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Genetics

Different mutator phenotypes in *Mlh1*- versus *Pms2*-deficient mice

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ABSTRACT **Deficiencies in DNA mismatch repair (MMR) result in increased mutation rates and cancer risk in both humans and mice. Mouse strains homozygous for knockouts of either the *Pms2* or *Mlh1* MMR gene develop cancer but exhibit very different tumor spectra; only *Mlh1*$^{-/-}$ animals develop intestinal tumors. We carried out a detailed study of the microsatellite mutation spectra in each knockout strain. Five mononucleotide repeat tracts at four different chromosomal locations were studied by using single-molecule PCR or an *in vivo* forward mutation assay. Three dinucleotide repeat loci also were examined. Surprisingly, the mononucleotide repeat mutation frequency in *Mlh1*$^{-/-}$ mice was 2- to 3-fold higher than in *Pms2*$^{-/-}$ animals. The higher mutation frequency in *Mlh1*$^{-/-}$ mice may be a consequence of some residual DNA repair capacity in the *Pms2*$^{-/-}$ animals. Relevant to this idea, we observed that *Pms2*$^{-/-}$ mice exhibit almost normal levels of Mlh1p, whereas *Mlh1*$^{-/-}$ animals lack both Mlh1p and Pms2p. Comparison between *Mlh1*$^{-/-}$ animals and *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ double knockout mice revealed little difference in mutator phenotype, suggesting that *Mlh1* nullizygosity is sufficient to inactivate MMR completely. The findings may provide a basis for understanding the greater predisposition to intestinal cancer of *Mlh1*$^{-/-}$ mice. Small differences (2- to 3-fold) in mononucleotide repeat mutation rates may have dramatic effects on tumor development, requiring multiple genetic alterations in coding regions. Alternatively, this strain difference in tumor spectra also may be related to the consequences of the absence of Pms2p compared with the absence of both Pms2p and Mlh1p on as yet little understood cellular processes.**

It is now well accepted that cancer results from clonal evolution of tumor cell populations and that genetic instability in any of several forms contributes to tumor development (1–3). Mutations in mismatch repair (MMR) genes result in elevated mutation rates and, in both humans and mice, an increased cancer risk (4–14). In humans, germ-line defects in MMR genes, primarily in *MSH2* or *MLH1*, lead to hereditary nonpolyposis colorectal cancer (HNPCC), a syndrome associated with increased frequencies of a variety of tumor types.

The construction of mouse strains carrying targeted mutations in MMR genes allows for the genetic manipulation of mutation rate *in vivo* and an assessment of the effect of mutation rate alterations on carcinogenesis. Mouse knockouts of the MMR genes *Msh2*, *Msh6*, *Pms2,* or *Mlh1* develop various neoplasias, including lymphoid, intestinal, skin, and other internal organ tumors (9–14). Interestingly, the tumor spectra are not identical in the different knockout mouse strains. For example, homozygousity for a null mutation in the murine *Pms2* gene results primarily in lymphoma, but no intestinal tumors. However, homozygousity for a null mutation in the *Mlh1* gene leads not only to lymphoma but also to intestinal cancer (12). The lack of intestinal tumors in *Pms2* knockout mice is surprising because Mlh1p and Pms2p function as a heterodimer *in vivo* (15, 16). The differences in tumor spectra may be related to differences in specific biological functions of *Mlh1* and *Pms2* that also may explain the relative paucity of *PMS2* mutation in HNPCC families.

In MMR-defective cells, insertion/deletion mutations in microsatellite sequences occur at a high frequency (4–8, 17–19). These mutations most likely are caused by the lack of repair of misaligned template or nascent strands that arise by slippage during DNA replication. Inactivation of genes by insertion/deletion in small mononucleotide repeat runs in the coding region are likely to be particularly relevant to tumorigenesis. Specific examples of this mechanism of mutation have been found in genes from human tumors showing microsatellite instability. These include transforming growth factor (TGF)-βRII, hMSH3, hMSH6, insulin-like growth factor-IIR, adenomatosis polyposis coli (APC), and BAX (20–27). Preliminary investigations of microsatellite mutation in mice lacking *Mlh1* or *Pms2* demonstrated generally elevated mutation rates in all tissues examined (9, 28–30). However, even small differences in mutation rates might have dramatic effects on the development of tumors that require multiple genetic alterations (31). To characterize more completely the mutator phenotype of *Mlh1*- and *Pms2*-deficient mice we conducted systematic studies of mononucleotide and dinucleotide repeat mutations in tissue from mice nullizygous for *Pms2*, *Mlh1*, or both genes (double knockouts, DKO). Surprisingly, the mononucleotide repeat mutation frequency in *Mlh1*$^{-/-}$ mice was consistently 2- to 3-fold higher than in *Pms2*$^{-/-}$ mice. The findings may provide one potential basis for understanding the greater predisposition to intestinal cancer exhibited by *Mlh1*$^{-/-}$ compared with *Pms2*$^{-/-}$ animals.

METHODS

Mutation Analysis. Microsatellite mutations were assayed by PCR of single target molecules. The amplification protocols were based on those used to amplify single haploid cells (32) and have been applied previously to mouse microsatellites (9, 12, 29). Equal amounts of toe DNA from nine *Mlh1*$^{-/-}$ mice (on average >93% C57BL/6J mouse DNA) or six *Pms2*$^{-/-}$ animals (on average >99% C57BL/6J genome) were mixed and diluted to slightly less than a single genome equivalent. On

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Abbreviations: MMR, mismatch repair; HNPCC, hereditary nonpolyposis colorectal cancer; DKO, double knockout.
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average, between 0.3 and 0.6 template molecules were present in each reaction. Each aliquot was amplified by using two PCR rounds in a heminesting strategy with three primers that flank the polymorphic marker. Three mononucleotide repeat markers were developed. The primers for Aa006036 (GenBank accession no.) were P1 (5′-ACG TCA AAA ATC AAT GTT AGG), P2 (5′-TTG CTG AAT TGG TGA GCT TC), and P3 (5′-F CAG CAA GGG TCC CTG TCT TA). At locus L24372 they were P1 (5′-GGG AAG ACT GCT TAG GGA AGA), P2 (5′-ATT GGA TAA GTA TGA GGT ACT), and P3 (5′-F ATT TGG CTT TCA AGC ATC CAT A). At locus U12235 they were P1 (5′-GCT CAT CTT CGT TCC CTG TC), P2 (5′-TAA CAC TGG AAG CCA TTC GG), and P3 (5′-F CAT TCG GTG GAA AGC TCT GA). PCR conditions were 94°C for 30 sec, 57–60°C for 1 min, and 72°C for 2 min for 25 cycles with primer P1 and P2 in 20 μl. Two microliters of PCR product were further amplified with primer P1 and P3 at 94°C for 30 sec, 60°C for 90 sec for 25 cycles in a 25-μl reaction. For all six loci, primer P3 is labeled at the 5′ end with fluorescein. The final PCR products were analyzed on an ALF DNA sequencer (Amersham Pharmacia Biotech). The methods of analysis of dinucleotide repeat markers D9Mit67 and D1Mit355 were reported previously (9, 29). Primers for locus D1Mit79 were P1 (5′-GAG GCA ACA TAA AAC TAA GAG AAA), P2 (5′-GGT GCA AAT GTA TCT ATG ATC C), and P3 (5′-FAGA ACC TCT GCC TTA TGG TG). The amplification conditions of this marker were the same as the two other dinucleotide repeat markers.

Methods for the analysis of mutation frequencies in the SupFG1 mutation assay system have been described (30). The 3340 supFG$_1$ transgenic mice (C57BL/6 mouse background) were produced as described for the 1139 mouse line by using standard technology (33) except that the supFG$_1$ gene (34) was substituted for supF in the transgene lambda vector. High molecular weight DNA from *Mlh1*$^{-/-}$;supFG$_1$$^{+/-}$ or *Pms2*$^{-/-}$; supFG$_1$$^{+/-}$ mice was prepared from selected tissues as described (33). Lambda vector rescue was carried out by using lambda *in vitro* packaging extracts (33, 35, 36). Packaging extracts were made as described (35), except that a new *Escherichia coli* lysogen, NM759 [*E. coli* K12 *recA56* Δ(*mcrA*) e14° Δ(*mrr-hsd-mcr*) (λ*imm434* c*I*ts *b2 red3 Dam15 Sam7*)/λ] was used instead of BHB2690 for the preparation of the sonicate extract (36). The mouse DNA was incubated in the lambda *in vitro* packaging extracts at a concentration of 0.05 μg/μl for 2 hr at 37°C. The packaged phage were adsorbed to PG901 [*E. coli* C1a *lacZ125* (am)], and plated in 0.6% top agar on LB plates in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside (1.6 mg/ml) and isopropyl β-D-thiogalactoside (1.3 mg/ml), as described (35). Phage with inactivating supFG$_1$ mutations produce colorless plaques.

Western Blot Analysis. Whole-cell lysates of mouse tissues were prepared from freshly harvested samples. The samples were weighed, minced finely with surgical scissors, and homogenized in a dounce homogenizer (Wheaton Scientific, with a B pestle) by using 10 ml of cell lysis buffer (50 mM Tris, pH 7.5/100 mM NaCl/1 mM EDTA/2.5 mM EGTA/0.05% NP-40/50 μg/ml PMSF/10 μg/ml leupeptin/10 μg/ml aprotinin) per gram of tissue. The mouse tissue lysates were diluted with an equal volume of 2× sample loading buffer before SDS/PAGE and transfer to poly(vinylidene difluoride) membrane (Millipore). mPms2p protein was detected with mAb Ab-1 (Oncogene), and mMlh1p was detected with mAb G168–15 (PharMingen). Proteins were visualized by using the ECL Western blotting detection system (Amersham Pharmacia). Samples from two male mice of each genotype aged 4–6 weeks were analyzed.

RESULTS

Mutations in "Long" Mononucleotide Repeat Tracts. Microsatellite mutation frequencies were studied by using DNA obtained from toe samples from 4- to 5-week-old mice. To minimize possible inter-individual genetic differences we pooled DNA samples obtained from individuals of the same strain (nine *Mlh1*$^{-/-}$ mice or six *Pms2*$^{-/-}$ animals) that had been backcrossed for at least four generations to C57BL/6J (>92% C57BL/6J). Each PCR sample contained less than one haploid genome equivalent of the target. A total of 2,647 molecules (including 452 wild type) were typed for the occurrence of length-altering mutations at three mononucleotide repeat loci. The data are shown in Table 1, and an example of the DNA sequencing gel trace is given in Fig. 1. Eighty-seven percent of all of the mutations were one-base insertions or deletions. There was no significant difference between the two knockout lines with respect to the distribution of mutations between one base and greater than one base changes ($P > 0.3$).

The mutation frequency in *Mlh1*$^{-/-}$ mice was approximately two times higher than in *Pms2*$^{-/-}$ animals at the three (A)$_n$ markers tested. A summary of the data averaged for all three loci is shown in Fig. 2, *Left*. The greater total frequency was caused entirely by a significantly higher frequency of contraction mutations at each locus [U12235, (A)$_{24}$ $P < 0.001$; Aa003063, (A)$_{23}$ $P < 0.005$; L24372, (A$_{27}$) $P < 0.001$]. Both lines showed similar (and low) frequencies of expansion mutation.

Mutations in "Short" Mononucleotide Repeat Tracts. The long mononucleotide repeat tracts (above) are not expected to occur frequently in coding regions. To study short mononucleotide repeat tracts (<10 bp) more likely to mimic those in coding regions, but which are expected to have very low mutation frequencies, we derived *Mlh1*$^{-/-}$ and *Pms2*$^{-/-}$ mice that carry a supF tRNA suppressor gene as a mutation reporter gene within a chromosomally integrated, recoverable phage

Table 1. Summary of individual mutation frequency data at three mononucleotide (A)$_n$ and three dinucleotide (CA)$_n$ loci in *Pms2*$^{-/-}$, *Mlh1*$^{-/-}$, and DKO mice

Mice	*N*	No. Exp.	Exp. freq.	No. Con.	Con. freq.	Total mutation freq.
(A)$_n$						
U12235						
Mlh1$^{-/-}$	360	7	0.02	67	0.19	0.21
Pms2$^{-/-}$	449	9	0.02	35	0.08	0.10
DKO	254	8	0.03	60	0.24	0.27
WT	452	0	0.00	1	0.002	0.002
Aa003063						
Mlh1$^{-/-}$	323	4	0.01	78	0.24	0.25
Pms2$^{-/-}$	366	6	0.02	57	0.16	0.17
DKO	237	4	0.02	57	0.24	0.26
L24372						
Mlh1$^{-/-}$	345	4	0.01	139	0.40	0.41
Pms2$^{-/-}$	352	15	0.04	83	0.24	0.28
(CA)$_n$						
D1Mit79						
Mlh1$^{-/-}$	450	44	0.10	62	0.14	0.24
Pms2$^{-/-}$	350	72	0.21	32	0.09	0.30
DKO	299	27	0.09	58	0.19	0.28
WT	142	0	0.00	0	0.00	0.00
D9Mit67						
Mlh1$^{-/-}$	234	13	0.06	23	0.10	0.15
Pms2$^{-/-}$	266	28	0.11	10	0.04	0.14
DKO	307	25	0.08	24	0.08	0.16
WT	103	0	0.00	0	0.00	0.00
D1Mit355						
Mlh1$^{-/-}$	349	25	0.07	54	0.15	0.23
Pms2$^{-/-}$	280	45	0.16	21	0.08	0.24
WT	290	0	0.00	3	0.01	0.01

Data on wild-type (WT) control samples at several of the loci are also included. Exp., expansions; Con., contractions.

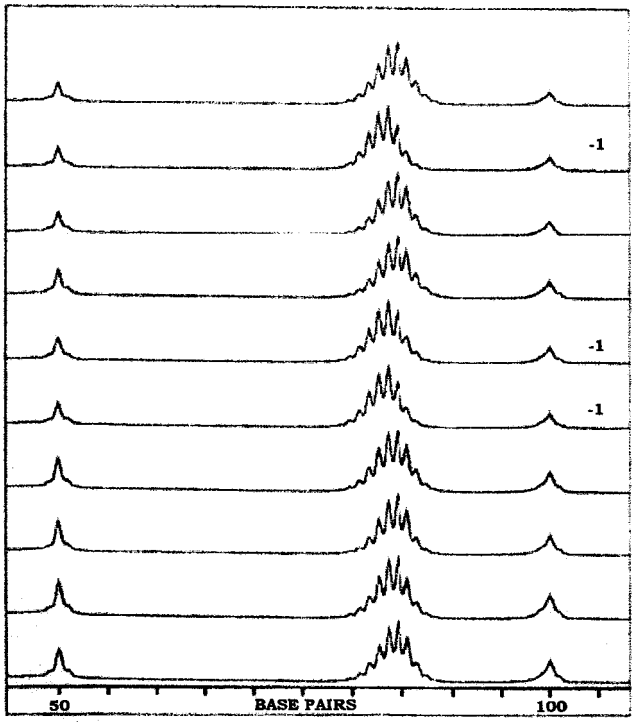

FIG. 1. Trace from DNA sequencing gel analysis of PCR products derived from single molecule amplification of mononucleotide repeat locus U12235. The molecular weight markers are shown as uniform peaks whereas the microsatellite PCR products exhibit typical PCR "stutter."

lambda shuttle vector (33, 35). By using lambda *in vitro* packaging extracts, the vector DNA can be identified, cut out, and packaged from DNA recovered from mouse tissues. The viable lambda particles are analyzed in bacteria for supF mutations that occurred in the animals (35, 36). Phage with functional supF genes suppress the nonsense mutation in the host bacteria β-galactosidase gene, allowing synthesis of active enzyme capable of metabolizing 5-bromo-4-chloro-3-indolyl β-D-galactoside, thereby yielding blue plaques. Phage with inactivating supF mutations produce colorless plaques (35, 36).

The mutation frequencies for knockout and wild-type strains are shown in Fig. 3. In *Pms2*$^{-/-}$ animals the mutation frequency in skin and colon is 2.2×10^{-3} and 2.6×10^{-3}, respectively (30). However, the mutation frequency was significantly higher in *Mlh1*$^{-/-}$ mice: 7.6×10^{-3} ($P < 0.001$) for skin and 6.7×10^{-3} for colon ($P < 0.001$). Based on sequence analysis of 155 SupF mutants from both strains, 97% were caused by single base insertions or deletions in a $(G)_7$ or $(C)_8$ mononucleotide repeat tract in the suppressor tRNA at positions 99–105 and 172–179, respectively (ref. 30 and data not shown). Like the "long" mononucleotide repeat data presented above, the 2- to 3-fold higher $supFG_1$ mutation frequency seen in *Mlh1*$^{-/-}$ mice was primarily the result of increased contraction mutations (65 one-base deletions and 16 one-base insertions) compared with *Pms2*$^{-/-}$ mice (16 one-base deletions and 24 one-base insertions). Based on the study of five mononucleotide tracts (both long and short) at four chromosomal positions, we conclude that *Mlh1* knockout mice have a higher mutation frequency (range 1.5- to 3.5-fold) compared with *Pms2*-deficient animals.

Mutations in Dinucleotide Repeats. Although dinucleotide repeats are less likely to be found in coding regions, we measured the mutation frequency at three loci in the two knockout strains. The data, representing 1,929 single molecules analyzed, are shown in Table 1. Eighty-five percent of the mutations were caused by insertion or deletion of a single 2-bp repeat. There was no significant difference between the two knockout strains with respect to the distribution of mutations between two base and greater than two base changes ($P > 0.8$).

The dinucleotide repeat mutation frequency (Table 1) did not differ significantly between the *Pms2*$^{-/-}$ and *Mlh1*$^{-/-}$ strains [D9Mit67, $(AC)_{22}$ $P > 0.7$; D1Mit79, $(AC)_{27}$ $P > 0.05$; D1Mit355, $(AC)_{33}$ $P > 0.75$]. However, the distribution of mutations between expansions and contractions differed dramatically (D9Mit67, $P < 0.01$; D1Mit79, $P < 0.005$; D1Mit355, $P < 0.005$). *Mlh1*$^{-/-}$ animals had a significantly higher frequency of contraction mutation at all three dinucleotide repeat loci compared with the *Pms2*$^{-/-}$ strain (D9Mit67, $P < 0.008$; D1Mit79, $P < 0.04$; D1Mit355, $P < 0.002$). On the other hand, *Pms2*$^{-/-}$ animals exhibited a significantly higher frequency of expansion mutation (D9Mit67, $P < 0.008$; D1Mit79, $P < 0.04$; D1Mit355, $P < 0.002$). A summary of the data averaged over all three loci is shown in Fig. 2, *Right*. A trend toward an increased expansion to contraction ratio in *Pms2*$^{-/-}$ compared with *Mlh1*$^{-/-}$ mice also was detected in the mononucleotide repeat data (U12235, $P < 0.001$; L24372, $P < 0.001$; Aa003063, $P > 0.20$; $SupFG_1$, $P = 0.05$) and studies on 2,800 single germ cell genomes ($P < 0.01$; data not shown).

Microsatellite Repeat Mutations in DKO Mice. Given the results on individual *Pms2*- and *Mlh1*-deficient lines, we examined microsatellite instability in *Pms2*$^{-/-}$; *Mlh1*$^{-/-}$ DKO mice. A total of 1,097 single molecules were analyzed by using pooled toe DNA samples from five 1-month-old animals (Table 1 and Fig. 2). The contraction mutation frequency at the two mononucleotide repeat loci was about the same (Aa003065, $P > 0.9$; U12235, $P > 0.13$) as in *Mlh1*$^{-/-}$ animals. Also, like the *Mlh1*$^{-/-}$ strain, the DKO had a significantly higher contraction mutation frequency (1.5-to 2.8-fold; Aa003065, $P < 0.015$; U12235, $P < 0.001$) compared with *Pms2*$^{-/-}$ mice. Analysis of two dinucleotide repeat loci in the DKO also showed the *Mlh1*$^{-/-}$ mutator phenotype. The DKO mice have a higher contraction mutation frequency when compared with *Pms2*$^{-/-}$ animals (D9Mit67, $P < 0.04$; D1Mit79, $P < 0.001$). The similar mutator phenotype for microsatellite repeats in *Mlh1*$^{-/-}$ and the DKO mice suggests that *Mlh1* deficiency alone is sufficient to yield a full MMR null phenotype.

Expression Levels of Pms2p and Mlh1p. The lower contraction mutation frequency in *Pms2*$^{-/-}$ compared with the *Mlh1*$^{-/-}$ and DKO mice might be explained by the presence of a residual repair activity in *Pms2*$^{-/-}$ animals. To address possible factors involved in any residual repair activity we examined the levels of Pms2p and Mlh1p in the two single knockout strains. In whole-cell lysates of thymus and testis isolated from *Mlh1*$^{-/-}$ mice, Pms2p levels were reduced 10- to 20-fold relative to the levels in wild-type tissues (Fig. 4). In contrast, Mlh1p levels were reduced only about 3-fold in extracts from the *Pms2*$^{-/-}$ tissues. In both knockouts, Msh2p and Msh6p levels were the same as in wild type (data not shown). Previous studies of human tumor cell lines show that *MLH1*-deficient cells have a reduced level of PMS2p but normal levels of *PMS2* RNA (37, 38). In contrast, the MLH1p level appeared to be unaffected by a *PMS2* gene deficiency (37). Our findings demonstrate that the presumed instability of Pms2p in the absence of Mlh1p is a general phenomenon in both long-term cultured cells and in tissues of the whole animal. The stability of Mlh1p may reflect formation of a complex with another protein.

DISCUSSION

Pms2p and Mlh1p function during MMR as a heterodimer (15, 16). In mice, deficiency for either gene results in a general increase in spontaneous mutation and increased cancer risk (12). However, the tumor spectrum of *Mlh1*$^{-/-}$, but not *Pms2*$^{-/-}$ mice, is more reminiscent of HNPCC patients. To

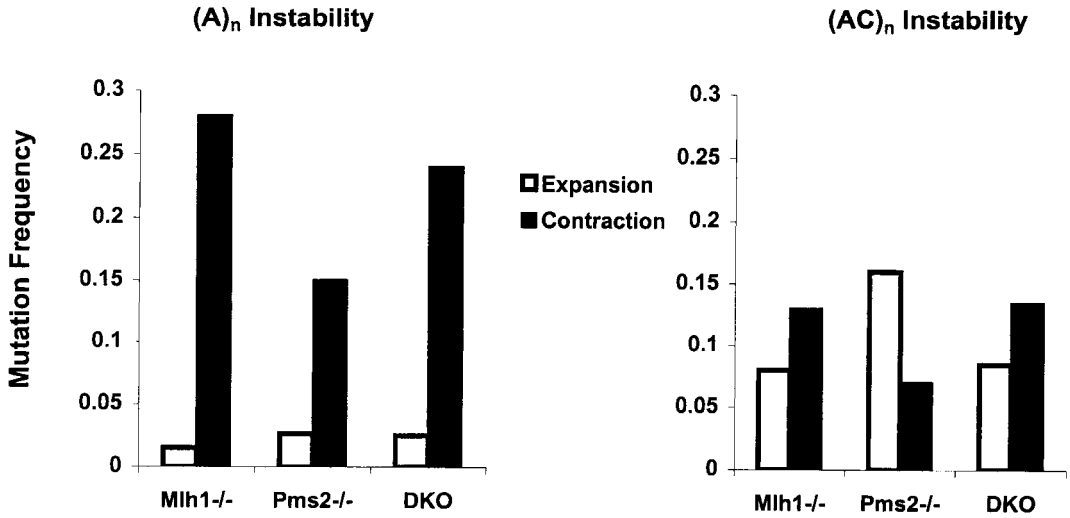

FIG. 2. Average microsatellite instability in *Pms2*$^{-/-}$, *Mlh1*$^{-/-}$, and DKO mice at mononucleotide (*Left*) and dinucleotide (*Right*) repeat loci.

identify potential differences in specific biological functions of *Mlh1* versus *Pms2*, we have characterized in greater detail the mutator phenotype in microsatellite repeat sequences of *Mlh1*$^{-/-}$, *Pms2*$^{-/-}$, and double mutant mice. We found that the *Mlh1*$^{-/-}$ and DKO mice consistently showed a 2- to 3-fold higher mutation frequency than *Pms2*$^{-/-}$ animals in mononucleotide repeat tracts. The increased mutation frequency was seen for multiple loci in several different tissues and can be accounted for by an increase in contraction mutation. For dinucleotide repeats, *Mlh1*- and *Pms2*-deficient mice had similar mutation frequencies, although the distribution of mutations among contractions and expansions was significantly different. Additionally, we found that *Pms2* protein was greatly reduced *in vivo* in tissues of *Mlh1*$^{-/-}$ mice, whereas the converse was not true, thus extending to tissues results from earlier studies on cultured cells.

The similarity between the mutation phenotypes of *Mlh1*$^{-/-}$ and the DKO mice indicates that inactivation of *Mlh1* alone is sufficient to completely inactivate MMR. Further, the lower contraction mutation frequency in *Pms2*$^{-/-}$ mice suggests two possibilities. First, *Pms2*$^{-/-}$ mice may have residual MMR activity not present in *Mlh1*$^{-/-}$ animals that reduces the frequency of contraction mutations. Residual MMR activity in *Pms2*$^{-/-}$ mice may depend on *Mlh1* because inactivation of *Mlh1* in addition to *Pms2* in the DKO mice increased the contraction mutations relative to that seen with the *Pms2* mutation alone. Additionally, Mlh1p is present at near normal levels in *Pms2*-deficient mice. In the absence of Pms2p, Mlh1p may be stabilized either by homodimer formation or via interaction with another protein, e.g., the MutL homolog Pms1p. In yeast, a MutL homolog, Mlh3p, appears to interact with Mlh1p and function in a minor mutation avoidance pathway acting on insertion/deletion-type mispairs (39). The fact that yeast Mlh3p is most similar to the human *PMS1* protein further supports the notion that mouse Pms1p is a possible "partner" for Mlh1p in *Pms2*$^{-/-}$ mice. An obvious prediction is that mice deficient in both *Pms1* and *Pms2* should mimic *Mlh1*-deficient mice in terms of both mutation levels and tumor spectrum.

Another explanation for the different mutation phenotypes of *Pms2*$^{-/-}$ and *Mlh1*$^{-/-}$ mice is that the frequency of slippage events on the nascent and template strands during DNA replication might be different in the two knockout strains. Expansion and contraction mutations most likely reflect DNA slippage events that result in single-strand loop formation on the nascent and template strands, respectively. MMR proteins, including Mlh1p, have been shown to form a complex with proliferating cell nuclear antigen, suggesting the possibility that the MMR apparatus may be intimately associated with the replication machinery (40). In turn, if the MutLα heterodimer (Mlh1p/PMS2p) is associated with the replication machinery,

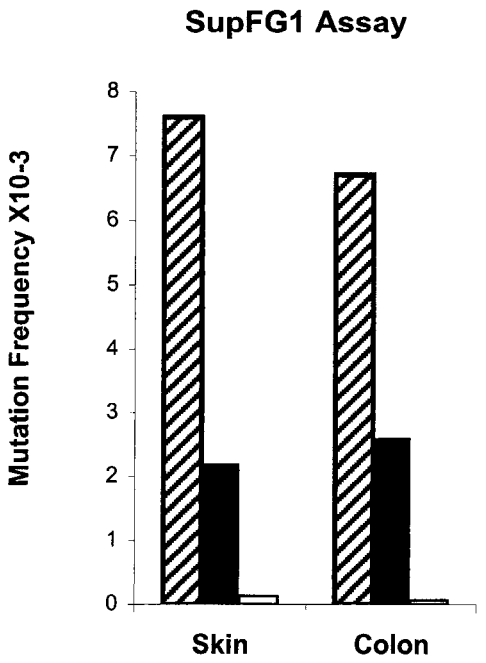

FIG. 3. SupFG$_1$ mutation frequency in skin and colon from *Pms2*$^{-/-}$, *Mlh1*$^{-/-}$, and wild-type mice.

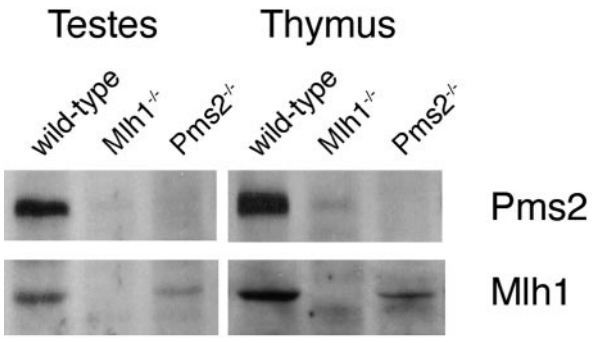

FIG. 4. Levels of Pms2p and Mlh1p in knockout mouse tissues.

the absence of one or the other protein may have differential influence on the slippage process itself. Perhaps a replication complex deficient in both Mlh1p and Pms2p ($Mlh1^{-/-}$ or the DKO mice) is more susceptible to slippage events, leading to contractions than a replication complex associated with Mlh1p. Similarly, an altered replication complex lacking Pms2p might yield more slippage events on the nascent strand (resulting in expansions) during replication of dinucleotide repeat tracts than a replication complex lacking both proteins. Influences of either pathway (residual repair or altering the slippage process) on the mutation phenotype of $Pms2^{-/-}$ and $Mlh1^{-/-}$ mice might be different for mononucleotide versus dinucleotide repeat mutations.

Regardless of the mechanism, the overall 2- to 3-fold greater mononucleotide repeat mutation frequency in $Mlh1^{-/-}$ compared with $Pms2^{-/-}$ animals may contribute to their different tumor spectra. In the $Mlh1^{-/-}$ strain, 83% of the animals developed at least one intestinal adenoma or adenocarcinoma between 6 and 12 months. On the other hand, no intestinal adenomas or adenocarcinomas were detected in $Pms2^{-/-}$ mice. Several genes with small mononucleotide repeat runs in the coding region have been implicated as relevant targets for inactivation because of insertion/deletion mutations during colon tumorigenesis in HNPCC patients (summarized above). Given a 2-fold difference in mutation rate, we can estimate the difference in the likelihood of intestinal tumor formation. If n independent mutations are required to develop a particular tumor in time t and the mutation rate per cell division for each locus is m, then the probability of a cell lineage experiencing all the required mutations is $\approx C(t)\, m^n$, where $C(t)$ is the term that takes the time of appearance of the tumor into consideration. If the mutation rate were doubled to $2m$ the probability of acquiring all the needed mutations would be $\approx C(t)\,(2m)^n = \approx C(t)\, m^n\, 2^n$. An x-fold increase in the mutation rate will increase the probability of tumor formation in time t by x^n: tumor incidence will increase exponentially with a linear increase in mutation rate. The more independent mutations (n) that are required for tumor formation, the greater the effect of any particular difference in mutation rate will be on the probability of tumor formation. As an example, if five independent mutations were required for the development of a particular tumor type, a 32-fold difference in tumorigenesis between $Pms2^{-/-}$ and $Mlh1^{-/-}$ mice is predicted by a 2-fold difference in mutation rate. If mice with a 2-fold lower mononucleotide repeat mutation frequency compared with the $Mlh1^{-/-}$ knockout animals could be generated (perhaps by a "knockin" mutation at the *Mlh1* locus), the chance of intestinal tumor formation during the life span of these animals might be significantly reduced. Alternatively, construction of $Pms2^{-/-}$ mice with appropriate inactivating mutations in relevant target genes should result in development of intestinal tumors similar to those seen in $Mlh1^{-/-}$ mice. In fact, deficiency for *Pms2* does enhance adenoma formation in mice already predisposed to frequent, early-onset, intestinal adenomas caused by mutation in the *APC* gene (41).

If small differences in the mutation rate of mononucleotide repeats do, in fact, influence the risk of tumorigenesis in the intestine, similar effects would be predicted for tumorigenesis in other tissues. Both $Mlh1^{-/-}$ and $Pms2^{-/-}$ mice develop lymphoma. However, the extent to which lymphoid tumor formation is affected by microsatellite mutations is unknown. If microsatellite mutation does, in fact, contribute significantly to lymphoma formation, tumors should appear earlier in $Mlh1^{-/-}$ animals than in $Pms2^{-/-}$ mice. Addressing this issue will require much larger numbers of animals and a more complex experimental plan than used in earlier studies (12).

Although the differences in mononucleotide repeat mutation may contribute to differences in intestinal tumorigenesis, we note that MMR proteins are believed to be important in other cellular processes, including apoptotic responses to certain cytotoxic DNA damaging agents, nucleotide excision repair, transcription-coupled repair, and the block to recombination between nonidentical sequences (10, 42–45). Residual MMR-related activity in these other pathways using Mlh1p (alone, or with Pms1p) may contribute to "protection" from intestinal tumorigenesis in *Pms2*-deficient animals. Additional investigations of mice with multiple deficiencies in *MutL* homologs should help to elucidate the basis of the difference in the mouse cancer phenotypes and perhaps explain only the rare occurrence of *Pms2* germ-line mutations in HNPCC kindreds.

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