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Mutational spectrum at the *Hprt* locus in splenic T cells of B6C3F$_1$ mice exposed to *N*-ethyl-*N*-nitrosourea

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ABSTRACT **We have determined the mutational spectrum of *N*-ethyl-*N*-nitrosourea (ENU) in exon 3 of the hypoxanthine (guanine) phosphoribosyltransferase gene (*Hprt*) in splenic T cells following *in vivo* exposure of male B6C3F$_1$ mice (5–7 weeks old) to ENU. *Hprt*$^-$ mutants were isolated by culturing splenic T cells in microtiter dishes containing medium supplemented with interleukin 2, concanavalin A, and 6-thioguanine. DNA was extracted from 6-thioguanine-resistant colonies and amplified by the polymerase chain reaction (PCR) using primers flanking *Hprt* exon 3. Identification of mutant sequences and purification of mutant DNA from contaminating wild-type *Hprt* DNA was accomplished by denaturing-gradient gel electrophoresis. Purified mutant DNA was then sequenced. Treatment of mice with ENU at 40 mg/kg of body weight produced a *Hprt*$^-$ mutant frequency of 7.3 × 10^{-5} in splenic T cells, ≈35-fold above background levels. Sixty-nine of the 521 *Hprt*$^-$ mutants analyzed contained mutations in exon 3 (13%). Tranversions and transitions at A·T base pairs dominated the spectrum; 62 of the 69 exon 3 mutations were at A·T base pairs (14 different sites). Thirteen of 14 thymine bases undergoing mutation (61 of 62 mutations at A·T bases) were located on the nontranscribed strand of exon 3. The majority of the remaining mutations (6 of 69) were transitions at a single G·C base pair. These results suggest the importance of thymidine alkylation in ENU-induced mutagenesis *in vivo*. The mouse *Hprt*$^-$ T-cell cloning/sequencing assay described here may represent a useful system for studying the molecular mechanism of chemically induced mutation occurring *in vivo* in an endogenous gene.**

Chemically induced mutation *in vivo* is the end result of a complicated cascade of events including compound uptake and distribution, metabolic activation/detoxification, compound interaction with DNA, DNA repair, and cell replication. The complexity of this pathway suggests that *in vivo* mutagenesis assays may be more relevant than *in vitro* systems for modeling possible mutagenic consequences in humans. *In vivo* mutation assays based on the cloning of hypoxanthine (guanine) phosphoribosyltransferase (HPRT)-negative T cells have been developed in the mouse (1–3), rat (4), monkey (5), and human (6, 7). Recently, transgenic mouse systems have also been described that contain bacterial transgenes as mutational targets (8, 9). These systems provide excellent opportunities to quantitate and compare the type and frequency of mutations occurring *in vivo* in different sequence contexts.

The type of base-pair changes produced in DNA can offer important insight regarding the specific DNA adducts and mutagenic mechanisms involved in the mutagenic event. To this end, our group has developed a method to sequence *Hprt* mutations induced *in vivo* in splenic T cells of B6C3F$_1$ mice, the strain presently used in the National Toxicology Program cancer bioassays. The approach utilizes denaturing-gradient gel electrophoresis (DGGE) (10–12) to purify mutant sequences for analysis. DGGE is based on the fact that the electrophoretic mobility of a DNA molecule in a polyacrylamide gel is considerably reduced as the molecule becomes partially melted (denatured). Mismatched heteroduplexes formed by annealing wild-type and mutant DNA sequences are always less stable than the corresponding perfectly base-paired homoduplexes and consequently melt at a lower concentration of denaturant. Therefore, any mutant/wild-type heteroduplex will always travel a shorter distance relative to wild-type homoduplexes in a gel containing a gradient of denaturant. It is predicted that heteroduplex formation and DGGE analysis will resolve any base substitution, frameshift, or small deletions (<10 base pairs) in the low-temperature melting domain of a DNA molecule (11). Also, since the addition of G+C-rich primers can modify the melting characteristics of the DNA molecule under study, all regions can be analyzed (12).

Methods to analyze mutations in the exon 3 region of the human HPRT gene by DGGE have been developed previously (13). Since the sequence of exon 3 and flanking intron regions of mouse *Hprt* have been determined (14), we sought to develop similar DGGE methods to analyze mutations isolated by the mouse *Hprt*$^-$ T-cell cloning assay (1–3). We have used these methods to determine the mutational spectrum of *N*-ethyl-*N*-nitrosourea (ENU) in B6C3F$_1$ mice.

ENU is a well-characterized ethylating agent that produces a variety of DNA modifications, including the potent promutagenic adducts O^6-ethylguanine and O^4-ethylthymine (15, 16). These lesions produce predominantly transition mutations at G·C and A·T base pairs (17, 18). Recently, the mutagenic potential of another ethyl adduct, O^2-ethylthymine, has been demonstrated in *Escherichia coli* and has been shown to result mainly in transversions (19). We report here that mutations induced by ENU *in vivo* are predominantly transversion and transition mutations at A·T base pairs, and we contrast these results with ENU spectral data obtained in other systems.

MATERIALS AND METHODS

Chemicals, Enzymes, and Medium Components. Materials were obtained from the indicated sources: fetal bovine serum (GIBCO); RPMI-1640, Hepes buffer, glutamine, MEM nonessential amino acids, penicillin/streptomycin mixture, and sodium pyruvate (Lineberger Cancer Research Center, Tissue Culture Facility, University of North Carolina at Chapel Hill); HL-1 medium (Ventrex Laboratories, Portland, ME); ENU (in aqueous 23% acetic acid as stabilizer) (Sigma); deoxynucleoside triphosphates (Pharmacia LKB), Sequenase, dideoxynucleoside triphosphates, and concanavalin A (United States Biochemical); AmpliTaq DNA polymerase (Perkin–Elmer/Cetus); Lympholyte M (Cedarlane Laboratories, Hornby, ON, Canada).

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Abbreviations: ENU, *N*-ethyl-*N*-nitrosourea; HPRT, hypoxanthine (guanine) phosphoribosyltransferase; DGGE, denaturing-gradient gel electrophoresis; 6TG, 6-thioguanine; PE, plating efficiency.

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Mice and ENU Treatment. Male B6C3F$_1$ mice (5–7 weeks old) were obtained from Charles River Breeding Laboratories (Raleigh, NC). The animals were fed standard chow and distilled water ad libitum and maintained in a 12-hr light/dark cycle. After a 10-day acclimation, mice were treated with ENU (40 mg/kg of body weight), delivered as a single i.p. injection of ENU diluted in dimethyl sulfoxide (9.0 mg/ml). After injection, the mice were maintained as described above for 6 weeks.

Mutant Isolation. Animals were killed by CO_2 asphyxiation followed by cervical dislocation and their spleens were removed aseptically. Spleens were crushed individually with the end of a 10-ml syringe plunger in 1 ml of RPMI-1640 medium plus Hepes buffer and 0.3% bovine serum albumin, drawn through a ⅝-inch 25-gauge syringe needle, and expelled gently into an additional 5 ml of RPMI-1640. The cell suspension from each spleen was then layered onto 5 ml of Lympholyte M and spun at room temperature for 20 min at 500 × *g*. Mononuclear cells at the interface were removed with a pipet and washed twice with 5 ml of the medium described above. Cells were then resuspended in RPMI-1640 supplemented with 10% (vol/vol) fetal bovine serum, 20% (vol/vol) HL-1 medium, 2 mM glutamine, MEM nonessential amino acids, 20 mM Hepes buffer, 1 mM pyruvate, 1× penicillin/streptomycin, 50 μM 2-mercaptoethanol, 4 μg of concanavalin A per ml, and 10% (vol/vol) lymphokine-activated killer T-cell treatment supernatant containing human interleukin 2 (a gift from Timothy Darrow, Duke University). Cells were enumerated with a Coulter Counter, adjusted to a concentration of 2×10^6 cells per ml, and then incubated for 24 hr at 37°C in a 6% CO_2 humidified incubator. A total of 4×10^7 mononuclear cells per spleen were routinely obtained. To determine plating efficiency (PE), a sample of the culture was diluted with complete medium to 80 cells per ml and plated in 0.1-ml aliquots in 96-well microtiter dishes in the presence of 4×10^4 lethally irradiated (10 krads; 1 rad = 0.01 Gy) mouse T cells per well. To isolate mutants, the original culture was diluted to a concentration of 4×10^5 cells per ml, supplemented with 6-thioguanine (6TG, 1 μg/ml), and seeded in 96-well plates at 0.1 ml per well. Plates were scored for colony growth at ×100 magnification on day 10. The number of clone-forming units (cfu) per well on the PE and 6TG plates was calculated by observing the fraction of negative wells and then applying Poisson statistics. The mutant fraction was calculated as follows: [(cfu per well on 6TG plates)(2×10^{-4})]/(cfu per well on PE plates).

Cells from positive wells were pipetted into Eppendorf tubes and washed with Dulbecco's phosphate-buffered saline (without magnesium). To isolate genomic DNA, cell pellets were resuspended in 20 μl of digestion mix [0.5% (vol/vol) Tween 20 and 0.1 mg of proteinase K per ml in 1× PCR buffer (see below)], incubated at 55°C for 1 hr, boiled for 10 min, and then frozen until needed.

PCR Conditions. To facilitate processing, aliquots of DNA samples were pooled in groups of two. Unless specified otherwise, all PCR volumes were 25 μl and were overlaid with 30 μl of mineral oil. The PCR mixtures contained 1× PCR buffer (15 mM Tris·HCl, pH 8.7/2.75 mM $MgCl_2$/60 mM KCl), each dNTP at 0.75 mM, each primer at 1 μM, and 1.0 unit of *Taq* polymerase. The thermal cycle consisted of 1 min at 94°C, a quick ramp to 45°C, 1 min at 45°C, a 2-min ramp to 72°C, and 30 sec at 72°C. Initial PCR amplification from genomic DNA used 2 μl of DNA solution and primers homologous to intron sequences immediately adjacent to exon 3 (5′ primer, 5′-CCTGATTTTATTTCTATAG-3′; 3′ primer, 5′-TTTAATTATAAGTAATTATACTTAC-3′). Thirty-five cycles were performed. To analyze the high-temperature melting domain of exon 3, the DNA from the initial PCR was gel-purified and then used as template for a second PCR amplification containing a GC-clamped primer (5′-CCGCGGGCGGGCCTCGCGCCGCGGGCCGGGAC-CCGCGGCCTGATTTTATTTCTATAG-3′) and a primer homologous to internal sequences of exon 3 (P3HI primer, 5′-TCCAGCAGGTCAGCAAA-3′). The melting characteristics of the full-length exon 3 fragment and of the GC-clamped, truncated fragment were determined by the melting algorithm of Lerman and found to be very similar to those reported for the human HPRT gene (13).

DGGE and Mutant Sequence Purification. Oil was removed from PCR mixture and the volumes were adjusted to 50 μl with water. Samples were extracted twice with phenol and twice with chloroform. Mutant/wild-type heteroduplexes of full-length exon 3 fragments were formed by heating samples at 98°C for 10 min and then holding at 37°C for >1 hr to allow reannealing. Mutant/wild-type heteroduplexes of GC-clamped, truncated exon 3 fragments were formed by denaturing the DNA at 98°C and holding overnight at 65°C. Samples were then vacuum-dried and resuspended in 10–20 μl of loading buffer.

One-millimeter-thick, 12.5% polyacrylamide gels (37.5:1 acrylamide/*N,N′*-methylenebisacrylamide weight ratio) containing linear gradients of denaturant were used. An 18–36% denaturant gradient was used to resolve mutations in the low-temperature domain and a 36–53% gradient for the high-temperature domain [100% is defined as 7 M urea plus 40% (vol/vol) formamide]. Samples were loaded onto gels submerged in a water bath at 60°C and run for 15 hr at 150 V in Tris/acetate/EDTA buffer. Gels were stained with ethidium bromide and visualized under UV light. Thin slices of gel containing mutant/wild-type heteroduplex bands were carefully excised from the gel and crushed, and DNA was eluted overnight at 4°C in 100 μl 10 mM Tris·HCl, pH 8.0/1 mM EDTA. Because the heteroduplexes isolated at this point were usually faint and contaminated with wild-type homoduplex DNA, an additional round of purification was performed. The supernatant from the crushed gel was diluted 25-fold into another PCR mixture and reamplified with 20 additional cycles. Samples were then processed as described above to form heteroduplexes and loaded onto another denaturing-gradient gel. Mutant/wild-type heteroduplexes were again excised from the gel and eluted as above.

The supernatant containing each heteroduplex was then diluted 25-fold into two PCR mixtures, one containing only the upstream primer and the other containing only the downstream primer; 15 cycles were performed. This reaction biased the concentration of one strand of the heteroduplex relative to the other strand. The appropriate second primer was then added and an additional 15 cycles were performed. To determine which PCRs produced the mutant homoduplex, samples from each were then mixed with an equimolar amount of known wild-type PCR product, processed to form mutant/wild-type heteroduplexes, and run in another denaturing-gradient gel. The appearance of pronounced mutant/wild-type heteroduplex bands indicated the presence of mutant homoduplex in the corresponding PCR mixture. PCR samples with mutant homoduplex DNA were diluted 50-fold into another PCR mixture (50 μl) containing the appropriate downstream primer (3′ primer or P3HI) and an upstream primer (5′+C primer) that contained a universal sequencing primer sequence attached to the 5′ end of the 5′ primer sequence (5′+C primer, 5′-CAGGAAGACATTCTTTC-CAGTTCCTGATTTTATTTCTATAG-3′). After 10 cycles, the products were ethanol-precipitated and run in an 8% polyacrylamide preparative gel. Bands were visualized with ethidium bromide and UV light, excised from the gel, electroeluted, and ethanol-precipitated.

DNA Sequencing. Single-stranded sequencing template was generated by adding half of the electroeluted material to a 50-μl PCR mixture with 0.5 μg of the appropriate downstream primer and processed for 25 cycles (94°C, 1 min; 44°C, 15 sec;

72°C, 30 sec). The reaction mixture was extracted twice with phenol and twice with chloroform, ethanol-precipitated, washed, and dried. DNA was sequenced with the Sequenase/dideoxy protocol specified by Applied Biosystems for use with their DNA sequencer and a fluorescently tagged primer (5'-CAGGAAGACATTCTTTCCAGTT-3').

Reconstruction Experiment. To test the quantitative nature of the mutant selection system, an experiment was performed to confirm that the PE of *Hprt*$^-$ cells in the presence of 6TG and 4×10^4 wild-type cells was the same as that of cells seeded in PE plates with irradiated feeder cells. T cells were isolated from an E14TG2a-derived, *Hprt*$^-$ transgenic mouse [(C57BL/6JLac × CBA/CaLac)F_2, generously provided by the laboratory of O. Smithies, University of North Carolina at Chapel Hill], primed in culture as described above, and then seeded at different concentrations onto standard PE plates with irradiated feeders and onto 6TG selection plates containing the standard number of wild-type (control) B6C3F_1 cells. The number of colonies appearing on the two types of plates was determined after incubation for 10 days.

RESULTS

The dose of ENU used in this experiment caused no overt toxicity to animals and the PE of isolated T cells from treated animals was indistinguishable from control values 6 weeks after treatment (0.5–2%). The *Hprt*$^-$ mutant fraction in control animals was $\approx 2 \times 10^{-6}$; this is similar to the values reported previously for the BALB/c (1) and C57BL/6 (2, 3) mouse strains. Mutant frequency in treated animals was ≈35-fold higher (7.3×10^{-5}), similar to previously reported values obtained with ENU (2). Reconstruction experiments showed that the PE of mutants on plates containing 6TG was indistinguishable from the PE on the PE plates (data not shown), thus demonstrating that the observed mutation frequencies measured in our experiments are good approximations of true mutation frequency existing in the T-cell populations.

A total of 521 *Hprt*$^-$ mutants from 24 ENU-treated animals were analyzed by DGGE. All mutants were first analyzed with the full-length, unclamped exon 3 fragment (bases 135–318) to investigate the low-temperature melting domain (bases 214–318). Mutants from single animals were also analyzed with the GC-clamped, 5' end of exon 3 (high-temperature melting domain; bases 135–213). Several of the mutants detected in full-length, unclamped molecules were ultimately shown to reside in the high-temperature melting domain of exon 3. The heteroduplexes formed with these mutants must have been sufficiently unstable relative to the wild-type homoduplex to permit resolution. These mutants also produced detectable heteroduplexes in the GC-clamped, truncated molecules; a few of these molecules were sequenced to confirm the mutation observed in the full-length molecule. Sixty-nine mutants were determined to reside in exon 3 of *Hprt* (13%). The fraction of ENU-induced mutants in exon 3 is consistent with that observed previously for the HPRT gene in human cells treated with ENU *in vitro* (20).

Mutations at A·T base pairs dominated the ENU spectrum in terms of both total mutants and number of sites mutated (Table 1). Both transition and transversion mutations were observed at A·T base pairs. Five A·T base pairs (146, 194, 216, 220, and 233) were observed to undergo more than one type of mutation. Mutations at G·C base pairs were observed at two sites. One site (base 151) underwent G·C → A·T transitions and was observed multiple times in several animals. The other mutation at a G·C base pair (base 190) was observed in one animal and was part of a double mutation that also involved a mutation at an A·T base pair (base 216). Since mutations at base 216 were seen separately in other animals, the possibility exists that the mutation at base 190 may have been an untargeted event.

In initial experiments, the T cells from several animals were pooled prior to plating (group P1). In this group, multiple isolates of an A·T → C·G transversion at base 233 were observed. This same mutation was not observed in any animal analyzed individually.

DISCUSSION

ENU has been shown to react with oxygens in DNA as well as with the ring nitrogens of guanine and adenine residues (15, 16, 21). Although many different ethylation products are produced, modification at the O^6 position of guanine and at the O^4 and O^2 positions of thymine have been shown to direct misincorporation by polymerase during DNA synthesis. O^6-Ethylguanine has been shown to induce G·C → A·T transition mutations (17), O^4-ethylthymine has been shown to induce A·T → G·C transitions (18), and O^2-ethylthymine has been shown to induce transversions (19). The mutations observed *in vivo* in ENU-treated B6C3F_1 mice are consistent with the persistence and mispairing potential of these promutagenic adducts.

Mutations at A·T base pairs accounted for 94% of all observed mutations (Table 2). It is possible that these mutations originated from spontaneous or glycosylase-mediated depurination of 7-ethyl- or 3-ethyladenine, with subsequent bypass replication of apurinic sites. This possibility is unlikely, however, given the production of similar levels of 7-ethyl- and 3-ethylguanine by ENU (21) and the lack of transversion mutations at G·C base pairs. Therefore, given the present data, we conclude that the transversions at A·T base pairs most likely result from miscoding directed by O^2-ethylthymine.

The paucity of mutations found at G·C base pairs relative to those found at A·T is probably due to the efficient repair of O^6-ethylguanine (22). In contrast, O^4- and O^2-ethylthymine adducts have been shown to be very persistent both *in vitro* in human cells (23) and *in vivo* in rats (24, 25). The persistence of thymine adducts would increase the probability of mutation at A·T base pairs relative to G·C. Furthermore, the rarity of mutations at G·C base pairs is probably not the result of selection bias, since a compilation of the literature reveals that 28 G·C and 28 A·T base pairs in exon 3 of the closely related human HPRT gene have been observed to mutate and result in the 6TG-resistant phenotype (26).

Thirteen of 14 thymine bases undergoing mutation (61 of 62 mutations at A·T base pairs) were located on the nontranscribed strand of exon 3. Such strand bias has been seen previously in *in vitro* mutagenesis studies with certain agents and has been attributed to strand-specific repair of the transcribed versus the nontranscribed strand (27, 28). If preferential repair of the transcribed strand *in vivo* is the reason for the observed strand bias with ENU, then one would assume that thymine modification and not adenine modification was responsible for the A·T mutations observed. Another explanation for the strand bias could be differential fidelity of leading and lagging strand replication of ENU-adducted DNA.

One mutation (base 233, A·T → C·G) was seen multiple times in a sample of pooled T cells from several animals but not in any animal analyzed individually. We interpret this phenomenon as the result of an *in vivo* clonal amplification of a mutant in one animal. If base 233 were a genuine mutagenic hotspot, it would have been detected in the animals analyzed individually. This emphasizes the importance of analyzing mutants separately from several animals while constructing spectra, to rule out biases due to clonal expansion. Such clonal expansion of HPRT$^-$ T cells *in vivo* has been documented previously in humans (29).

The mutational spectrum of ENU has been reported in a variety of systems. In *E. coli*, transition mutations at both G·C and A·T base pairs dominated the spectra observed in both the *gpt* and *lacI* genes (30, 31), in contrast to the spectra presented

Table 1. Base-pair substitution induced *in vivo* in ENU-treated mice in exon 3 of *Hprt*

Base	Mutation	P1	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M14	M15	M16	Total Exon 3 Mutants
146	AT►GC	1												1				2
	AT►CG															1		1
151	GC►AT	4					1	1										6
158	AT►TA					1	1										1	3
170	AT►TA					1						1		3		1		6
(190)	GC►TA				(1)													(1)
194	AT►GC			1								1			1			3
	AT►CG															1		1
203	AT►GC		2								2		1			1		6
214	AT►CG													1				1
216	AT►CG				1		1		1									3
	AT►TA													1				1
220	AT►GC							1							1			2
	AT►TA					1												1
221	AT►GC	1																1
233	AT►GC											1						1
	AT►CG	12																12
	AT►TA					1					1				1	1		4
236	AT►GC						1											1
271	AT►TA														1			1
299	AT►TA				1					1			2		1	3	2	10
318	AT►TA												1			1		2
Total Exon 3 Mutants		18	2	1	3	4	4	2	1	1	3	3	4	6	5	9	3	69

Base 135 is defined at the first base in exon 3. The number of isolates observed at each base position is given. Five hundred twenty-one *Hprt*$^-$ mutations from 15 individual mice (M1–M16) and a group of 9 mice (P1) were screened for exon 3 mutations. Each of the mutations observed would produce a change in an amino acid residue of HPRT protein. Four mutants from mouse M15 gave ambiguous sequencing results and are not included.

here. These data suggest that in *E. coli*, O^6-ethylguanine and O^4-ethylthymine are the most important lesions for mutagenesis. This difference implies that extrapolation of mutational specificity data from *E. coli* to mammalian systems may not always be a straightforward exercise. However, Eckert *et al.* (32) observed that an ENU-treated plasmid inserted into an SOS-induced *E. coli* gave rise to a large fraction of transversions at A·T base pairs (46%). The authors suggested that error-prone bypass of DNA-blocking lesions such as 3-ethyladenine, O^2-ethylthymine, or apurinic sites could have given rise to the transversion mutations. This spectrum is very similar to the one observed in B6C3F$_1$ mice.

Table 2. Types of base substitution mutations induced by ENU in B6C3F$_1$ mice

Mutation	No.
G·C → A·T	2 (4%)
G·C → T·A	1 (2%)
G·C → C·G	0 (0%)
A·T → G·C	14 (27%)
A·T → C·G	6 (12%)
A·T → T·A	28 (55%)

Data are taken only from mice analyzed individually (no pooled samples).

The spectra of ENU in normal and repair-deficient human lymphoblastoid cell lines in culture have been constructed previously by our group (20). Comparison of mutations observed in exon 3 reveals a striking similarity in the sites mutated by ENU in culture and *in vivo* and in the types of mutations induced. Five of the 16 sites seen in the present study were also recovered *in vitro* in normal cells after ENU treatment. It should be noted that the spectra obtained *in vitro* were constructed by using the entire cDNA sequence and were not "saturated" for mutations in exon 3 as in the present study; therefore, one would anticipate that even more overlap actually exists between the two systems. Also, the *in vitro* spectra contained a large fraction of transversion mutations at A·T base pairs, similar to that observed in the present study. Thus, with ENU good concordance was observed between results obtained *in vitro* and *in vivo* in cells of lymphoid origin.

The mutational spectrum of ENU has also been studied in a nontranscribed bacterial gene inserted into a transgenic mouse strain (8). The spectrum obtained in splenic DNA in this

system appears to be qualitatively different from the results reported here for a native gene. Six of 9 mutations (67%) observed were at G·C base pairs (3 G·C → T·A and 3 G·C → A·T), compared with 7 of 69 (10%) observed in the present study (6 G·C → A·T and 1 G·C → T·A). Only 3 of 9 mutations (33%) were at A·T base pairs in the transgenic mice. However, these preliminary data from transgenic mice are not sufficient to draw strong conclusions concerning similarities or differences between the two *in vivo* mutagenesis systems.

The most prevalent type of mutation observed in this study, A·T → T·A transversions, has been previously associated with exposure of rats and mice to ENU. In rats, activation of the *neu* oncogene by a A·T → T·A transversion has been demonstrated in ENU-induced tumors of the peripheral nervous system (33). Also, a mutation in the β-globin gene of an ENU-exposed mouse has been attributed to an A·T → T·A transversion by extrapolation from the amino acid substitution in the altered gene product (34). It is interesting that the thymine base undergoing mutation in both of these studies is located in the nontranscribed strand.

Due to the long phenotypic expression time associated with the 6TG-resistant phenotype (35) and the marked toxicity of 6TG to wild-type cells, the mouse T-cell cloning assay as performed here probably selects only mutants that have been fixed and expressed *in vivo*. Nevertheless, at the present time we cannot exclude the possibility that some of the mutations observed could have resulted from *in vitro* replication of DNA containing extremely persistent DNA adducts. However, this seems unlikely given the stable mutation frequency observed in mice up to a year after ENU exposure (2). If replication of promutagenic lesions *in vitro* were contributing a substantial fraction to the total number of observed mutations, then one would anticipate that the mutation frequency would decrease with time as adducts were slowly repaired.

The majority of *Hprt*$^-$ mouse T-cell clones isolated with the present plating technique have a limited growth potential *in vitro*. Positive wells on 6TG plates rarely contain a number of mutant cells that approaches the number of wild-type cells originally seeded into them. The limited growth potential of mouse T cells was a serious technical impediment to the analysis of *Hprt*$^-$ mutations. However, DGGE provided a means of purifying mutant sequences from contaminating wild-type sequences, thus permitting unambiguous sequence determination. The limited growth of mouse *Hprt*$^-$ T-cell clones is unlike that of human T cells, which are capable of undergoing several generations in culture after isolation. The extended growth potential of human T cells permits the establishment of viable mutant clonal populations that are amenable to molecular analysis of the HPRT gene by a cDNA/PCR approach (36).

The B6C3F$_1$ mouse T-cell cloning/sequencing assay for *Hprt* described here may represent a useful system for studying the molecular mechanism of chemically induced mutation occurring *in vivo* at an endogenous gene. It will be essential to compare results from this assay with other systems to uncover similarities and differences with other marker genes in other species, both those which exist naturally in the animal and those introduced in transgenic animals. It should be noted that the analysis scheme presented here should be amenable for use with any tissue cell type capable of even limited growth *in vitro*. For those tissues which cannot be grown in culture, the spectrum for a given compound can first be "saturated" in splenic T cells to reveal the important mutagenic events in the HPRT gene. These mutations can then be specifically detected and quantitated in other tissues by nonselective approaches analogous to those described by Cerutti *et al.* (37) and/or Kumar *et al.* (38).

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