ABSTRACT **The mutational response of mismatch repair-deficient animals to the alkylating agent *N*-methyl-*N*-nitrosourea was evaluated by using a transgenic *lacI* reporter system. Although the mutations detected in *MSH2* heterozygotes were similar to those of controls, *MSH2*$^{-/-}$ animals demonstrated striking increases in mutation frequency in response to this agent. G:C to A:T transitions at GpG sites, as opposed to CpG sites, dominated the mutational spectrum of both *MSH2*$^{+/+}$ and *MSH2*$^{-/-}$ *N*-methyl-*N*-nitrosourea -treated animals. Extrapolating to humans with hereditary non-polyposis colorectal cancer, the results suggest that *MSH2* heterozygotes are unlikely to be at increased risk of mutation, even when exposed to potent DNA methylating agents. In contrast, mismatch repair-deficient cells spontaneously arising within individuals with hereditary non-polyposis colorectal cancer would likely exhibit hypermutability in response to such mutagens, an outcome predicted to accelerate the pace of tumorigenesis.**

Kindreds with hereditary non-polyposis colorectal cancer (HNPCC) carry germ-line mutations in various human orthologs of the bacterial DNA mismatch repair (MMR) genes *mutS* and *mutL* (1). The gene most frequently involved in HNPCC, *hMSH2*, encodes a component required for DNA mismatch recognition (2, 3). Although heterozygosity for *hMSH2* confers a high risk of colonic and endometrial neoplasms, malignancies at other sites, such as stomach, ovary, and urinary tract, also occur with increased frequency. Such tumors are accompanied by somatic mutations that inactivate the normal allele. The mutator phenotype arising from the combination of polymerase errors and a lack of MMR is thought to hasten the acquisition of alterations within key growth control genes, thus driving the multistep process that culminates in neoplasia (4).

To generate a model of human HNPCC, mice harboring a disruption of the *MSH2* locus were generated (5, 6). However, rodents heterozygous for *MSH2*, unlike humans with HNPCC, failed to show an increased rate of tumor formation. Mice lacking MSH2, on the other hand, developed thymic lymphomas with high frequency. In addition, although small intestinal adenomas and adenocarcinomas were seen, colonic tumors were rarely observed in these mice (5–7). Interestingly, MMR deficiency because of a lack of MSH2 or PMS2 was compatible with normal murine growth and development (5, 6, 8), despite the presence of an elevated mutation frequency in all tissues evaluated (9, 10). Thus, the restricted spectrum of tumors in *MSH2*$^{-/-}$ animals was not attributable solely to differences in tissue-specific spontaneous mutation frequencies.

Although mice heterozygous for *MSH2* did not demonstrate a mutator phenotype (10), it was conceivable that heterozygous cells might accrue mutations at a higher rate after mutagen exposure, owing to reduced levels of MSH2 protein. To determine whether specific mutagen administration would result in abnormal increases in mutation frequency in *MSH2*$^{+/-}$ and *MSH2*$^{-/-}$ tissues, we investigated the effects of an S_N1 class DNA alkylating agent on MSH2-deficient animals. Such chemicals react with DNA to form various lesions, of which *O*^{6}-alkylguanine (*O*^{6}-alkylG) appears to be the most highly mutagenic (11). Studies of *Escherichia coli* and mammalian cell lines exposed to such methylating agents have shown that mispairing of *O*^{6}-methylguanine (*O*^{6}-meG) with thymine can result in G:C to A:T transitions (12) because of DNA polymerase base misincorporations that may occur opposite this lesion (13, 14).

MMR proteins recognize O^6-meG:T base pairs, thus initiating long patch repair of these lesions (15). However, repetitive cycles of repair after DNA polymerase-mediated misincorporations of thymine opposite *O*^{6}-meG sites has been postulated to result in cell death, likely as a result of the presence of excessive DNA strand breaks (16). MMR deficiency thus has been associated with tolerance to alkylation damage in terms of cell viability (16). In keeping with this, *MSH2*$^{-/-}$ embryonic stem cells demonstrated tolerance to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), as measured by increased cell survival after exposure to this agent (6).

The mutational response to DNA alkylation of tissues from MSH2-deficient animals has not been reported. Furthermore, although *MSH2* heterozygous cells do not appear to demonstrate resistance to alkylation-induced cell death (6), the specific mutational response of normal *MSH2*$^{+/-}$ tissues to DNA alkylation has not been explored. The experiments with *N*-methyl-*N*-nitrosourea (MNU) described herein provide an *in vivo* assessment of the mutational consequences of alkylation tolerance. Furthermore, because alkylating agents have the potential to be either formed endogenously or ingested, it was important to establish whether *MSH2*$^{+/-}$ and/or *MSH2*$^{-/-}$ animals demonstrated increased sensitivity to such agents when compared with controls.

To examine the response of MMR-deficient animals to DNA alkylating agent exposure, a transgenic *lacI*-bearing lambda shuttle phage line (BC-1), previously developed for *in vivo* mutation detection (17), was crossed onto the *MSH2*$^{-/-}$ background (5). This enabled us to evaluate the effects of specific mutagen exposure on the tissue-specific *lacI* mutation frequency and spectrum. In this study, the consequences of MNU administration to *MSH2*$^{+/+}$, *MSH2*$^{+/-}$, and *MSH2*$^{-/-}$ mice were assessed.

Table 1. Spontaneous and MNU-induced mutation frequencies for BC-1 controls, BC-1/MSH2$^{+/-}$ and BC-1/MSH2$^{-/-}$

		BC-1 controls					
		Untreated control animals			MNU-treated animals		
Tissue	Animal	Total pfu	No. of mutants	Mutation frequency, $\times 10^{-5}$	Total pfu	No. of mutants	Mutation frequency, $\times 10^{-5}$
Small intestine	a	286,860	11	3.8	211,384	106	50.1
	b	261,540	4	1.5	191,800	102	53.2
	c	265,320	11	4.1	179,380	210	117.1
	Mean			3.1 ± 1.4			74 ± 38
Thymus	a	292,890	13	4.4	206,580	13	6.3
	b	264,180	8	3.0	203,720	65	31.9
	c	285,680	5	1.8	195,680	54	27.6
	Mean			3.1 ± 1.3			22 ± 14
Heart	a	244,960	10	4.1	202,620	29	14.3
	b	229,400	30	13.1	158,220	14	8.8
	Mean			8.6 ± 6.4			12 ± 4
		BC-1/MSH2$^{+/-}$					
		Untreated control animals			MNU-treated animals		
Tissue	Animal	Total pfu	No. of mutants	Mutation frequency, $\times 10^{-5}$	Total pfu	No. of mutants	Mutation frequency, $\times 10^{-5}$
Small intestine	a	253,560	7	2.8	217,044	91	41.9
	b	299,340	7	2.3	217,660	78	35.8
	Mean			2.6 ± 0.4			39 ± 4
Thymus	a	259,080	5	1.9	201,550	61	30.3
	b	257,900	17	6.6	214,040	39	18.2
	Mean			4.3 ± 3.3			24 ± 9
		BC-1/MSH2$^{-/-}$					
		Untreated control animals			MNU-treated animals		
Tissue	Animal	Total pfu	No. of mutants	Mutation frequency, $\times 10^{-5}$	Total pfu	No. of mutants	Mutation frequency, $\times 10^{-5}$
Small intestine	a	282,640	93	32.9	221,340	330	149
	b	269,980	99	36.7	200,420	687	343
	c	246,440	80	32.5	202,640	613	303
	Mean			34 ± 2			265 ± 102
Thymus	a	300,460	89	29.6	203,420	394	194
	b	295,020	166	56.3	214,920	652	303
	c	281,640	155	55.0	200,840	566	282
	Mean			47 ± 15			260 ± 58
Heart	a	215,740	34	15.8	252,360	64	25.4
	b	96,080	16	16.7	88,660	42	47.4
	c	196,980	54	27.4	94,480	60	63.5
	Mean			20 ± 6			45 ± 19

pfu, plaque forming units.

MATERIALS AND METHODS

Transgenic Mice. *MSH2*$^{+/-}$ heterozygotes generated by gene targeting (5) were bred with BC-1 transgenic mice (17) to obtain *MSH2*$^{-/-}$/BC-1, *MSH2*$^{+/-}$/BC-1, and *MSH2*$^{+/+}$/BC-1 mice. Genotypes were determined as previously described (7, 10).

One male and two female animals, approximately 21 days of age, were chosen from each of the *MSH2*$^{+/+}$, *MSH2*$^{+/-}$, and *MSH2*$^{-/-}$ genotypes. Mice were given intraperitoneal injections of 50 mg/kg MNU (Sigma) on 2 consecutive days, followed by a 3-week recovery period to ensure that the repair of DNA lesions had occurred and that mutations had become fixed. MNU was selected as a prototypic S_N1 alkylating agent, a class known to efficiently generate O^6-alkylguanine DNA lesions. Animals were sacrificed by carbon dioxide inhalation, and tissues were flash-frozen in liquid N_2. DNA was extracted from small intestine, thymus, and heart and then packaged and plated as previously described (18).

Determination of *lacI* Gene Mutation Frequency and Spectrum. Tissue isolation and transgenic lambda phage rescue were carried out as described (18, 19). Briefly, phage genomes within high-molecular-weight BC-1 transgenic mouse DNA were excised and packaged by using a highly efficient phage packaging extract, Transpack (Stratagene). Rescued phage were then plated in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) on an SCS-8 (Stratagene) bacterial cell lawn, and *lacI* mutant frequency was established by determining the ratio of mutant (blue) to nonmutant (colorless) plaques. *LacI* mutations were verified as previously described (17). Following phage rescue and the isolation of single mutant clones, *lacI* genes were amplified by PCR of phage templates from randomly selected mutants. Templates were then directly sequenced by using primers spanning the *lacI* gene (17) and an ABI 388 sequencer (Applied Biosystems).

RESULTS

Mutation Frequencies. Mutation frequencies were determined for *lacI* genes rescued from small intestine, thymus, and heart DNA of $MSH2^{+/+}$, $MSH2^{-/+}$, and $MSH2^{-/-}$ animals. The number of *lacI* mutant (blue) plaques and the total number of plaque forming units (pfu) counted for the tissues of each animal are presented in Table 1. In repair proficient animals, MNU treatment resulted in increased *lacI* mutation frequencies in small intestine (74×10^{-5}) and thymus (22×10^{-5}), but much less so in heart (12×10^{-5}). Treated *MSH2* heterozygotes demonstrated mutation frequency inductions similar to those of the controls for small intestine (39×10^{-5}) and thymus (24×10^{-5}). MSH2-deficient animals, in contrast, revealed striking *lacI* mutation frequency increases above the already elevated backgrounds in all three tissues, with the highest increases occurring in small intestine (265×10^{-5}) and thymus (260×10^{-5}). A comparison of the mean mutation frequencies for each tissue from the three *MSH2* genotypes is illustrated in Fig. 1, where the excess mutations resulting specifically from MNU treatment can be seen superimposed on the spontaneous background frequencies of $MSH2^{-/-}$, $MSH2^{+/-}$, and $MSH2^{+/+}$ mice. Thus, the induction of mutations by MNU was greatest in *MSH2* nullizygous mice. Mutation frequency inductions were tissue-specific, with heart being far lower than tissues containing substantial proliferative cell populations, as exemplified by small intestine and thymus. Furthermore, with respect to the latter two tissues, the inclusion of nonproliferative cell populations such as thymic stromal cells or intestinal smooth muscle cells in the DNA preparation suggests that the mutation frequency increases measured underestimate the inductions within the proliferating cell populations.

Spectrum of Alkylation-Induced Mutations. The spectrum of mutations within small intestinal and thymic DNA obtained from MNU-treated $MSH2^{+/+}$ and $MSH2^{-/-}$ animals are presented in Table 2. The types of mutations observed were similar for both small intestine and thymus (data not shown) and thus were combined. Mutations isolated more than once per tissue per animal were included only once in the mutation frequency and spectrum data presented. This was done to eliminate any potential bias arising from the clonal expansion of cells harboring a particular mutation.

In untreated controls, G:C to A:T and A:T to G:C transitions were both present (50% and 13%, respectively) and together formed the most frequent class of mutation, followed by transversions (28%) (Table 2). MNU treatment of controls strikingly altered the distribution of *lacI* transition mutations, these being restricted to G:C to A:T mutations (74%).

The spontaneous *lacI* mutation spectrum of MMR-deficient mice was similar to that of controls, in that transitions, both G:C to A:T and A:T to G:C, predominated (42% and 25%, respectively) (Table 2). The proportion of frameshift mutations was increased and transversions were decreased in *MSH2*-deficient mice as compared with controls. Similar to controls treated with MNU, the spectrum shifted, with 82% of all mutations being G:C to A:T transitions. *LacI* mutants sampled from MNU-treated MMR-deficient mice also showed no A:T to G:C transitions.

Sequence Specificity of Mutations. In addition to a shift in mutation type, the site specificity of G:C to A:T transitions was altered in MNU-treated mouse tissues (Fig. 2). In untreated controls, 80%, and in untreated $MSH2^{-/-}$ animals, 60%, of G:C to A:T transitions occurred at CpG sites. In contrast, after MNU treatment, the majority of G:C to A:T transitions were at GpG sites in both $MSH2^{+/+}$ and $MSH2^{-/-}$ mice (59% and 65%, respectively).

The dramatic alteration in mutation spectrum of MNU-treated animals confirmed that mutation frequency inductions observed were mutagen-specific and also eliminated the possibility that high endogenous background mutation frequencies had been present in some of the animals.

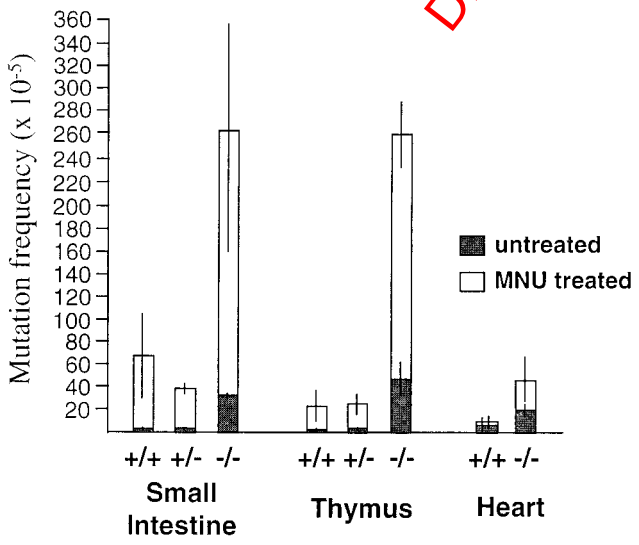

FIG. 1. *LacI* mutation frequencies (and standard errors) from tissues from $MSH2^{+/+}$, $MSH2^{+/-}$, and $MSH2^{-/-}$ animals, untreated and treated with MNU.

DISCUSSION

To determine the mutability of mice heterozygous or homozygous for a mutation in the *MSH2* mismatch repair gene, $MSH2^{-/-}$, $MSH2^{+/-}$, and $MSH2^{+/+}$ animals carrying a mutational reporter system were challenged with the alkylating agent MNU. We show that mice heterozygous for *MSH2* demonstrated *lacI* mutation frequency inductions similar to those of controls, whereas mice deficient in *MSH2* were hypersensitive to mutation induction by this agent.

Prior studies on alkylation-induced mutations in MMR-deficient systems have been limited to analyses of tumor cell lines such as MT-1, which is deficient in hMSH6/GTBP (20), a subunit of the hMSH2α complex (21, 22). MNNG-induced mutations of *hprt* genes in MT-1 cells were predominantly G:C to A:T transitions (20). The use of *lacI* transgenic mice, however, has permitted an *in vivo* assessment of mutations in a passive reporter gene within MMR-deficient hosts after exposure to an alkylating agent.

Previously, we demonstrated that spontaneous mutation frequencies in $MSH2^{+/-}$ mice were similar to those of controls, whereas mutation frequencies in $MSH2^{-/-}$ mice demonstrated 5- to 15-fold elevations as compared with controls in the tissues examined (10). Herein, we have shown that MNU administration led to an increase in mutation frequency in $MSH2^{+/-}$ animals that was similar to that of the $MSH2^{+/+}$ animals. The responses of these animals were also in keeping with studies employing "Big Blue" *lacI* transgenic mice, as well as rat cell lines (23–25). The lower inductions seen in heart, as compared with small intestine and thymus, were consistent with the reduced mutation frequency increases observed in brain (23), another tissue having relatively low levels of cell turnover in the adult animal.

In contrast to the results obtained in the $MSH2^{+/-}$ and $MSH2^{+/+}$ mice, MNU treatment of $MSH2^{-/-}$ mice resulted in a dramatic mutation frequency increase over the already elevated spontaneous backgrounds, reaching *lacI* mutation frequencies of $>250 \times 10^{-5}$ for small intestine and thymus (Fig. 1). Thus, when compared with controls, $MSH2^{-/-}$ animals proved exquisitely sensitive to mutation induction by this DNA methylating agent. Alkylation tolerance is a likely factor contributing to the greatly elevated mutation frequencies

Table 2. Mutation spectra from small intestine and thymus of MNU-treated and untreated control and MSH2$^{-/-}$ animals corrected for clonality

	Untreated MSH2$^{+/+}$		MNU-treated MSH2$^{+/+}$		Untreated MSH2$^{-/-}$		MNU-treated MSH2$^{-/-}$	
Mutation	No.	%	No.	%	No.	%	No.	%
Transitions								
G:C −> A:T	16	50	29	74	15	42	31	82
A:T −> G:C	4	13	0	0	9	25	0	0
Transversions	9	28	7	18	6	17	1	3
Deletions	1	3	0	0	1	3	2	5
Frameshifts	2	6	3	8	5	14	4	11
Total	32	100	39	100	36	100	38	100

observed in *MSH2*$^{-/-}$ tissues, because *MSH2*$^{-/-}$ cells experiencing high levels of DNA damage would be predicted not to undergo apoptosis, thus contributing to the tissue-specific mutation frequency data obtained.

When background *MSH2*$^{-/-}$ mutation frequencies were subtracted from MNU-induced frequencies, the resulting increase because of alkylation varied from tissue to tissue, with thymus and small intestine demonstrating much greater inductions (5- to 8-fold) than were present in heart. Although these differences might have resulted from multiple factors, such as differences in drug distribution or differences in O^6-methylguanine-DNA-methyltransferase levels in the different tissues (16), they likely reflect primarily differences in tissue-specific mitotic activity (23).

MNU-induced mutations in repair-proficient cells (Table 2, Fig. 2) were similar to those obtained with "Big Blue" transgenic mice, where 91% of all MNU mutations sequenced were G:C to A:T transitions, with 71% occurring at the 3′ G of GpG sequences (23). This was also in keeping with studies in bacteria that indicated that mispairing of O^6-meG with T was the principal mutagenic mechanism of MNU-induced DNA base alkylation (12, 26–32). MNU-induced mutations of *hprt* were also dominated by G:C to A:T transitions at A/GpG sites, further suggesting O^6-meG as the principal premutagenic lesion stemming from MNU exposure (33, 34).

The increased sensitivity of the *MSH2*$^{-/-}$ animals to alkylating agents and the similarity of the induced *lacI* mutant spectrum to that of MNU-treated controls suggested that *MSH2*$^{-/-}$ mice may prove to be useful adjuncts in evaluating the mutagenic potential of chemicals with potential DNA alkylating activity.

Do alkylating agents play a role in the genesis of colonic tumors in individuals with HNPCC? These compounds may be endogenously produced in the intestine from dietary nitrates that are reduced to nitrites in the proximal colon by bacteria, resulting in *N*-nitroso compounds with alkylating activity (35–37). In addition, amines and amides arising from protein catabolism are substrates for nitrosation by nitric oxide, with the resulting alkylating *N*-nitroso compounds being capable of DNA alkylation (38, 39). Other sources of methylating agents in the colon potentially include activated subepithelial macrophages (40) or nitrosated bile acids in the bowel lumen (41). Indeed, O^6-meG-containing DNA has been demonstrated in both normal and malignant colonic epithelia of humans (42), indicating that sources of alkylating compounds exist in the normal colon. Also, MNU-induced murine lymphomas typically contain activating mutations of K-*ras* within the GpG of codon 12 (43, 44), a site frequently involved in human colorectal cancers, including HNPCC (45, 46). Although the K-*ras* mutations could arise by other mechanisms, their presence suggests a potential role for agents capable of alkylating O^6-G in this process. It could be speculated that an alkylating agent(s) gradient exists in the human colon, with the highest concentrations being present in the proximal colon, as a way of explaining the tendency of HNPCC lesions to arise in the proximal bowel.

Whether MMR-deficient cells arise spontaneously in the normal colonic mucosa of HNPCC carriers, and whether these mutational events tend to predominate in the proximal colon or are distributed equally throughout the colon, is not known. However, given the presence of a mutagen gradient as suggested above, it might be predicted that the second *MSH2* allele would be at increased risk for mutational inactivation in the proximal colon, with such cells subsequently undergoing the increased mutation rates typical of MMR deficiency. There are very few reports on the nature of the second somatic MMR gene mutation in HNPCC colon tumors, and therefore there is insufficient evidence at present to exclude DNA alkylation damage as an etiologic factor in these mutations (2, 3, 47–49). If MMR deficient cells were to occur sporadically throughout the colon, our results suggest that in the presence of the putative alkylating agent gradient these cells would be at a greatly increased risk for G:C to A:T transition mutations within the proximal colon.

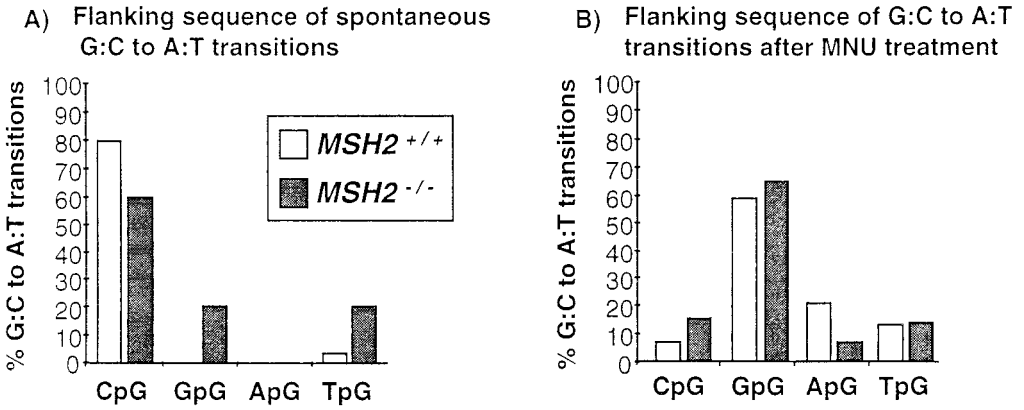

FIG. 2. Flanking sequence of G:C to A:T transitions observed in untreated *MSH2*$^{+/+}$ and *MSH2*$^{-/-}$ animals (*A*) and MNU-treated *MSH2*$^{+/+}$ and *MSH2*$^{-/-}$ animals (*B*).

MSH2 nullizygous but not heterozygous mice demonstrated an increased incidence of small intestinal tumors but were not predisposed to lesions of the large bowel, even on a $Min^{-/-}$ background (7). This difference from human HNPCC likely stems from a host of interspecies variables, including differences in life span, diet composition and mutagen content, intestinal flora and level of gut colonization, as well as the ability of the rodent to generate endogenous ascorbic acid (50).

We have determined the *in vivo* mutational frequencies and spectra of three tissues from $MSH2^{+/+}$, $MSH2^{+/-}$, and $MSH2^{-/-}$ animals. On exposure to MNU, heterozygosity for *MSH2* resulted in mutation frequency inductions similar to those of control animals. In contrast, MSH2-deficient mice were highly sensitive to mutation induction by this agent. MNU treatment resulted almost exclusively in G:C to A:T transitions in both $MSH2^{+/+}$ and $MSH2^{-/-}$ animals, in keeping with the miscoding properties of O^6-meG (13). These results suggest that HNPCC carriers may not be at increased risk for alkylation-induced mutations. However, spontaneously arising MMR-deficient cells would be predicted to accumulate mutations at a greatly accelerated rate on exposure to specific types of alkylating agents.

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