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Tumors of DNA mismatch repair-deficient hosts exhibit dramatic increases in genomic instability

AGNES BAROSS-FRANCIS, SUSAN E. ANDREW, JANICE E. PENNEY, AND FRANK R. JIRIK*

Center for Molecular Medicine and Therapeutics, and Department of Medicine, University of British Columbia, Vancouver, BC Canada V5Z 4H4

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ABSTRACT **DNA mismatch repair (MMR) deficiency is associated with an increased mutational burden and predisposition to certain malignancies. Relatively little is known, however, about gene-specific mutation frequencies within MMR-deficient primary tumors. Thymic lymphomas from *Msh2*$^{-/-}$ mice were thus analyzed by using a *lacI*-based transgenic shuttle-phage mutation detection system. All tumors exhibited greatly elevated *lacI* gene mutation frequencies, ranging from 3.2- to 17.4-fold above the ≈15-fold elevations present within normal *Msh2*$^{-/-}$ thymi. In addition, *lacI* genes harboring multiple changes, including clusters of mutations, were found in thymic tumor DNA. The results suggest that an additional mutator activity, such as an error-prone DNA polymerase, leads to increased genomic instability in these MMR-deficient tumors.**

Because the spontaneous mutation rate of human cells appears to be insufficient to account for the multiple mutations that are required for the evolution of most malignancies (1), it has been proposed that tumor evolution requires a mutator phenotype, such as would result from disruption of the genes encoding DNA repair proteins (2). In keeping with this prediction, highly penetrant mutations in human mismatch repair (MMR) genes (primarily the *mutS* homolog *hMSH2* and the *mutL* homologs *hMLH1*, *hPMS2,* and *hPMS1*) have been found to be responsible for most cases of the familial cancer syndrome, hereditary nonpolyposis colorectal cancer (HNPCC) (3). Affected individuals inherit a germ-line mutation in one allele and in association with the loss of the second allele develop malignancies, predominantly of the proximal colon, endometrium, and ovary. Interestingly, mice with homozygous germline deficiencies of specific MMR genes generate only a limited spectrum of tumor types with varying frequencies and latencies (4–7). *Msh2*$^{-/-}$ mice, for example, develop neoplasms with a high frequency, but the spectrum is dominated by thymic lymphomas (5). This suggests that the increased mutation rate afforded by MMR deficiency may be necessary but not always sufficient for malignant transformation to occur.

Tumors and cell lines lacking MMR almost invariably demonstrate microsatellite instability and malignant lines generally exhibit greatly elevated mutation rates of specific indicator genes (8–17). Such studies have limitations, however, because MMR-deficient tumor lines may contain uncharacterized mutations able to potentiate genomic instability and also because mutation rates can be greatly influenced by cell culture conditions (18). Other than by the semiquantitative method of measuring instability at microsatellites, little is known about gene-specific mutation frequencies (or spectra) in primary MMR-deficient tumors. Mice with induced deficiencies of specific MMR components provide a unique opportunity for quantifying genomic instability, both in normal tissues and spontaneously arising tumors. To this end, shuttle phage-based mutation detection systems (19, 20) have been used to investigate tumor-specific mutation frequencies. Thus, adenocarcinomas arising in bitransgenic mice carrying a *lacI* reporter and the polyomavirus middle-T oncogene failed to show an increase in mutation frequency (21). Similarly, of eight thymic lymphomas in p53$^{-/-}$ Big Blue *lacI* transgenic mice (22, 23), only one demonstrated a 2.3-fold *lacI* mutation frequency increase compared with normal thymus. In contrast, we found that thymic lymphomas arising within *Msh2*$^{-/-}$ BC-1 murine hosts uniformly demonstrate remarkable increases in *lacI* gene mutation frequency compared with normal *Msh2*$^{-/-}$ thymi.

MATERIALS AND METHODS

Transgenic Mice. *Msh2*$^{+/-}$ heterozygotes (5) were bred with the BC-1 transgenic line to generate BC-1/*Msh2*$^{-/-}$ mice. Hemizygous BC-1 mice carry ≈30 copies of a λ-shuttle-phage transgene with the *lacI* reporter gene (24). For genotype testing, 0.5-cm tail clips were obtained from anesthetized mice and digested in 1.2 mg/ml proteinase K, 50 mM Tris·HCl (pH 8.0), 10 mM EDTA, O.1% SDS, and 100 mM NaCl. Mice carrying the *lacI* transgene and also homozygous for *Msh2*$^{-/-}$ were identified with a PCR assay (5, 24). BC-1/*Msh2*$^{-/-}$ mice were euthanized when moribund, and the thymic origin of the tumors was established by necropsy and histological examination.

Determination of *lacI* Gene Mutation Frequency and Spectrum. Tissue isolation and transgenic λ-phage rescue were carried out as described (25, 26). Briefly, phage genomes within high molecular weight BC-1 transgenic mouse chromosomal DNA were excised and packaged by using Transpack (Stratagene) phage-packaging extract. Rescued phages were plated in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside on SCS-8 (Stratagene) bacterial cell lawns. *LacI* mutation frequencies were established by the ratio of mutant (blue) to nonmutant (colorless) plaques. Phage containing *lacI* mutations were verified as described (24). Following isolation of single mutant clones, *lacI* genes were amplified by PCR of phage templates. Templates, obtained from randomly selected *lacI* mutant phage, were directly sequenced by using primers spanning the *lacI* gene (24) with an ABI 388 automated DNA-sequencing instrument (Applied Biosystems).

RESULTS

Increased Mutation Frequency in *Msh2*$^{-/-}$ Thymic Lymphomas. Mutation frequencies were determined for six thymic lymphomas, which arose spontaneously in 3- to 4-month-old *Msh2*$^{-/-}$ mice (Table 1, I). Tumor-specific frequencies were

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Abbreviation: MMR, mismatch repair.
*To whom reprint requests should be addressed at: Center for Molecular Medicine and Therapeutics, Room 2020, 950 West 28th Avenue, Vancouver, BC Canada V5Z 4H4. e-mail: jirik@cmmt.vbc.ca.

Table 1. Spontaneous mutation frequencies for *Msh2*$^{-/-}$ thymic lymphomas (I), *Msh2*$^{-/-}$ normal thymi (young mice) (II), *Msh2*$^{-/-}$ normal thymi (older mice) (III), and *Msh2*$^{-/-}$ normal brains from mice with thymic lymphomas (IV)

Tissue	Mice	Age, days	Sex	Total pfu*	No. of mutants	Mutation frequency, $\times 10^{-4}$	Mutation frequency, 100% clonality†
I. Thymic lymphoma	A	115	F	138,820	211	15.2	13.9
	B	121	F	234,760	759	32.3	24.2
	C	139	F	214,460	447	20.8	19.0
	D	132	F	207,740	581	28.0	25.6
	E	123	F	211,620	1729	81.7	44.9
	F	81	M	210,140	1105	52.6	32.4
	Mean					38 ± 23‡	27 ± 10
II. Normal thymus	G	19	F	295,020	166	5.6	2.6
	H	19	F	300,460	89	3.0	2.6
	I	26	M	281,640	155	5.5	4.0
	Mean					5 ± 1.2	3 ± 0.7
III. Normal thymus	J	132	F	185,100	109	5.9	
	K	103	M	249,180	104	4.2	
	Mean					5 ± 0.8	
IV. Normal brain	E	123	F	220,080	89	4.0	
	F	81	M	215,300	95	4.4	
	Mean					4 ± 0.2	

*pfu = plaque-forming units.
†See Table 2. for clonality.
‡Mean ± SD.

elevated compared with those of normal thymi from 3- to 4-week-old *Msh2*$^{-/-}$ animals (*t* test, $P = 0.0039$). The latter already demonstrate an ≈15-fold elevation compared with the thymi of DNA repair-proficient animals (27) (Table 1, II; Fig. 1). When compared with *Msh2*$^{-/-}$ and to *Msh2*$^{+/+}$ thymi, the average mutation frequency increase in the tumors was 8.2- and ≈120-fold, respectively. There was variation in the increases among the tumors (3.2- to 17.4-fold over the mean value of normal thymi). It was important to establish whether the elevations resulted from clonal expansions of cells harboring specific *lacI* gene mutations. To establish the approximate level of clonality for each of the tumors, as well as the mutation spectrum, a total of 100 *lacI* mutants from *Msh2*$^{-/-}$ thymic lymphomas and 58 *lacI* mutants from the normal thymi of 3- to 4-week-old *Msh2*$^{-/-}$ mice were analyzed (Table 2; Fig. 2). From the sequencing data (Table 2) it was possible to establish the approximate level of clonality for each sample (Table 1). A total of 19 different mutations were observed to occur more than once among the various thymic lymphomas and control thymi. In three cases [G → A at 93, frameshift at an $(A)_5$ repeat at 135–139, G → A at 180], the sites corresponded to putative "hot spots" previously identified in *lacI* genes recovered from normal *Msh2*$^{-/-}$ tissues (27). In spite of the fact that a number of the recurrent mutations likely arose independently of each other, mutations observed more than once per sample were eliminated to arrive at the corrected frequencies. To estimate the contribution of clonality to the mutation frequency data, 11 to 24 *lacI* mutants were sequenced per tumor (Table 2). After correction for clonality, a comparison of the mutation frequencies of thymic lymphomas and normal *Msh2*$^{-/-}$ thymi demonstrated an 8.6-fold (compared with 8.2-fold before correction) elevation in the tumors when the mean values were compared (*t* test, $P = 0.00064$; Table 1). In addition, after correction for clonality, the frequency variation observed between the different tumors was greatly reduced (Fig. 1).

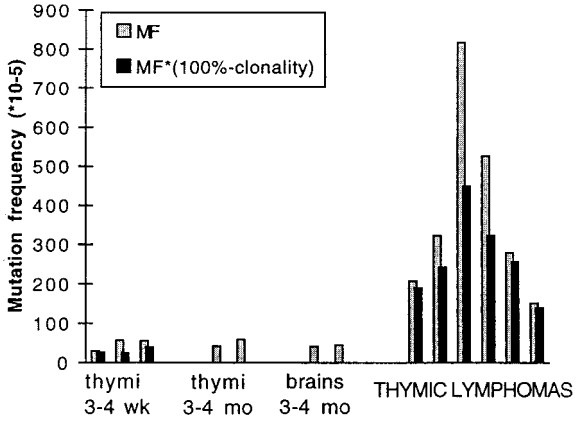

FIG. 1. *lacI* gene mutation frequency. Raw mutation frequencies of individual control and tumor tissues were determined from plating data (gray bars) and the corresponding mutation frequencies after correction for clonality (black bars).

Table 1, I and II, show the mutation frequencies of thymic lymphomas from 3- to 4-month-old *Msh2*$^{-/-}$ mice compared with data from *Msh2*$^{-/-}$ normal thymi obtained at 3–4 weeks of age. To determine whether the frequency elevations of the tumors might simply be a function of age, two normal thymi were isolated from 3.5- and 4-month-old *Msh2*$^{-/-}$ mice. The frequency data obtained (Table 1, III; Fig. 1), however, was not significantly different (*t* test, $P = 0.8$) from that of normal 3- to 4-week-old *Msh2*$^{-/-}$ thymi. Thus, the increased mutation frequencies of the thymic tumors were not attributable to age-related effects. Were the *lacI* mutation frequencies of the lymphomas solely a property of the tumors, or did lymphomas tend to arise in hosts with elevated mutational frequencies in all tissues? Brain, in contrast to most other tissues (such as liver, kidney, and spleen), demonstrated little, if any, histological evidence of infiltrating lymphoma cells. The brains of two *Msh2*$^{-/-}$ mice whose lymphomas exhibited the highest elevations in mutation frequency (Table 1, IV, mouse E and mouse F; Fig. 1) were analyzed. Mutation frequencies for the two brains were considerably lower than for the corresponding lymphomas in these same animals (*t* test, $P = 0.0053$), and were not significantly different from those of normal *Msh2*$^{-/-}$ brains or thymi (27) (a comparison of the combined E and F brain mutation frequencies with those of 3–4-week-old and 3-

Table 2. *lacI* mutation spectrum of *Msh2*$^{-/-}$ thymic lymphomas (I) and *Msh2*$^{-/-}$ normal thymi (II)

Animal	Transitions GC → AT AT → GC	Transversions GC→TA GC→CG AT→TA AT→CG	Frameshifts, +1 or −1 nucleotide	Insertion/deletion, >1 nucleotides	No. of mutations	Clonality, %*
I. *Msh2*$^{-/-}$ thymic lymphomas						
A	16/17† (3/3 CpG)	3/3	3/4	0	22/24	8
B	9/11 (2/4 CpG)	0	5/8	1/1	15/20	25
C	6/7 (1/1 CpG)	1/1	3/3	0	10/11	9
D	4/5 (2/3 CpG)	4/4	3/3	0	11/12	8
E	8/17 (2/4 CpG)	2/2	1/1	0	11/20	45
F	3/3 (1/1 CpG)	4/4	1/6	0	8/13	38
Total	46/60 (11/16 CpG)	14/14	16/25	1/1	77/100	23
Distribution of independent mutations‡	60% (24% CpG)§	18%	21%	1%	100%	
II. Msh2$^{-/-}$ normal thymi						
G	8/20 (3/15 CpG)	1/1	1/1	0	10/22	54
H	9/11 (2/3 CpG)	2/2	4/4	1/1	16/18	11
I	6/6 (1/1 CpG)	6/11	1/1	0	13/18	28
Total	23/37 (6/19 CpG)	9/14	6/6	1/1	39/58	33
Distribution of independent mutations	59% (26% CpG)	23%	15%	3%	100%	

*Clonality = number of clonal (total-independent) mutations/total number of mutations.
†n/m = *n* independent mutations of *m* mutations found.
‡Based on numbers of independent mutations.
§Number of independent transitions occurring at CpG sites per total number of independent transitions.

to 4-month-old thymi by *t* test was $P = 0.68$ and $P = 0.58$, respectively). Thus, the highly elevated *lacI* mutation frequencies appear to be not only intrinsic to but also characteristic of the *Msh2*$^{-/-}$ thymic lymphomas.

Mutation Spectrum of *Msh2*$^{-/-}$ Tumors. After correcting for clonality, we compared the numbers of transitions (including those falling at CpG sites), transversions, frameshifts (+1 or −1), insertions, and deletions (more than one nucleotide) between the tumors and the normal tissues (Table 2). A comparison of the pooled *lacI* mutation spectra of normal *Msh2*$^{-/-}$ thymi and the tumors (Fig. 2) did not show any significant difference (χ^2 test, $P > 0.5$). Mutations within individual mice did show variability, raising the possibility that *lacI* mutation spectrum might differ somewhat from tumor to tumor. However, because a relatively low number of *lacI* mutants were obtained from each tumor, the apparent individual differences in spectrum may not be significant. In keeping with the paucity of repetitive sequences in the *lacI* gene, transitions dominated the mutation spectrum in both groups (Table 2). The percentage of transitions falling at CpG sites (potentially resulting from the deamination of 5-methylcytosine) was similar in normal and malignant tissues, accounting for ≈25% of transitions in both. The percentage of transitions falling at CpG sites was lower than observed previously, where they accounted for >50% of total transitions in normal *Msh2*$^{+/+}$ liver (24), *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ small intestine and brain (27), and the combined spectrum of testis, ovary, and spleen in the *lacI* genes of Big Blue mice (25). Also, in Big Blue thymi, >50% of transitions fell at CpG sites (23). The decreased percentage of transitions at CpG sites in the *Msh2*$^{-/-}$ thymi and lymphomas may be because of lower levels of CpG methylation of the BC-1 *lacI* transgene in this tissue. Frameshifts (±1) were the second most common mutation type in both. Frameshifts (≈60% for tumors and ≈80% for normal thymi) were observed at mono- or dinucleotide repeats of 3 or more units. The proportion of frameshift mutations was modestly elevated in the tumors (21 vs. 15% in the *Msh2*$^{-/-}$ thymi). Transversion mutations varied considerably between animals; however, because the numbers were low, the significance of this observation is questionable (Table 2). Insertions or deletions (>1 bp) were rare in both normal and tumor DNA, with only one such *lacI* mutation found in each.

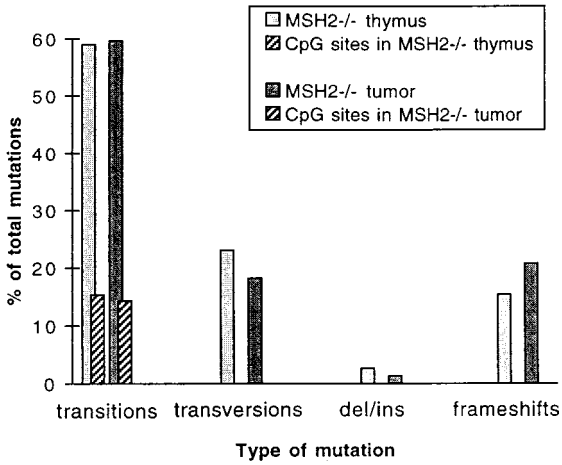

FIG. 2. *lacI* gene mutation spectrum. Mutation spectrum of the *lacI* gene mutants rescued from normal *Msh2*$^{-/-}$ thymi and *Msh2*$^{-/-}$ thymic lymphomas. The patterned bars represent the numbers of transitions occurring at CpG sites.

Multiple Mutations in Single *lacI* Genes. There were five examples of *lacI* genes having more than one mutation (Table 3). In addition, mouse F yielded a complex *lacI* mutation C_{131}GGGAAAAAGT → C_{131}GGAAAAAAT that could have resulted from one −1 frameshift and one transition or from one −1 frameshift and two transitions. In contrast, in the *lacI* genes sequenced from normal tissues of wild-type BC-1 and BC-1/*Msh2*$^{-/-}$ mice, in this study and previously (24, 27) we found only one mutant harboring more than one mutation (Table 3). In several *lacI* genes recovered from the tumors, clustering of mutations was observed; this was highlighted by the *lacI* mutant having four T → C transitions at positions 451, 547, 549, and 552.

Table 3. Multiple mutations occurring in one *lacI* gene from *Msh2*$^{-/-}$ thymic lymphomas (A, B, and E) and an *Msh2*$^{-/-}$ normal thymus (G)

Tissue	Animal	No. of mutations per *lacI* gene	Type of mutation, coding sequence	Site of mutation
Thymic lymphoma	A	2	T → A	703
			T → C	726
		4	T → C	451
			T → C	547
			T → C	549
			T → C	552
	B	2	A → G	320
			ΔA	350–352
	E	2	C → G	755
			A → C	858
		2	T → C	87
			T → C	162
Thymus	G	2	C → T	161
			G → A	222

DISCUSSION

DNA repair genes, such as *MSH2*, have been described as "caretaker" genes, responsible for the maintenance of genomic integrity. The inactivation of such genes results in increased genetic instability, which in turn leads to an increased rate of mutation in "gatekeeper" genes that regulate cell proliferation and death (28). The adenomatous polyposis coli (*APC*) and *BAX* genes, for example, belong to the latter category (3, 29). In mice, Msh2 deficiency appears to play a role in accelerating intestinal tumorigenesis, an effect attributable in part to *APC* mutations in *Min*$^{+}$ mice (6). With regard to lymphomagenesis in *Msh2*$^{-/-}$ mice, are the increased levels of genomic instability within the normal thymi (27) sufficient to account for the increased incidence of these tumors, or is an additional mutational rate increase necessary for this process? Because all *Msh2*$^{-/-}$ lymphomas examined demonstrated significant increases in mutation frequency compared with normal *Msh2*$^{-/-}$ thymi, a superimposed mutator phenotype might have been required to facilitate either tumor genesis or progression. Alternatively, the mutation frequency increases might be simply indicative of a "mitotic clock", registering cell divisions as a function of *lacI* gene mutations. Given that the *Msh2*$^{-/-}$ thymic lymphomas demonstrate histological evidence of apoptosis, including a classical "starry sky" appearance because of cells containing phagocytosed apoptotic cell bodies, it is difficult to estimate the number of tumor cell divisions that have transpired by the time an animal is moribund. Thymic lymphomas in p53$^{-/-}$ mice have also been studied by using Big Blue mice containing a *lacI* transgene similar to ours (22, 23). If we assume that similar numbers of cell divisions occur in the p53$^{-/-}$ thymic lymphomas, it is remarkable that with one exception these tumors failed to show an increase in *lacI* gene mutation frequency. It is clear that proliferating *Msh2*$^{-/-}$ cells should acquire mutations at a greater rate than MMR-proficient cells; however, if the *lacI* mutation frequency increases in the *Msh2*$^{-/-}$ tumors were simply a function of errors accumulated during cell division, then some consistent evidence of *lacI* mutation frequency induction would have been anticipated in all of the p53$^{-/-}$ lymphomas. Perhaps the single p53$^{-/-}$ lymphoma showing a 2.3-fold *lacI* mutation induction might reveal evidence of MMR deficiency. It should be noted that *lacI* transgene mutation frequencies observed by using either system may not accurately reflect mutational events within a given endogenous gene; thus, additional studies should be undertaken to establish mutation frequencies in loci such as *HPRT* in these lymphomas.

The comparison of the pooled BC-1 *lacI* mutations obtained from the *Msh2*$^{-/-}$ lymphomas and the *Msh2*$^{-/-}$ thymi (Fig. 2) did not reveal any gross differences in spectrum, except for the trend toward increased frameshifts in the tumors. A greater number of mutants would have to be characterized to unveil tumor-specific differences in mutation spectrum or in the position of the mutations on the *lacI* gene. The findings, however, do suggest that the mutagenic mechanisms responsible for the normal thymic *lacI* mutation spectrum may be similar to those operating within the lymphomas. Thus, except for a possible frameshift increase, the pooled *lacI* mutation spectra of the lymphomas did not suggest the existence of new, tumor-specific mutagenic mechanisms such as were observed in solid tumor xenografts and in hypoxic cells in culture (30). Perhaps lymphomas are better vascularized than solid tumors and thus undergo less hypoxia during their growth. The spectrum of the lymphomas mirrored that of the *Msh2*$^{-/-}$ thymi, whose spectrum is undoubtedly dominated by errors arising during DNA synthesis. Thus, the *Msh2*$^{-/-}$ tumors exhibited a mutator phenotype more like that observed in *MSH2*$^{-/-}$ cells grown under restrictive conditions (18).

The nonrandom distributions of mutations, as seen within several of the *lacI* genes, are unlikely the result of a generalized increase in mutation rate. Indeed, alkylating agent treatment of *Msh2*$^{-/-}$ mice led to 5- to 8-fold mutation frequency inductions that were not accompanied by the isolation of multiple mutants (38 *lacI* mutants characterized) (31). Clustered mutations have also been observed after *in vitro* DNA synthesis across templates with oxidative lesions (32), as well as in adenine phosphoribosyl transferase gene mutants in both MMR-proficient and -deficient colorectal cancer cell lines (33, 34). Interestingly, tumor suppressor genes with multiple mutations were recovered from the colorectal cancers of two patients with HNPCC (35). As suggested in these reports, the presence of clustered mutations is highly suggestive of a focal mutagenic mechanism, such as an error-prone DNA polymerase involved in DNA replication across damaged templates or in patch repair.

An additional mutator mechanism may be at work within the cells of the *Msh2*$^{-/-}$ tumors. The combination of a greatly increased mutation frequency and the absence of any major shift in spectrum (compared with normal *Msh2*$^{-/-}$ thymi) raises the possibility that an alteration in DNA polymerase fidelity may be a possible factor in tumor development. Furthermore, an error-prone repair polymerase could account for the clustering of mutations observed in some of the *lacI* mutants rescued from the *Msh2*$^{-/-}$ lymphomas (Table 3). Error-prone polymerases might arise from mutations in polymerase genes (or their associated subunits) (36), mutations of genes regulating the accuracy of mRNA translation, such as tRNA gene mutations responsible for the *mutA* and *mutC*

mutator phenotypes (37), or from *in vivo* microenvironmental abnormalities that generate tumor stress, including inadequate oxygenation or deficiencies of growth factors and nutrients (18, 38).

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