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Differing patterns of genetic instability in mice deficient in the mismatch repair genes *Pms2, Mlh1, Msh2, Msh3* and *Msh6*

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Defects in genes associated with DNA mismatch repair (MMR) have been linked to hereditary colon cancer. Because the MMR pathway includes multiple factors with both overlapping and divergent functions, we sought to compare the impact of deficiencies in each of several MMR genes on genetic instability using a collection of knock-out mouse models. We investigated mutation frequencies and patterns in MMR-deficient mice using two transgenic reporter genes, *supFG1* and *cII*, in the context of mice deficient for *Pms2, Mlh1, Msh2, Msh3* or *Msh6* or both *Msh2* and *Msh3* or both *Msh3* and *Msh6*. We found that the mean mutation frequencies of all of the MMR-deficient mice were significantly higher than the mean mutation frequencies of wild-type mice. *Mlh1*-deficient mice and *Msh2*-deficient mice had the highest mutation frequencies in a comparison of the single nullizygous mice. Of all the mice studied, mice nullizygous for both *Msh2* and *Msh3* and those nullizygous for both *Msh3* and *Msh6* displayed the greatest overall increases in mutation frequencies compared with wild-type mice. Sequence analysis of the mutated reporter genes revealed significant differences between the individual groups of MMR-deficient mice. Taken together, our results further characterize the functions of the MMR factors in mutation avoidance and provide *in vivo* correlation to biochemical models of the MMR pathway.

Introduction

The DNA mismatch repair (MMR) system in humans is essential in order to maintain genomic stability. MMR deficiency has been shown to result in an increased risk of developing cancer, particularly hereditary non-polyposis colorectal cancer (HNPCC). Cells deficient in MMR exhibit genetic instability manifested as microsatellite instability (MSI) and point mutations. MSI, and thus the loss of MMR, has been seen in a variety of sporadic as well as familial cancers, including endometrial, lung, breast, pancreatic, gastric and prostate (1). The MMR system functions to preserve genomic stability not only by base mispair correction but also by stimulating apoptosis in response to DNA damage caused by physical and chemical agents (2).

In mammalian cells there are multiple homologs of the *Escherichia coli* MutS and MutL proteins. The MutS homologs, MSH2, MSH3 and MSH6, are crucial for mismatch recognition. MSH2 forms heterodimers with either MSH6 (MutSα) or MSH3 (MutSβ). The MutL homologs include MLH1, PMS1 and PMS2. Both of the MutS complexes interact with the MutLα heterodimer, which consists of MLH1 and PMS2. The MutLα complex appears to play the key role in the process of mismatch correction by linking the mismatch recognition of MutSα and MutSβ with the excision of mutations and resynthesis of corrected bases (3). More recently, the novel MMR gene *Mlh3* has been shown to interact with *Mlh1* and contribute to MMR as well (4–6). In addition, a number of homologs, such as MSH4 and MSH5, have been identified that have not been linked to hereditary cancer, and may participate in meiosis and other cellular functions besides mismatch recognition (7). MLH3 has also been shown to play a crucial role in mammalian meiosis (8).

Since there is such a variety of MutS and MutL homologs in mammalian cells, it has been proposed that there is both divergence as well as redundancy of function. For example, the MutSα complex recognizes single base pair point mutations and small insertion/deletion loops (IDLs) and the MutSβ complex recognizes base pair mismatches and large IDLs (3,7).

In the human syndrome of HNPCC, affected individuals primarily have mutations in *MSH2* or *MLH1*, but defects in *MSH6, PMS1* and *PMS2*, although less common, have also been shown to be a factor (3,9–11). Many sporadic cancers have also been found to display MMR deficiency due to the silencing of the *MLH1* gene via promoter hypermethylation (10).

A number of studies have examined genetic instability in the setting of MMR deficiency using both cell culture and mouse models (6,12–17). The initial mouse studies have begun to define *in vivo* patterns of mutations in the presence of MMR deficiency (6,15–17). However, most previous studies have focused on individual genotypes or on pairwise comparisons. To more systematically compare the functional roles of the MutS and MutL homologs in mammalian cells, we utilized a series of knock-out mouse models to examine patterns of genetic instability *in vivo* due to a deficiency in *Pms2, Mlh1, Msh2, Msh3, Msh6, Msh2* and *Msh3*, or *Msh3* and *Msh6*. On the basis of the studies mentioned above, as well as the fact that *MSH2* and *MLH1* are the genes most commonly associated with HNPCC-affected individuals, we hypothesized that the $Mlh1^{-/-}$ and $Msh2^{-/-}$ mice would have the highest mutation frequencies. We also hypothesized that the $Pms2^{-/-}$, $Msh6^{-/-}$ and $Msh3^{-/-}$ mice would have intermediate levels of mutations, with the relative pattern potentially providing insight into the association of these genotypes with cancer predisposition.

Abbreviations: MMR, mismatch repair; SEM, standard error of the mean.

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This work was facilitated by the use of transgenic mice carrying either the *supFG1* or the *cII* mutation reporter genes within chromosomally integrated, recoverable lambda shuttle vectors. These reporter genes and lambda vector constructs serve as powerful tools for directly studying mutagenesis *in vivo* and their relatively short lengths, sensitivity and low background of spontaneous mutation frequencies allow accurate determination of the frequencies or types of mutations due to MMR deficiency.

We found that the extent to which genetic instability, measured by the frequencies of mutations in two different reporter transgenes, is affected in mice deficient in the MMR genes varied considerably, but that all the groups of MMR-deficient mice had mean mutation frequencies significantly higher than the mean mutation frequencies of wild-type mice. Among the MMR single nullizygous mice, *Mlh1* and *Msh2* deficiency produced the greatest instability, whereas *Msh3* deficiency generated the least. Compared with wild-type, the double mutant mice deficient for both *Msh2* and *Msh3* or deficient for both *Msh3* and *Msh6* displayed the largest increases in mutation frequencies of all the groups. In addition, examination of the mutation patterns indicated differences that may provide insight into the role of the various MMR proteins in mismatch correction. The results presented here also add to the increasing amount of evidence that the MMR factors play both distinctive and redundant roles in DNA repair.

Materials and methods

Transgenic mice and genotyping

Mice nullizygous for the *Pms2* (18), *Mlh1* (19), *Msh2* (20), *Msh3* (21) or *Msh6* (22) genes were crossed with transgenic mouse lines carrying either the *supFG1* (3340 strain) or *cII* (Muta™ Mouse strain, Covance of Denver, PA, USA) mutation reporter genes within chromosomally integrated, lambda shuttle vectors to create $MMR^{+/+}$/*supFG1*, $MMR^{+/-}$/*supFG1*, $MMR^{-/-}$/*supFG1*, $MMR^{+/+}$/*cII*, $MMR^{+/-}$/*cII* and $MMR^{-/-}$/*cII* offspring. All mice were derived from the C57BL/6 mouse background, and construction of the *supFG1* and *cII* lambda shuttle vectors has previously been described (13,23). Offspring that contained either the *supFG1* or *cII* mutational reporter genes and heterozygous for each of the above-listed MMR genes were bred to create sibling sets of wild-type and nullizygous mice to be studied for mutational analysis. Heterozygotes were used for breeding to continue to create sibling sets of wild-type and nullizygous mice. All genotypes were determined by polymerase chain reaction (PCR) of tail DNA as described previously for *Pms2* (18), *Mlh1* (19), *Msh2* (20), *Msh3* (21) and *Msh6* (21). The presence or absence of the *supFG1* or *cII* reporter gene was also determined by PCR as described previously (13,23,24).

For both the *supFG1* and *cII* lines, $Msh2^{+/-}$ mice were bred with $Msh3^{+/-}$ in order to generate mice heterozygous for both MMR genes. These double heterozygotes were then bred to generate mice deficient for both *Msh2* and *Msh3*. The same procedure was followed to produce mice deficient for both *Msh2* and *Msh6* and for both *Msh3* and *Msh6*. However, we were unable to obtain mice deficient for both *Msh2* and *Msh6* in spite of 37 breeding attempts and analysis of 218 pups. Genotypes were determined as described above.

λ Shuttle vector rescue and mutagenesis assays

Sibling mice either wild-type or nullizygous for each of the five MMR genes and containing either the *supFG1* or *cII* reporter genes were killed at age 10–12 weeks by cervical dislocation. Skin (epidermis plus superficial dermis) and proximal colon (smooth muscle layers plus epithelium) samples were collected, quick frozen in liquid nitrogen and stored at −80°C. A total of 2–4 mice were killed for each of the 10 genotypes.

The procedure described above was also performed with $Msh2^{-/-}$/$Msh3^{-/-}$ mice and $Msh3^{-/-}$/$Msh6^{-/-}$ mice containing either the *supFG1* or *cII* reporter genes. A total of 2–3 mice were killed for each of these four genotypes.

High molecular weight DNA was prepared from the skin and colon samples as described previously (24). We chose to use the skin and colon because previous work in our laboratory has shown these tissues to provide good DNA preparation for efficient λ *in vitro* packaging. λ *in vitro* packaging extracts were made as described previously and were used to rescue phage vectors from skin and colon DNA (13,24–27). Rescued phage were analyzed for *supFG1* and *cII* mutations as described previously (13,24). Briefly, functional *supFG1* reporter genes suppress the nonsense mutation in the host bacteria β-galactose gene, yielding blue plaques, whereas mutations inactivating the *supFG1* gene produce colorless phage plaques (13). The plates were screened for colorless plaques and the total number of plaques per plate was calculated. For the *cII* reporter gene, at 24°C wild-type phage form lysogens and therefore do not form plaques. However, phage with inactivating mutations can be detected through lytic plaque production after 48 h at this temperature (28). The mutants produced at 24°C were counted and the total number of packaged phage was determined by titers plated at 37°C, which allows lytic plaque production regardless of *cII* status (28).

Mutant sequencing

A portion of mutant plaques from each of the 14 groups of knock-out mice were picked immediately upon detection and plaque-purified. The plaque-purified mutants were amplified by PCR and purified by the Qiagen PCR purification kit. Sequence analysis was performed as described (13). Primers used to amplify the *supFG1* or *cII* region from the DNA and for sequencing have been described previously (13,23).

Statistics

Mutation frequencies were defined as the number of mutant plaques per the total number of plaque forming units from skin and colon DNA of each mouse. The raw data of the wild-type mice (separately for each of the two reporter genes) were combined and the means of the skin and colon mutation frequencies were used to compare the MMR nullizygous mice. Statistical differences among mutation frequencies were determined by the unpaired *t*-test (Prism Version 4, GraphPad Software). The mutation frequency for each group of mice is expressed as the mean ± standard error of the mean (SEM). Statistical significance was determined as $P < 0.05$.

Results

Generation of mice deficient in selected MMR factors

Mice were bred as described in Materials and methods in an attempt to generate a series of transgenic mice containing either the *supFG1* or *cII* mutation reporter gene (in a chromosomally recoverable lambda phage vector) along with specific genotypes at MMR loci as follows: (i) *WT*; (ii) *Pms2* null; (iii) *Mlh1* null; (iv) *Msh2* null; (v) *Msh3* null; (vi) *Msh6* null; (vii) *Msh2* and *Msh3* double null; (viii) *Msh2* and *Msh6* double null; and (ix) *Msh3* and *Msh6* double null. All desired genotypes were obtained except for *Msh2* and *Msh6* double nulls, in spite of numerous attempts.

Mutation frequencies in supFG1 mice

The mean mutation frequencies were determined for the skin and colon DNA of the *supFG1*/wild-type, *supFG1*/$Pms2^{-/-}$, *supFG1*/$Mlh1^{-/-}$, *supFG1*/$Msh2^{-/-}$, *supFG1*/$Msh3^{-/-}$, *supFG1*/$Msh6^{-/-}$, *supFG1*/$Msh2^{-/-}$/$Msh3^{-/-}$ and *supFG1*/$Msh3^{-/-}$/$Msh6^{-/-}$ mice. The skin and colon sub-totals for the mutants/plaques were combined since there were no significant differences found between the two tissues (data not shown). Table I conveys the total number of mutants per the total number of plaques counted for each of the 12 groups of mice, including wild-type (Supplementary Table IV presents the mutants/plaques and mutation frequencies for each individual animal), and Figure 1 presents a graphical comparison of the mean mutation frequencies. The overall average frequency of mutations in the skin and colon DNA of the wild-type *supFG1* mice (Figure 1) is consistent with baseline mutation frequencies previously observed in such transgenic animal systems (13,28).

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Table I. Mutation frequencies in wild-type and MMR-deficient mice in the *supFG1* and *cII* reporter genes

MMR gene deficiency	Number of mice	Wild-type		Nullizygous	
		Mutants/total plaques	Frequency ($\times 10^{-5}$)[a]	Mutants/total plaques	Frequency ($\times 10^{-5}$)[a]
supFG1					
Pms2	3 WT, 3 KO	32/381 363	9.2	664/277 756	220.7
Mlh1	3 WT, 4 KO	59/525 640	11.3	4560/635 452	616.8
Msh2	2 WT, 3 KO	35/331 299	10.4	2820/467 502	555.1
Msh3	3 WT, 3 KO	55/680 415	8.2	84/557 240	14.5
Msh6	2 WT, 3 KO	8/210 291	3.8	469/409 987	116.0
Msh2/Msh3	3 KO/KO			4065/507 039	777.8
Msh3/Msh6	3 KO/KO			4419/490 062	873.4
cII					
Pms2	2 WT, 3 KO	18/530 354	3.4	186/788 314	23.6
Mlh1	3 WT, 4 KO	122/1 952 754	6.4	381/1 163 005	34.5
Msh2	4 WT, 3 KO	53/1 106 760	6.1	329/904 323	35.7
Msh3	4 WT, 3 KO	56/1 121 690	5.0	78/784 595	9.7
Msh6	3 WT, 2 KO	51/1 103 242	4.6	33/395 894	9.4
Msh2/Msh3	2 KO/KO			849/1 296 294	53.9
Msh3/Msh6	3 KO/KO			1399/1 465 170	90.5

[a]Mutation frequency was determined as the average of the means.

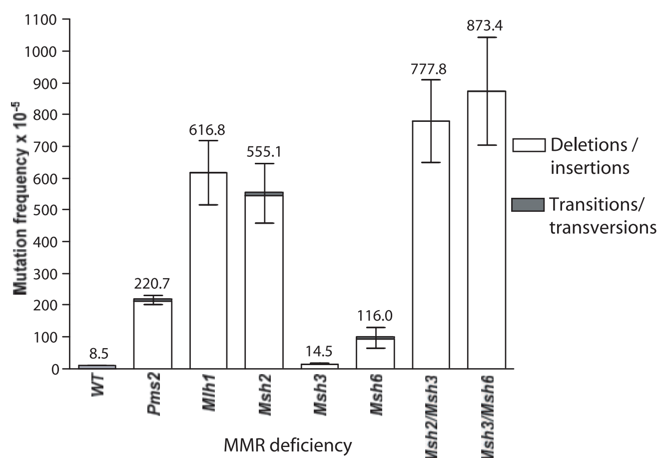

Fig. 1. Comparison of *supFG1* reporter gene mutation frequencies in wild-type and MMR-deficient mice. The mutation frequency for each group of mice is expressed as the mean ± SEM. The wild-type mutation frequency is the mean ± SEM of the skin and colon mutation frequencies of all the wild-type mice listed in Table I. The absolute frequencies of the types of mutations are indicated by the shaded segments of each bar. A total of 74 mutants were analyzed for the *Pms2*$^{-/-}$ mice, 81 for the *Mlh1*$^{-/-}$ mice, 59 for the *Msh2*$^{-/-}$ mice, 64 for the *Msh3*$^{-/-}$ mice, 62 for the *Msh6*$^{-/-}$ mice, 24 for the *Msh2*$^{-/-}$/*Msh3*$^{-/-}$ mice and 24 for the *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice.

With respect to the MMR-deficient mice, all the groups exhibited significantly higher mutation frequencies than the wild-type mice (pairwise statistical comparisons between each genotype are shown in Table II). *Mlh1*-deficient mice exhibited the highest mutation frequencies among the *supFG1* single nullizygous mice, >72 times the mutation frequencies of wild-type mice (Figure 1). The mutation frequencies of the *Msh2*-deficient mice were also quite elevated, with a 65-fold increase above wild-type (Figure 1). Of the *supFG1*/MMR-deficient mice, the *Msh3*-deficient mice exhibited the lowest, yet still significantly elevated, mutation frequencies (Table II and Figure 1).

The mutation frequencies observed in the *Msh2*$^{-/-}$/*Msh3*$^{-/-}$ double knock-out mice were significantly greater than those of the wild-type mice (by over 90-fold), as well as those of the *Pms2*$^{-/-}$, *Msh6*$^{-/-}$ and *Msh3*$^{-/-}$ mice (Table II and Figure 1). The mutation frequencies of the *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ double knock-out mice were >100-fold higher than the wild-type mutation frequencies and also significantly higher than those of the *Pms2*$^{-/-}$, *Msh6*$^{-/-}$ and *Msh3*$^{-/-}$ mice (Table II and Figure 1).

Mutation frequencies in cII mice

The mean mutation frequencies were determined for the skin and colon DNA of the *cII*/wild-type, *cII/Pms2*$^{-/-}$, *cII/Mlh1*$^{-/-}$, *cII/Msh2*$^{-/-}$, *cII/Msh3*$^{-/-}$, *cII/Msh6*$^{-/-}$, *cII/Msh2*$^{-/-}$/*Msh3*$^{-/-}$ and *cII/Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice. Similar to the *supFG1* mice, the skin and colon mutants/plaques were combined since there were no significant differences found between the two tissues (data not shown). Table I presents the total number of mutants per the total number of plaques counted for each of the mouse groups (Supplementary Table V displays the sub-totals of mutants/plaques and mutation frequencies for each individual animal) and Figure 2 presents a graphical comparison of the mean mutation frequencies. The overall average of the skin and colon DNA mutation frequencies of the wild-type *cII* mice (Figure 2) is, as in the case of the *supFG1* gene, consistent with previous studies of baseline *cII* mutation frequencies in other studies of lambda vector-containing transgenic mice (28).

On the basis of the pairwise statistical comparisons shown in Table II, all of the groups of *cII* mice exhibited significantly higher mutation frequencies than the wild-type mice. The *Msh2*-deficient mice and the *Mlh1*-deficient mice exhibited the highest mutation frequencies among the single nullizygous mice (Figure 2), with both having mutation frequencies >7-fold greater than the wild-type mice. The *Msh3*-deficient and *Msh6*-deficient mice presented the lowest mutation frequencies of the *cII* mice, but these were still significantly higher than those of the wild-type mice (Table II and Figure 2).

The mutation frequencies seen in the double nullizygous *Msh2*$^{-/-}$/*Msh3*$^{-/-}$ mice were significantly greater than the mutation frequencies of the wild-type, *Pms2*$^{-/-}$, *Msh6*$^{-/-}$ and *Msh3*$^{-/-}$ mice (Table II). Interestingly, the mutation

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Table II. Pairwise tests of statistical significance in comparisons of *supFG1* and *cII* reporter gene mutation frequencies in wild-type and MMR-deficient mice

	Msh3/Msh6	*Msh2/Msh3*	*Msh6*	*Msh3*	*Msh2*	*Mlh1*	*Pms2*
supFG1							
WT	**<0.001**	**<0.001**	**<0.001**	**0.044**	**<0.001**	**<0.001**	**<0.001**
Pms2	**0.007**	**0.004**	**0.038**	**<0.001**	**0.014**	**0.009**	
Mlh1	0.205	0.344	**0.001**	**<0.001**	0.675		
Msh2	0.134	0.201	**0.002**	**<0.001**			
Msh3	**<0.001**	**<0.001**	**0.017**				
Msh6	**<0.001**	**<0.001**					
Msh2/Msh3	0.663						
cII							
WT	**<0.001**	**<0.001**	**0.031**	**0.027**	**<0.001**	**<0.001**	**<0.001**
Pms2	**0.0011**	**0.0322**	**0.0397**	**0.0341**	0.3321	0.2165	
Mlh1	**0.0056**	0.2070	**0.0270**	**0.0174**	0.6840		
Msh2	**0.0028**	0.1067	**0.0205**	**0.0139**			
Msh3	**0.0006**	**0.0080**	0.8512				
Msh6	**0.0017**	**0.0159**					
Msh2/Msh3	0.1101						

Probability values were obtained with the unpaired *t*-test.
Statistically significant results are in bold ($P < 0.05$).

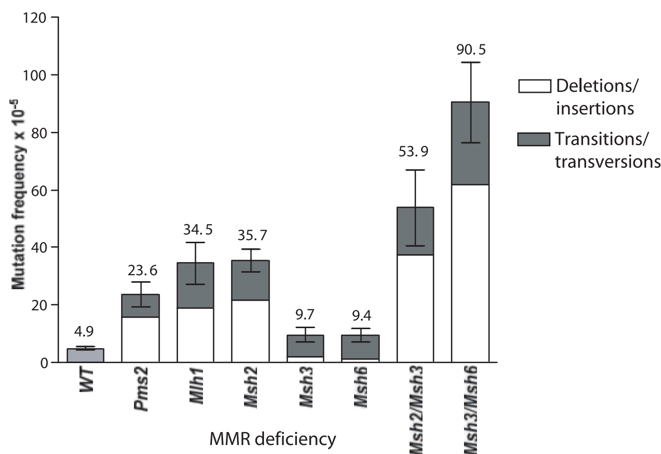

Fig. 2. Comparison of *cII* reporter gene mutation frequencies in wild-type and MMR-deficient mice. The mutation frequency for each group of mice is expressed as the mean ± SEM. The wild-type mutation frequency is the mean ± SEM of the skin and colon mutation frequencies of all the wild-type mice listed in Table I. The absolute frequencies of the types of mutations are indicated by the shaded segments of each bar. A total of 27 mutants were analyzed for the *Pms2*$^{-/-}$ mice, 33 for the *Mlh1*$^{-/-}$ mice, 31 for the *Msh2*$^{-/-}$ mice, 28 for the *Msh3*$^{-/-}$ mice, 15 for the *Msh6*$^{-/-}$ mice, 23 for the *Msh2*$^{-/-}$ /*Msh3*$^{-/-}$ mice and 22 for the *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice.

frequencies of the *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice were significantly higher than all of the single nullizygous groups of mice (Table II and Figure 2).

Sequence analysis of supFG1 and cII mutants

A sample of the mutants from each of the MMR-deficient mice, for both the reporter genes, was analyzed by DNA sequencing, and the patterns of mutations were determined. No differences were detected between the mutants of skin and colon for either of the reporter genes (data not shown). In the case of each genotype, the absolute frequencies of deletions/insertions and transitions/transversions were calculated for each group by multiplying the percentages of each type by their respective mutation frequencies (Figures 1 and 2).

The majority of the *supFG1* mutations were either −1 deletion or +1 insertion mutations, with the majority being deletions (Table III). There were only slight differences in the types of mutations analyzed among the different genotypes, as only a small proportion of the *Msh2*$^{-/-}$, *Pms2*$^{-/-}$, *Msh3*$^{-/-}$ or *Msh6*$^{-/-}$ *supFG1* mutations consisted of base substitutions; most were deletions or insertions (Table III and Figure 1). This prevalence of deletion and insertion mutations in the *supFG1* mice was not surprising due to the two G : C bp stretches (of lengths of 7 and 8 bp) in the *supFG1* gene. Those sites are mutation hotspots and account for the majority of the insertion and deletion mutations detected (data not shown).

When compared with the *supFG1* mutants, the *cII* mutants showed a more diverse spectrum of mutation types, although there were still larger numbers of deletions and insertions than of base substitutions in the *Pms2*$^{-/-}$, *Mlh1*$^{-/-}$, *Msh2*$^{-/-}$, *Msh2*$^{-/-}$/*Msh3*$^{-/-}$ and *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice (Table III and Figures 1 and 2). However, the majority of the *cII* mutations in both the *Msh3*$^{-/-}$ and *Msh6*$^{-/-}$ mice were transitions and transversions (Table III and Figure 2).

Discussion

We have reported here a comprehensive comparison of mutation frequencies and patterns in wild-type and MMR-deficient mice in the absence of genotoxic insults. The spontaneous mutation patterns found in these MMR-deficient mice reveal a hierarchy in the extent to which each of the MMR factors contributes to genome stability, with the highest mutation frequencies among the single nullizygous mice seen in the *Mlh1*-deficient and *Msh2*-deficient mice, and the least in the *Msh3*-deficient mice. These comparisons were performed in the context of two different reporter genes, yielding similar relative values, although the absolute mutation frequencies were different, reflective of the nature of the reporter genes. In this regard, earlier studies have shown that the *supFG1* reporter gene is particularly prone to mutagenesis in the absence of MMR owing to two long mononucleotide repeats in the coding sequence (28). Therefore, it was not surprising that the MMR-deficient

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Table III. Analysis of the mutants sequenced from each group of MMR-deficient mice

Mutation type	*Pms2*$^{-/-}$	*Mlh1*$^{-/-}$	*Msh2*$^{-/-}$	*Msh3*$^{-/-}$	*Msh6*$^{-/-}$	*Msh2*$^{-/-}$/*Msh3*$^{-/-}$	*Msh3*$^{-/-}$/*Msh6*$^{-/-}$
supFG1							
Deletions/insertions	71	81	58	57	58	24	24
−1 nucleotide	47	65	46	39	29	18	14
+1 nucleotide	24	16	12	18	29	6	10
Transitions	3	0	1	4	0	0	0
CG→TA	2		1	4			
TA→CG	1						
Transversions	0	0	0	3	4	0	0
CG→AT				2	2		
CG→GC				1			
AT→CG					2		
cII							
Deletions/insertions	18	18	19	6	2	16	15
−1 nucleotide	14	16	14	2	2	13	14
+1 nucleotide	4	2	5	2	0	2	
> −1 nucleotides				2		1	1
Transitions	4	13	10	17	10	6	7
CG→TA	2	10	4	12	6	6	6
TA→CG	2	3	6	5	4		1
Transversions	5	2	2	5	3	1	0
CG→AT		2	1	2	1		
CG→GC	3		1	2	2		
AT→TA	2			1		1	

supFG1 mice were found to have mutation frequencies that were considerably elevated when compared with the MMR-deficient *cII* mice. Nonetheless, similar trends were seen in the comparison of MMR genotypes in both the *supFG1* and *cII* reporter lineages.

A number of previous studies have also utilized mutation reporter genes to investigate genome instability in MMR-deficient mice (13,15–17,29–31). The data presented here expand upon this prior body of work and provide further validation of these earlier findings. For example, it was previously shown that *Msh2*-deficient mice exhibit mutation frequencies higher than *Pms2*-deficient mice in the *supF, lacI* and *cII* reporter genes (15). Other studies also have revealed elevated mutation frequencies in *Mlh1, Pms2* and *Msh6* nullizygous mice, with relative levels consistent with our findings (15,17,29,31). Additionally, *Mlh1*-deficient mice have shown higher *supFG1* and *lacI* mutation frequencies than *Pms2*-deficient mice (16,30), and recently MSI analysis showed that *Mlh3*$^{-/-}$/*Pms2*$^{-/-}$ mice have higher mutation frequencies than *Pms2*$^{-/-}$ mice, but the same as *Mlh1*$^{-/-}$ mice (6). However, the present work also provides a combined analysis of these and additional MMR genotypes, allowing a comprehensive comparison among these MMR factors.

In addition, the work reported here includes an analysis of double nullizygous mice in two different reporter systems. Although *Msh3*/*Msh6*-deficient mice have previously been examined (21,29), this is the first characterization of mice deficient for both *Msh2* and *Msh3*. The *Msh2*$^{-/-}$/*Msh3*$^{-/-}$ and *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice had the highest mutation frequencies of all the genotypes studied, suggesting that disrupting more than one of the MMR genes escalates the accumulation of mutations, leading to considerably increased mutation frequencies. A similar effect has also been observed in an analysis of *hprt* gene mutations of colon adenocarcinoma cells with concomitant *MLH1* and *MSH6* inactivation (31). Although statistically significant only in the case of the *cII Msh3*$^{-/-}$/*Msh6*$^{-/-}$ data and not in the *supFG1* data, the increased mutation frequencies that the *Msh2*$^{-/-}$/*Msh3*$^{-/-}$ and *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice displayed over the *Msh2*$^{-/-}$ mice raise the possibility that the absence of *Msh2* may not completely eliminate MMR *in vivo*. However, numerous studies support a critical role for *Msh2* in mismatch recognition, and there is not yet any biochemical evidence to suggest that there may be an alternative factor. Since MSH3 and MSH6 overlap in function, and MSH2 has been suggested to partition between available pools of the two (32), the higher frequencies of the *Msh3*/*Msh6*-deficient mice may suggest an increased level of disruption in that there are not other factors to take their place. In any case, the key role of *Msh2* in both the MutSα (Msh2/Msh6) and MutSβ (Msh2/Msh3) heterodimers is borne out in the comparison among the MutS homologs. In contrast, elimination of either *Msh3* or *Msh6* alone still maintains some functional MMR activity, consistent with the persistence of the MutSα or MutSβ heterodimers, respectively. Similarly, a non-redundant role for *Mlh1* among the MutL homologs is also supported by the data, as presented here and elsewhere (6).

The greater mutation frequencies of the *Msh6*$^{-/-}$ mice compared with *Msh3*$^{-/-}$ mice supports the previous conclusion that *Msh6* may be more important to the maintenance of genomic stability than is *Msh3*. This is also reflected in previous work that has shown that *Msh3* deficiency plays a critical role in survival of mice predominantly when *Msh6* is also deficient (21). Also, the loss of *Msh6* has been shown to cause a strong predisposition to intestinal tumors in APC^{1638N} mice, whereas the loss of *Msh3* did not (33). The apparently greater impact of *Msh6* deficiency versus *Msh3* deficiency may partly explain why our attempts to create *Msh2*$^{-/-}$/*Msh6*$^{-/-}$ mice were unsuccessful. However, the simplest explanation is that the close proximity of these loci to each other on mouse chromosome 17 means that they are too closely linked to allow any reasonable chance of the required cross-over events within the scope of the number of progeny evaluated in this work. In any case, in the single

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nullizygous *Msh3*$^{-/-}$ mice we did detect a mutator phenotype. Although lowest among all the MMR-deficient mice studied, the frequencies in the *Msh3*$^{-/-}$ mice were still significantly elevated above that of the wild-type mice. In addition, the study mentioned above regarding the comparison of *Msh6* versus *Msh3* deficiency also reported that *Msh3*$^{-/-}$/*Msh6*$^{-/-}$ mice display a cancer predisposition phenotype that is indistinguishable from *Mlh1* or *Msh2* deficiency (21). Our results are in keeping with these findings.

Analysis of the types of mutations in the various mice revealed a predominance of deletions in all of the MMR-deficient mice in the context of the *supFG1* reporter gene. This reporter gene is most sensitive to +1 or −1 mutations, and so the predominance of −1 mutations suggests a propensity for such replication errors at this locus or a biased role for MMR in fixing products of template slippage leading to −1 deletions. Analysis of the *cII* mutations revealed a more diverse pattern, but there was still an overall predominance of deletions in five of the seven genotypes examined. Since this gene is well established to report all possible point mutations with less bias than *supFG1*, the predominance of deletions in the *cII* mouse assay suggests that such mutations are the primary *in vivo* result of MMR deficiency.

Taken together, the results reported here establish a rank order of the severity of MMR deficiency *in vivo* across five single and two double nullizygous MMR genotypes. All of the MMR genes studied were found to be important in preserving genome stability, with a hierarchy of effect consistent with their proposed single or multiple roles as components of MMR heterodimeric complexes.

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