *Xpa*$^{-/-}$ clones, and 0.90 for C57BL/6 clones. Thus, hypermutation of the V_κOx1 gene in *Msh2*$^{-/-}$ mice is vastly skewed towards targeting G·C pairs compared to the other strains.

Discussion

Msh2$^{-/-}$ mice do not appear to be immunodeficient for hypermutation by the following criteria. First, these mice possess the same percentage of PNA$^+$ B cells as *Msh2*$^{+/+}$ mice, suggesting that the B cells passaged through germinal centers (23). Second, the frequency of mutation between *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ C57BL/6 clones was identical, indicating that there was active hypermutation at the V_κOx1 locus. Third, B cells in both strains of mice underwent affinity maturation as codons 34 and 36 were selected for the mutations that produce high affinity antibodies to oxazolone (20, 21). It is quite possible that *Msh2*$^{-/-}$ mice have other immune defects; for example, they may have fewer germinal centers. However, it has been shown that hypermutation and affinity maturation can proceed in the absence of germinal centers (24).

Unexpectedly, the frequency of mutation was not higher in V_κ genes from MSH2-deficient mice compared to wild-type mice. Jacobs et al. (25) also reported a normal frequency of mutation in the V_λ1 gene from *Msh2*$^{-/-}$ mice. Perhaps the hypermutation frequency in V genes is already so high at 10^{-2} mutations/bp that the molecule cannot tolerate higher loads of mutation because the antibody protein will be nonfunctional. Since MSH2 is necessary for canonical mismatch repair, the data strongly suggest that this repair pathway is not required to generate somatic hypermutation in V genes. We observed a high level of mutation (0.9% mutations/bp) in the V_κOx1 gene from mice defi-

Table 2. *Pattern of Substitutions in V_κOx1 Genes from DNA Repair–deficient Mice*

	Msh2$^{-/-}$		*Pms2*$^{-/-}$		*Xpa*$^{-/-}$		C57BL/6	
Substitution	%	(No.)	%	(No.)	%	(No.)	%	(No.)
A to G	0	(0)	17	(13)	19	(23)	19	(17)
A to T	5	(5)	8	(6)	8	(10)	16	(15)
A to C	1	(1)	8	(6)	6	(7)	11	(10)
T to C	1	(1)	7	(5)	8	(10)	9	(8)
T to A	1	(1)	4	(3)	7	(9)	2	(2)
T to G	1	(1)	5	(4)	2	(2)	1	(1)
C to T	25	(27)	12	(9)	17	(21)	10	(9)
C to A	5	(5)	3	(2)	3	(4)	2	(2)
C to G	1	(1)	9	(7)	2	(2)	2	(2)
G to A	44	(48)	19	(15)	19	(24)	17	(16)
G to T	5	(5)	1	(1)	3	(4)	4	(4)
G to C	11	(13)	7	(5)	6	(7)	7	(6)

Mutations in the 5′ flanking and V_κOx1 coding region are listed. Mutations in codons 34 and 36 were excluded because of strong immunological selection for these mutations. Data for *Pms2*$^{-/-}$, *Xpa*$^{-/-}$, and C57BL/6 clones (18) were generated the same way as in this *Msh2*$^{-/-}$ study.

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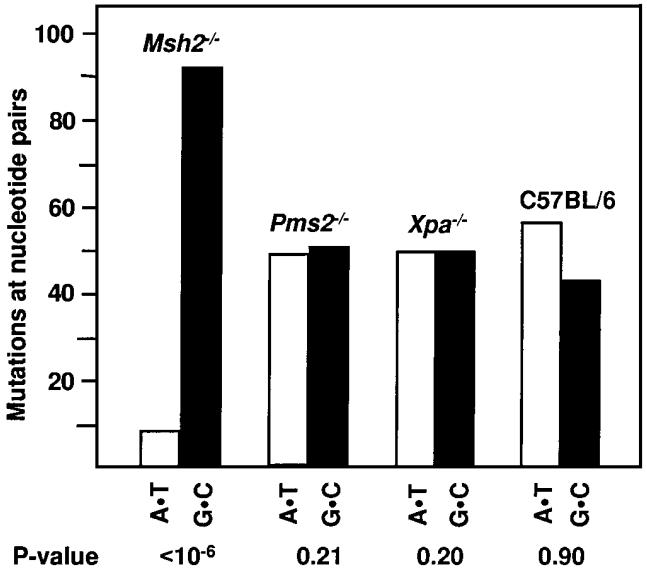

Figure 3. Bias for mutations at G·C pairs in *Msh2*$^{-/-}$ clones. Frequencies of mutation of germline nucleotide pairs to any other base were calculated after correction for base composition. *P* values for whether the mutation frequency in A·T pairs is equal to the mutation frequency in G·C pairs are shown.

cient for another mismatch repair protein, PMS2 (18), further confirming that mismatch repair does not generate mutations in immunoglobulin genes. On the other hand, Cascalho et al. (26) reported a decreased frequency of mutation in *Pms2*$^{-/-}$ quasimonoclonal mice and suggested that mismatch repair is involved in fixing mutation once it is generated. Since many factors can influence the frequency of mutation, such as quality of antigen stimulation, rate of transcription (27), and germinal center formation (24), decreased frequencies should be interpreted with caution.

Furthermore, localized inactivation of the mismatch repair pathway is not solely responsible for permitting large numbers of mutations to remain around the rearranged V gene. Indeed, an active mechanism for increasing the base substitution frequency must be used to generate the observed frequency of 10^{-2} mutations/bp. Simple inactivation of mismatch repair may reveal frequent insertions and deletions of nucleotides that are produced by slippage of DNA polymerase during normal semiconservative DNA replication (8, 28). Insertions and deletions would be most evident in the noncoding flanking sequences where they would not cause a frameshift. In this study, there were no insertions or deletions in the 5′ flanking or coding regions of V genes from *Msh2*$^{-/-}$ mice. However, occasional insertions and deletions that are templated by adjacent nucleotides have been identified in a recent study (29), which suggests that slippage of DNA polymerase occurs infrequently.

Tandem mutations of two in a row were observed at a high frequency in another mismatch repair–deficient strain lacking PMS2 (18), suggesting that the PMS2 component of the mismatch repair pathway is involved in repairing doublet mutations. To determine if the MSH2 protein is also used to repair tandem mutations, we analyzed the mutational pattern. Three tandem mutations in *Msh2*$^{-/-}$ clones were observed (Table 1) compared to 1.8 expected by chance, and exact Poisson calculations indicate no significant excess of tandem mutations ($P = 0.56$). In contrast, there were 11 tandem mutations observed in *Pms2*$^{-/-}$ clones compared to only 1.2 expected ($P < 10^{-6}$). Thus, repair of tandem mutations introduced by the hypermutation mechanism is dependent on PMS2, but appears largely independent of MSH2, suggesting a pathway for repair of certain mismatches that does not involve all the canonical components of mismatch repair.

MSH2 appears to play a dominant role in modifying the spectrum of mutations formed during hypermutation. A very different mutational pattern was seen in the *Msh2*$^{-/-}$ clones, with the vast majority of mutations occurring at germline G and C nucleotides compared to A and T nucleotides ($P < 10^{-6}$). We noticed that data from Jacobs et al. (25) had a similar bias for mutation at G·C pairs in rearranged V_λ genes from an independently derived MSH2-deficient mouse (7). Our calculations of *P* values for their data showed that *Msh2*$^{-/-}$ clones had a significant excess of mutations at G·C pairs ($P = 0.001$) unlike clones from wild-type and other DNA repair–deficient mice in their study ($P > 0.4$). Although the effect of driving mutation at G·C pairs produced new hot spots of mutation with corresponding amino acid changes in the V_κOx1 gene (Fig. 2), the G·C bias did not affect selection for high affinity antibodies. Thus, infrequent mutations of A at nt 104 in codon 36 were strongly selected because they change the tyrosine codon to phenylalanine, which confers a 10-fold increase in affinity on the antibody molecule (20, 21).

The skewed mutational pattern in *Msh2*$^{-/-}$ clones predicts that during hypermutation, either G or C are chemically modified to cause mispairing, or an error-prone polymerase frequently introduces a wrong base opposite these nucleotides. For convention, all the mutations in Table 2 were recorded from the coding strand, although it is not known on which strand mutations actually occur. Chemical modifications of G and C can occur by several methods. First, G can be oxidized to 8-oxyguanine (30) that, if not removed by the base excision repair pathway, preferentially pairs with A to cause a G to T transversion on one strand or the corresponding C to A transversion on the opposite strand. Germinal centers express a high level of 8-oxoguanine glycosylase (31), suggesting there is a high level of oxygen radicals in this tissue. However, this cannot be a major mechanism of somatic hypermutation in V genes since G to T and C to A changes only account for 1–5% of the substitutions in all four strains of mice shown in Table 2. Second, 5-methyl C in a CpG motif can undergo deamination to produce the C to T transition (32). However, none of the mutations occurred at CpG dinucleotides. Third, C could spontaneously deaminate to produce uracil, leading to a C to T transition (33), or C could be oxidized to 5-hydroxy cytidine (34), but these damaged bases are usually removed

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by uracil-DNA glycosylase and endonuclease III type enzymes. It is possible that some of these enzymes are deficient in germinal centers from *Msh2*$^{-/-}$ mice, which could produce the altered pattern. Alternatively, an error-prone polymerase could frequently introduce a wrong base opposite G or C. One of the known DNA polymerases may be modified to become more error prone (for review see reference 35), or a novel polymerase may be expressed in germinal centers to generate the high frequency of substitutions.

Concomitantly, the data predict that MSH2 preferentially corrects mismatches of G or C relative to mismatches of A or T. For example, if a DNA polymerase puts T opposite a germline G, the G·T mismatch will be recognized by a heterodimer of MSH2 and MSH6 proteins (36, 37). In the absence of MSH2, G·T mispairs would remain and produce an increased frequency of G to A mutations. In the presence of MSH2, the disproportionate number of mismatches at G or C would be corrected, and the final frequency of mutation at germline-encoded G·C and A·T pairs would be equal. MSH2-dependent repair of G and C mismatches in V genes appears to be independent of PMS2, since a G·C bias was not observed in *Pms2*$^{-/-}$ clones. As MSH2 normally forms a heterodimer with the MSH6 or MSH3 proteins during conventional mismatch repair (38), it will be interesting to determine the phenotype of mutation in mice deficient for the latter two proteins.

A bias for mutations at G·C pairs has been reported in IgM molecules from *Xenopus* and horned shark (39–41), raising the possibility that this phenotype indicates low or absent levels of the MSH2 protein in some cold blooded vertebrates. Mismatch repair has not been studied in these species, and it would be interesting to see if they are deficient in repair of G and C mismatches. A bias for mutations at G and C bases was also noted in a murine pre–B cell line (42). Similarly, humans with defective *Msh2* genes, as identified by susceptibilty to colorectal and other cancers (43), may have altered patterns of hypermutation that affect the ability of their antibodies to bind antigen efficiently.

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