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Genetics

Characterization of mutations induced by ethylnitrosourea in seminiferous tubule germ cells of transgenic B6C3F$_1$ mice

(*lacI*/sequence spectra/*in vivo* mutagenesis testing)

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ABSTRACT **Transgenic B6C3F$_1$ mice carrying a *lacI* target gene were exposed to acute and multiple doses of ethylnitrosourea (ENU), and germ cells from the seminiferous tubules were assayed for mutation 3 and 90 days after treatment. Relative to untreated controls, the mutation frequency increased 3.2- and 19.9-fold at 3 and 90 days after treatment, respectively. Mutant *lacI* genes recovered from untreated and treated groups were sequenced, and the spectra of mutations were determined. Eighty-five percent (11/13) of the spontaneous mutations resulted in G·C → A·T transitions, all of which occurred at CpG dinucleotides. Fifteen of 22 sites (68%) found mutated 3 days after ENU treatment occurred at G·C base pairs, although some of these are expected to be spontaneous mutations. Ninety days after treatment, 13 of 19 sites (68%) found mutated occurred at A·T base pairs. The mutation spectra seen are consistent with proposed mechanisms of ENU mutagenesis and correlate with the *in vivo* spectra seen in ENU studies by using transmissibility assays and the *hprt* gene. These findings represent significant progress toward defining the *in vivo* spectra of ENU mutagenesis in mammalian germ cells.**

Specific information about mutations in mammalian germ cells is of considerable interest to those involved in genetic risk assessment. Assays such as the mouse visible specific-locus test, described by William Russell (1) and similar transmissibility assays have contributed to the foundation on which heritable genetic risk assessment is based today. The value of such assays lies in their ability to detect transmittable mutations affecting the heritable gene pool. Transgenic mouse mutagenesis assays potentially complement these important transmissibility assays by providing a means of measuring mutagenesis and determining mutational spectra *in vivo*. Spontaneous and induced mutations from pools of cells isolated from the seminiferous tubules have been quantified and characterized (2). In addition, information about mutagenic response to such factors as chemical dose, administration frequency, route of exposure, and expression time has been obtained by using λ/*lacI* transgenic mice (2–5). However, transgenic assays offer the additional practical advantage of a readily recoverable source of tissue-independent mutant DNA that can be further characterized through sequence analysis (2, 4, 5).

Ethylnitrosourea (ENU) induces a broad spectrum of promutagenic lesions *in vivo* (6, 7) and has been studied by using a variety of transmissibility assays (8–15). Although ENU has been well characterized as a mouse germ cell mutagen, the spectrum of ENU-induced mutations in mouse germ cells remains largely undefined. To date, only six germ-line mutations identified in ENU-treated mice offspring have been characterized at the nucleotide level and reported, all of which occur at either adenine or thymine residues (10–13, 16). The experiments described here characterize the mutagenic response of transgenic mouse testicular germ cells to acute- and multiple-dose ENU exposure and outline the spectra of mutants recovered after these exposures. These findings represent progress toward defining the *in vivo* spectrum of ENU mutagenesis in germ cells and explore the relationship to the *in vivo* somatic cell spectrum in the endogenous *hprt* gene.

MATERIALS AND METHODS

Transgenic Mice. Transgenic B6C3F$_1$ hybrid mice were generated from natural matings (performed at Taconic Farms) of inbred female transgenic hemizygous λ/*lacI* C57BL/6 mice (2) and male nontransgenic C3H mice. DNA was prepared from tail tips of the resulting offspring and screened for the transgene by dot-blot DNA hybridization, as described (2). Animals were maintained on a 12-hr light schedule in cages containing heat-sterilized wood chips and were provided with F4 rodent block food (Harlan Bioproducts, Haslett, MI) and tap water ad libitum.

Mutagen Treatment. ENU (Sigma, Lot no. 20H0369) was dissolved in Dulbecco's phosphate-buffered saline (pH 6.0) (GIBCO) and administered within 30 min of solution preparation. Four 8- to 10-week-old male transgenic B6C3F$_1$ mice (Big Blue mouse, Stratagene) were given three i.p. injections of 100 mg (± 2%) of ENU per kg of body weight at 7-day intervals for a cumulative multiple dose of 300 mg of ENU per kg. Ninety days after the initial injection, DNA was prepared from seminiferous tubules after collagenase and protease digestion and phenol–chloroform extraction, as described (2). In a separate (acute) experiment, four 8- to 10-week-old male B6C3F$_1$ mice were injected i.p. with 250 (± 2%) mg of ENU per kg of body weight. Three days later DNA was prepared from the seminiferous tubules as described above. Four age-matched, untreated animals served as negative controls in each experiment.

Mutation Screening. The transgenic shuttle vector was recovered from the mouse genomic DNA by using *in vitro* λ packaging extract (Transpack, Stratagene), as described (2). Mutations are detected as blue *lacI*$^-$ λ phage plaque-forming units on McrA$^-$ McrBC$^-$ Mrr$^-$ HsdR$^-$ *Escherichia coli* K-12 lawns (strain SCS-8, Stratagene) using 250 ml of NZY bottom agar (1.5%) and 35 ml of top agarose (0.7%) containing 52.5 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per 25-cm^2 assay tray.

Mutant Characterization. The criteria used for selecting phage plaques to be sequenced from the 3-day expression study were as follows. (*i*) Blue mutant phage plaques were separated from colorless nonmutant plaques. (*ii*) Phage from plaques meeting the first criterion did not exhibit a mosaic (mixed population of mutant and nonmutant phage) pheno-

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Abbreviations: O^6-eG, O^6-ethylguanine; MF, mean frequency; ENU, ethylnitrosourea.

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type when cored from the agar, eluted into buffer, used to reinfect *E. coli*, and replated in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside. Mutant phage plaques exhibiting a mosaic phenotype were considered *ex vivo* mutations (4); *ex vivo* mutations are defined as mutations occurring after DNA isolation and include mutations from *E. coli*-directed semiconservative replication and erroneous repair of DNA damage. Because <3% of the mutant phage plaques from the multiple-dose-exposure study were mosaic (see *Results*), mutant phage characterized were selected at random from the pool of all mutant plaques recovered in this study. Sequence data from the acute-exposure study were derived from plaques chosen at random from the pool of plaques meeting the preceding outlined criteria with the described sequencing methods (2).

Statistics. The logarithm to base 10 ($\log_{10}$) for all mutant frequencies was calculated to control for variances between samples. *P* values were calculated by using the $\log_{10}$-transformed data and a *t* test for two samples with equal variances (Microsoft EXCEL software).

RESULTS

Mutant Frequency. In all cases, a minimum of 300,000 plaque-forming units per mouse were screened for mutations. The individual and the mean mutant frequency (MF) are shown in Table 1. The mean spontaneous MF in the seminiferous tubules of the untreated controls is 1.1×10^{-5} (±0.2 SD) for the 3-day expression and 1.0×10^{-5} (±0.4 SD) for the 90-day expression animals. The mean MF for the acute exposure (250 mg of ENU per kg) samples after 3 days of expression is 3.8×10^{-5} (±1.0 SD). However, when screened for mosaicism, 5 of the 29 mutant plaques characterized (17%) were determined to be mosaic. The *ex vivo* MF was subtracted from the observed MF of each animal to yield an adjusted mean MF of 3.2×10^{-5} (±0.8 SD). The observed induced MF for the multiple-dose exposure (three injections of 100 mg of ENU per kg at 7-day intervals) 90-day expression study is 20.5×10^{-5} (±7.5 SD). Only one out of the 39 mutants (2.6%) tested for mosaicism from the 90-day expression time group was mosaic, resulting in an adjusted mean MF of 19.9×10^{-5} (±7.3 SD).

Spontaneous Mutant Spectrum. Sequence data from a total of 13 spontaneous mutations were used for comparison with treated samples (Table 2). These 13 mutations were located at nine different sites within the *lacI* gene and were obtained from seven different animals. All 13 mutations were unique by position, mutation type, or animal. However, 4 of 13 mutations, all derived from separate animals, occurred at position 42 of the *lacI* gene as numbered by Farabaugh (17). Two of 13 mutations occurred at position 56, and the remaining sites were unique. Eleven of 13 mutations sequenced from untreated samples were G·C → A·T transitions, all occurring at CpG dinucleotides. One G·C → T·A transversion and a frameshift mutation at A·T were also observed.

ENU Mutant Spectrum. Table 2 outlines the spectra of germ-cell spontaneous and ENU-induced mutations. A total of 26 mutants from the multiple-dose 90-day study and 23 mutants from the acute 3-day study group were sequenced, using a minimum of 5 mutants from each animal. For the ENU-treated samples in the acute 3-day study, 14 out of 23 of the mutations were G·C → A·T transitions (43% of which occurred at CpG dinucleotides), and 7 out of 23 mutations were A·T → T·A transversions. One G·C → T·A transversion and one frameshift mutation at C·G were also observed. Of the 26 mutants sequenced in the multiple-dose 90-day study, 11 were transitions (7 G·C → A·T and 4 A·T → G·C), and 15 were transversions (3 G·C → T·A and 12 A·T → T·A). Multiple identical mutations within a single animal were prevalent in every animal in the multiple-dose 90-day group, which was not seen in the acute-dose 3-day study (Table 3).

DISCUSSION

In a previous study of mutation spectra in transgenic C57BL/6 λ/*lacI* mice (2), germ-cell spontaneous mutation frequencies were consistently lower than those seen in somatic tissues. In addition, 3 days after acute ENU exposure

Table 1. ENU-induced mutant frequency in germ cells of seminiferous tubules

Animal	Total pfu rescued	Mutants, no.	MF, no. × 10^5
Control			
Day 3			**1.1 ± 0.2**
B9	341,900	4	1.2
B10	504,000	4	0.79
B11	346,800	4	1.1
B12	332,600	4	1.2
Day 90			**1.0 ± 0.4**
235	564,500	5	0.89
239	382,500	5	1.3
241	665,200	3	0.45
242	342,600	5	1.4
ENU (250 mg/kg)			
Day 3			**3.8 ± 1.0**
B1	403,400	18	4.5
B2	387,400	12	3.1
B3	361,300	10	2.8
B4	557,100	27	4.8
Day 3 (adjusted for *ex vivo* mutations)			**3.2 ± 0.8**
ENU (100 mg/kg per week for 3 weeks)			
Day 90			**20.5 ± 7.5**
133	556,900	64	11.5
136	561,700	168	29.9
143	571,000	118	20.7
146	574,400	114	19.8
Day 90 (adjusted for *ex vivo* mutations)			**19.9 ± 7.3**

MF values in boldface type represent the mean MF for that group ± SD. pfu, Plaque-forming units.

Table 2. Summary of spontaneous and ENU mutation spectra

	Mutation, % (*n*)		
		ENU	
	Spontaneous	Day 3	Day 90
Transitions			
A·T → G·C	0 (0)	0 (0)	15 (4)
G·C → A·T	85 (11)	61 (14)	27 (7)
CpG sites*	(11/11)	(6/14)	(2/7)
Transversions			
G·C → T·A	8 (1)	4 (1)	12 (3)
G·C → C·G	0 (0)	0 (0)	0 (0)
A·T → C·G	0 (0)	0 (0)	0 (0)
A·T → T·A	0 (0)	30 (7)	46 (12)
Other†	8 (1)	4 (1)	0 (0)
Total	≈100 (13)	≈100 (23)	≈100 (26)

Values in parentheses represent the number of mutations seen in that class.
*Mutations from potential methylcytosine deamination at CpG sites (eukaryotic methylation sites).
†Single-base frameshift mutations.

(250 mg/kg), relatively low induction over background was observed, considering the reported potency of ENU as a germ-cell mutagen (8). The conditions of that study were repeated by using the B6C3F$_1$ hybrid strain to determine whether strain-specific differences in spontaneous and induced mutant frequencies exist. To test the effect of expression time on induction and sequence spectrum, a 90-day expression time was used after initial ENU exposure. In addition, multiple exposures at subacute dose concentrations (three 100 mg/kg injections at 7-day intervals) were used because this treatment increases induction over spontaneous background in mouse germ cells while reducing toxicity (15).

In these studies, the frequency of *ex vivo* mosaic phage plaques was determined, and mosaic plaques were excluded from the spectral analysis. *Ex vivo* mutations are defined as mutations that arise outside the mouse, although they may arise from promutagenic lesions initiated within the mouse. Indeed, the phenomenon of mosaic mutations has been observed in transmissibility assays (18), presumably as the result of postfertilization events, thereby underscoring the potential importance of unrepaired promutagenic lesions. Although the specific mechanism for the formation of mosaic plaques was not investigated in this study, several potential mechanisms exist in the transgenic shuttle vector system. One class of *ex vivo* mutations analogous to the mosaic mutations seen in transmissibility assays could result from semiconservative replication of unrepaired, mutagen-induced DNA damage (e.g., adducts) in the bacterial recovery cells. Replication products derived from heteroduplex template strands would result in a mosaic plaque demonstrating a mutant phenotype and consisting of a population of mutant and nonmutant daughter phage. Because these mutations would reflect fixation of the mutation in *E. coli* and not necessarily in the mouse, they were excluded in the spectral analysis. However, the potential presence of nonmosaic *ex vivo* mutant plaques from mechanisms other than replication-derived mutations cannot be completely ruled out in the samples studied.

In repair-proficient germ cells the repair rate of ENU-induced damage depends on the type of adduct modifying the DNA. For example, the $t_{1/2}$ for removal of the ENU-induced O^6-ethylguanine adduct (O^6-eG) in mouse testes is reported to be ≈38 hr (19). Therefore, ≈25% of the O^6-eG adducts should remain unrepaired at the end of a 3-day expression period, whereas essentially none would remain unrepaired after 90 days. Because the mispairing capabilities of O^6-eG have been implicated as one of the primary mechanisms for ENU-induced mutation in replicating *E. coli* (20), the presence of O^6-eG on recovered transgenic DNA could contribute to the *ex vivo* mutant frequency. This hypothesis is consistent with the observation that 17% of the mutant plaques detected 3 days after acute ENU exposure are mosaic, whereas <3% are mosaic in the multiple-dose 90-day samples. In addition, adducts in postspermatogonial germ cells in the acute 3-day study may also contribute to *ex vivo* mutagenesis because DNA replication and repair may not occur during these cell stages (21). Mosaic mutations have been detected as late as 27 days after similar ENU exposures in transmissibility assays (18).

The observed low spontaneous mutant frequencies in germ cells relative to somatic cells (e.g., liver = 4.3×10^{-5}) (4) may reflect more stringent repair mechanisms, greater fidelity of replication, reduced deamination due to hypomethylation in germ cells (22–24), the protected environment afforded by the Sertoli cell barrier, or a combination of some or all of these factors. The relatively low but significant ($P<0.001$) induction over background observed in the B6C3F$_1$ strain after acute ENU exposure and 3-day expression was consistent

Table 3. Incidence and position of ENU-induced mutations

		Day 3				Day 90			
		B1	B2	B3	B4	133	136	143	146
Base*	Mutation†								
−8‡§	T → A	1			1				
42	C → T							1	
56	G → A			2					
72	T → C							1	
74	T → A			1					
79§	T → A					2	1		1
80	C → T	1							
83	A → G						2		
87	T → A				1				
89	T → C					1			
90	C → T	1							
92	C → T		1						
95	G → A		1						
101	A → T	1							
102	A → T						1		
104	C → T		1						
134	G → T						1		
140§	G → A			1	1				
183	T → A								1
191	C → T					2			
198	C → T						1		
201	G → A				1				
221	G → T								2
270	C → T	1							
276	T → A				1				
329	C → T	1							
381	G → T			1					
406	T → A		1						
484	Loss of C			1					
540	T → A								1
573	T → A								1
634	T → A					1			
710	C → T							3	
747	C → T		1						
843	G → A		1						
871	T → A						2		
874	T → A							1	
Total		6	6	6	5	6	8	6	6 (=49)

B1–B4 and 133–146 represent nos. of individual mice.
*Base-pair numbering system was that of Farabaugh (17).
†Base change is on sense strand of *lacI*.
‡This position is located in *lacI* promoter region.
§This site of mutation was seen in multiple animals.

with previous results in C57BL/6 mice (2) and, therefore, does not appear to be strain specific. The low induction is more likely a function of the short expression time used and reduced replication and repair in postspermatogonial cell stages (19). Thus, only the replication- and repair-proficient spermatogonial and prespermatogonial stages are likely to incur fixed mutations 3 days after ENU exposure. Spermatogenesis requires ≈45 days to be completed (25), and the significantly increased ($P<0.0001$) mutant frequency seen in the multiple-dose study is likely a function of the extended 90-day expression time. Because germinal stem cells present at exposure are susceptible to ENU mutagenesis, all resulting daughter cells are potential carriers of induced *lacI* mutations.

Exposures to identical doses of ENU have yielded germ-cell mutation inductions from ≈50- to 200-fold above background when the visible specific-locus test is used (15, 26), and induced mutant frequencies can vary from ≈10- to >140-fold above background when individual loci are examined separately (26). Few ENU-induced mutations detected in the visible specific-locus test have been characterized; whether the presence of hotspots for ENU within the seven loci plays a role in these high inductions is unknown. It has been suggested that because the specific-locus test is limited to detection of only recessive visible mutations, this test leads to an exaggerated mutation rate relative to other end points, such as the dominant cataract assay (9, 27, 28). Thus, higher or lower detectable mutation frequencies may exist between loci of the specific-locus test and the transgenic mouse target gene (*lacI*), given the precedent for induction variations when using different end points. Interestingly, a recent comparison of the endogenous *Dlb1* locus in the small intestine (29) and the transgenic *lacI* gene showed similar ENU-induced mutagenic responses.

Table 2 shows that spontaneous mutations were observed frequently (85%) at CpG dinucleotides in germ cells, presumably due to the deamination of methylcytosine (30), resulting in G·C → A·T transitions. The high frequency of G·C → A·T mutations at CpG sites in the spontaneous set is consistent with the observation that one-third of single-site mutations in inherited human diseases is of this class (31) and that this mutation class has also been implicated as carcinogenic in p53 tumors (32). The strong bias of spontaneous mutations resulting in G·C → A·T transitions at CpG dinucleotides in control groups suggests that spontaneous mutants in treated samples most probably occur in this mutation class.

The spectrum of mutations 3 days after ENU exposure consists primarily of G·C → A·T transitions (≈61%) and A·T → T·A transversions (30%). Based on fold induction (3.8-fold above controls; 3.2-fold adjusted for *ex vivo* mutations), approximately one-third of this spectrum is affected by the presence of spontaneous mutations. Six of the G·C → A·T transitions occur at CpG sites and, therefore, likely originated spontaneously. In addition, the single-base frameshift seen in the acute-exposure 3-day samples may also be spontaneous in derivation because a similar mutation was seen in control groups, and none was seen in the multiple-dose 90-day study. Although which are the spontaneous mutations is uncertain, the six mutations at CpG sites and the single-base frameshift account for roughly one-third of the 23 original mutations and are consistent with the distribution of mutations in the spontaneous spectrum (Table 2). After correction for the proposed contribution of spontaneous mutations at CpG sites, the remaining eight G·C → A·T transitions in the acute-dose 3-day study sample account for 50% of all mutations analyzed. This contrasts with the multiple-dose 90-day samples, where 27% of the mutations seen were G·C → A·T transitions. However, only 5% of the mutants sequenced in the multiple-dose study are probably spontaneous, due to the ≈20-fold induction over controls. In this case only one of the two G·C → A·T transitions at CpG dinucleotides is probably spontaneous, yielding an adjusted frequency of 23% G·C mutations. In addition, four A·T → G·C transitions in the multiple-dose 90-day study were detected, whereas none were seen in the spontaneous or acute 3-day study (Table 2).

The observed spectra in the separate experiments are likely a function of ENU mutagenesis and adduct persistence and repair. For example, the presence of G·C → A·T transitions at non-CpG sites in both treated groups is consistent with *in vivo* mispairing at ENU-induced O^6-eG adducts (19, 20, 33). In addition, the ENU-induced adducts O^4-ethylthymine and O^2-ethylthymine induce A·T → G·C transitions (34) and A·T → T·A transversions (35), respectively. Slow repair of O^2- and O^4-ethylthymine adducts has been suggested to contribute to increases in observed mutations at A·T base pairs over time (36, 37) and, therefore, should be detected at 90 days, as seen in our studies (Table 2). This result suggests that the observed spectra result from adduct-specific repair rates and the mutagenic potential of the ENU-induced adducts.

In contrast to one study where ENU-induced mutations at thymine residues demonstrated a bias for the nontranscribed strand of the *hprt* gene of B6C3F$_1$ mice (37), 14 of 17 sites (82%) of mutation involving thymine in *lacI* were in the sense strand (Table 3). Strand bias can be a function of transcription where DNA repair is more efficient on the transcribed strand (38). However, previous studies of the *lacI* gene have shown that ≈75% of all known sensitive thymine residues occur on the sense strand (2, 39–41) and, therefore, the results of this study suggest that there is little strand preference in the *lacI* gene. This is consistent with the fact that no known eukaryotic transcriptional promoters are present on the transgenic shuttle vector, and this result indicates that the *lacI* target is not actively transcribed in the mouse germ cells from cryptic promoters or read-through transcription from flanking promoters.

Other factors that may contribute to the observed spectra include the ratio of G·C- versus A·T-sensitive sites within target genes. Historical data derived from the detection of *lacI* mutations colorometrically using 5-bromo-4-chloro-indoyl β-D-galactoside and P-gal (refs. 2 and 39–41, and Barry Glickman, personal communication) indicate that mutationally sensitive sites occur more frequently at G·C base pairs (60%) relative to A·T base pairs (40%). Spectral sensitivity information such as this may be useful in normalizing data for comparing spectra in different target genes at the primary sequence level. For example, in one study of T cells in ENU-treated B6C3F$_1$ mice, 94% of mutations observed occurred at A·T base pairs in exon 3 of the *hprt* locus (37). These authors point out that the distribution of selectable base-pair sites in exon 3 is essentially even (28 A·T and 28 C·G sites). In germ cells of *lacI* transgenic mice, 62% of all mutations observed in the multiple-dose 90-day study also occurred at A·T base pairs (Table 2). When identical site mutations are scored only once to eliminate potential influences from clonal expansion, the A·T mutant frequency from this group is 13 of 19 sites, or 68%. Furthermore, when normalizing this data to account for the relative paucity of sensitive A·T sites in *lacI*, ≈85% of mutations can be attributed to A·T sites. These results are similar to the 94% A·T mutant frequency seen in exon 3 of the *hprt* gene. Other variables—such as tissue type, chromosomal position, and transcription state—probably account for the remaining differences. Although different tissues are analyzed in these two studies, the similarity in the ENU-induced mutation spectra is supportive evidence that the mutations observed reflect *in vivo* processes.

The occurrence of identical mutations within the same animal suggests that some of the mutations arise clonally (Table 3). Specifically, in the multiple-dose treatment group,

6 out of 19 sites contain >1 identical mutation, compared with the acute-exposure group, where only 1 out of 22 sites contained identical mutations. Possibly the relatively large number of clonal mutations at the 90-day expression time reflects acute cell toxicity and subsequent replenishment of the germ-cell population from a relatively small number of stem cells. This hypothesis is supported by the lack of a documented mechanism in germ cells for preferential clonal expansion, such as that found in T-cell populations. Finally, the observation that 4 of 13 spontaneous mutations (G·C → A·T) occur at base 42 (CpG site) and that these mutations are derived from different animals suggest that this position may be a spontaneous mutagenic hotspot.

Investigation of tissue-specific sequence spectra in both treated and untreated samples will assist in dissection of the relevant mechanisms leading to observed mutations. Tissue-specific differences in DNA-repair rates and pathways may provide important clues in the investigation of such mechanisms. In addition, a broad understanding of the sensitivity and limitations of the target genes used will allow interpretation of mutant frequency and spectral comparisons. Such information is invaluable in leading to more accurate models of mutagenic mechanisms and a clearer understanding of somatic and heritable genetic risk and its ultimate impact on the human condition.

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