Genotype-based screen for ENU-induced mutations in mouse embryonic stem cells

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The ability to generate mutations is a prerequisite to functional genetic analysis. Despite a long history of using mice as a model system for genetic analysis, the scientific community has not generated a comprehensive collection of multiple alleles for most mouse genes. The chemical mutagen of choice for mouse has been N-ethyl-N-nitrosourea (ENU), an alkylating agent that mainly causes base substitutions in DNA, and therefore allows for recovery of complete and partial loss-of-function alleles1. Specific locus tests designed to detect recessive mutations showed that ENU is the most efficient mutagen in mouse with an approximate mutation rate of 1 in 1,000 gametes2,3. In fact, several genome-wide6–7 and region-specific8–10 screens based on phenotypes have been carried out. The anticipation of the completion of the human and mouse genome projects, however, now emphasizes genotype-driven genetics—from sequence to mutants. To take advantage of the mutagenicity of ENU and its ability to create allelic series of mutations, we have developed a complementary approach to generating mutations using mouse embryonic stem (ES) cells. We show that a high mutation frequency can be achieved and that modulating DNA-repair activities can enhance this frequency. The treated cells retain germline competency, thereby rendering this approach applicable for efficient generation of an allelic series of mutations pivotal to a fine-tuned dissection of biological pathways.

To assess the mutagenic effect of ENU in ES cells and to test the feasibility of a strictly genotype-based screen, we characterized the mutation frequency and spectrum at the X-linked hypoxanthine phosphoribosyl transferase (Hprt) locus, which is hemizygous in male ES cells. Cell viability decreased with increasing ENU dose and treatment time (Fig. 1). We chose an ENU dose between 0.3 and 0.4 mg/ml on the basis of pilot results that suggested a higher mutation frequency for this dose. We sequenced the coding region of Hprt in 1,650 clones mutagenized with 0.35 mg/ml ENU to identify any that harboured mutations regardless of their cellular phenotype. We identified eight mutants. Four clones showed 6-thioguanine (6-TG) resistance, a loss-of-function Hprt phenotype consistent with the non-conservative missense mutations and splicing mutations revealed by sequence analysis (Tables 1 and 2). The remaining four clones contained silent or conservative amino acid changes and were sensitive to 6-TG, indicating that Hprt function was not abolished by such mutations. The sequencing results revealed an overall mutation frequency around 1 in 200 at the Hprt locus.

We next applied 6-TG selection to a larger sample of mutagenized clones to assess the frequency of loss-of-function mutations at the Hprt locus. We designed a 96-well sub-culturing scheme to ensure that each mutant surviving selection represented a unique mutation event. Our data indicated that ENU was able to induce loss-of-function Hprt mutants in ES cells at a frequency of 1 in 1,000 (Table 1). To examine the locus variability of this result, we determined the mutation frequency at an autosomal transgenic Herpes Simplex Virus thymidine kinase (HSV-tk) locus by selecting mutagenized cells with 1,2′-deoxy-2′-fluoro-β-D-arabinofuranosyl-5-iodouracil (FIAU). The mutation frequency at the HSV-tk locus was 1 in 1,200 (Table 1). Both frequencies were comparable to that of a typical whole-animal ENU screen for recessive mutations in specific locus tests2,3.

Sequence analysis of all Hprt mutants (38) produced by ENU treatment of ES cells provided information as to the mutation spectrum at the Hprt locus (Table 2). Most mutations were single-base substitutions (28) that resulted in missense and nonsense mutations. Among them, mutations affecting AT base pairs predominated (21 affected AT base pairs (75%); 7 affected GC base pairs (25%)). This base-pair bias has been previously seen in ENU-generated mutations in rat skin fibroblasts11 and mouse testicular germ cells12. In addition to amino acid substitution mutations, more than one-quarter of the mutations (10; 26.3% of total mutations) affected mRNAsplicing, transcription or stability. The occurrence of the pre-mutagenic lesions (21; 75%) seemed to be skewed toward the non-transcribed strand.

So far we have produced high-contribution chimaeras that are now being bred. One of the cell lines, E5/8-11, has undergone germline transmission, providing evidence that ES cells exposed to high concentrations of ENU retain their ability to populate the germ line. G1 animals derived from ES cells were viable and have been intercrossed to generate G2 animals. We have recovered G2 litters which were smaller than normal (one or two pups per litter).
ter), suggesting a high load of recessive lethal mutations in the genome of G1 animals. Thus, these animals can also be used for a phenotype-based screen, which supports the results presented in the accompanying paper.13

O6-alkylguanine-alkyltransferase (Agt) is the only identified mammalian alkyltransferase capable of removing various alkyl adducts from the O6 position of guanine bases in DNA (ref. 14). We performed an assay to measure the alkyltransferase activity in ES cell extracts and the effect of O6-benzylguanine (O6-BG), a specific inhibitor of Agt enzyme activity. Consistent with the expression data (Fig. 2a), ES cells and primary fibroblasts displayed detectable levels of Agt activity that were inhibited by O6-BG (Fig. 2b). Consistent with this observation, O6-BG pretreatment increased the frequency of loss-of-function mutations at the Hprt locus by more than twofold, to 1 in 450 (Table 2). To prove the statistical significance of the effect of O6-BG on mutation frequency, we chose the transgenic autosomal Hprt-tk locus as an independent target. The fact that tk mutants can be selected without passages after ENU treatment allowed us to screen a large number of mutagenized cells. The sample treated with O6-BG showed a statistically significant 3.6-fold increase in mutation frequency (Table 2). These data are consistent with the idea that

Table 1 • Mutation frequency influenced by O6-BG

| Treatment       | Number of clones screened | Number of drug-resistant clones | Hprt 6-TG  FIAU  Hprt  FIAU  Fold increase |
|-----------------|---------------------------|--------------------------------|--------|--------|-----------------|
| ENU alone       | 4,109                     | 10,800                          | 4      | 9      | 1/1,027         |
| O6-BG+ENU       | 2,276                     | 15,500                          | 5      | 47     | 1/330           |

*The sample size was limited by the 96-well sub-culturing scheme to reach statistical significance. 
**P=5.3×10^{-5}, as calculated using Fisher's exact test. The ENU concentration was 0.35 mg/ml.

Table 2 • Mutation spectrum at the Hprt locus

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Nucleotide position</th>
<th>Nature of the mutation</th>
<th>Strand</th>
<th>Codon and amino acid change</th>
<th>6-TG resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>5D1</td>
<td>–</td>
<td>splice mutation</td>
<td>–</td>
<td>undetectable RT–PCR product</td>
<td>yes</td>
</tr>
<tr>
<td>4B1</td>
<td>–</td>
<td>splice mutation</td>
<td>–</td>
<td>undetectable RT–PCR product</td>
<td>yes</td>
</tr>
<tr>
<td>D7H1</td>
<td>–</td>
<td>deletion of exon 2, 3b</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>E6/10-1</td>
<td>–</td>
<td>deletion of exon 2, 3b</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>B5-H6</td>
<td>–</td>
<td>deletion of exon 2, 3c</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>E5/4-2</td>
<td>–</td>
<td>multiple truncated RT–PCR products</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>E5/1-1</td>
<td>–</td>
<td>deletion of exon 6</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>E6/6-1</td>
<td>–</td>
<td>deletion of exon 6</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>7B3</td>
<td>–</td>
<td>deletion of exon 7</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>E5/9-2</td>
<td>–</td>
<td>deletion of 5 amino acids, frameshift nonsense mutation at base 714</td>
<td>yes</td>
<td>yes</td>
<td></td>
</tr>
</tbody>
</table>

The adenine A in the start codon ATG is designated as position 1. **Two RT–PCR products were present: exon 3 deletion and exons 2 and 3 deletion. **Three RT–PCR products were present: exon 3 deletion, exon 2 deletion and exons 2 and 3 deletion. n, non-transcribed; t, transcribed. A polymorphism in CT129Sv cells changes the ATG in the published sequence23 to GAT, substituting Asn with Asp.
abolishing the activity of Agt in ES cells can increase mutation frequency. This trend is supported by studies in other cell systems. For example, a two- to threefold increase in ENU mutagenicity has been observed in normal human keratinocytes pretreated with O6-BG (ref. 15).

Functional genome analysis relies on the availability of multiple mutant alleles, which are the building blocks for genetic analysis. Alleles of different strength are particularly valuable reagents as they enable a fine-tuned dissection of gene function and match what is generally found in the human population. The mutagenic nature of ENU in the mouse germ line has encouraged large-scale efforts to recover novel alleles in whole-animal mutagenesis programs,16,17. Despite rewarding results, these screens are costly and time consuming and it is difficult to achieve saturation6. The ENU mutagenesis strategy that we have used allows efficient generation of an allelic series in genes or domains of interest in ES cells. The capacity to induce, characterize and type of mutations produced, coupled with advancements in reagents as they enable a fine-tuned dissection of gene function and match what is generally found in the human population.

Methods
ENU mutagenesis of ES cells. We used CT129/Sv ES cells21 in the Hprt studies. We used IR4-B2 ES (J. Rivera and T.M., unpublished data) cells targeted with a construct expressing the Herpes Simplex Virus thymidine kinase (HSV-tk) to one of the NCAM alleles on chromosome 9 in the HSV-tk studies. Cells were cultured on primary feeder layers or gelatin-coated plates at 37 °C in a humidified incubator with 5% CO2. The culture medium consisted of MEM-α, 15% fetal calf serum, β-mercaptoethanol (0.1 mM) and leukemia inhibitory factor (1,000 units/ml). The mutagenesis protocol was as follows. ENU (Sigma) was dissolved in equal portions of 95% ethanol and PCS buffer (50 mM sodium citrate, 100 mM sodium phosphate dibasic, pH 5.0). O6-BG was dissolved in dimethyl sulphoxide (DMSO). All chemicals used here were diluted to the desired concentration with the growth medium and filter sterilized before they were added to cells. Before ENU treatment, we treated cells grown to ~80% confluence with O6-BG (10 µM) or 0.1% DMSO alone for 12 to 16 h. The pre-treated cells were then trypsinnized and incubated in suspension with the desired concentration of ENU in the presence of O6-BG (10 µM) or 0.1% DMSO at 5×10⁶ cells/ml for 2 h with constant rocking at 37 °C. Treating cells in suspension allows homogeneous exposure to ENU and accurate assessment of the mutation frequency. At the end of the incubation, the cells were washed, trypsinnized again to dissociate any cell aggregates formed during ENU treatment and counted. The mutagenized cells were either plated by limiting dilution directly into 96-well plates to allow fewer than 5 surviving colonies per well or plated onto 100-mm plates and isolated colonies were hand-picked into 96-well plates. We added O6-BG to the growth medium of cells pretreated with O6-BG during the first 24 h of post-ENU treatment to deplete any newly synthesized alkylguanine alkyltransferases. Triplicates of each clone were made to allow selection for Hprt-null mutants, sequence analysis of the non-selected clones and the generation of frozen stocks of mutagenized cells. The number of colonies formed per 1,000 non-mutagenized cells plated was used to determine the plating efficiency. After being normalized against the plating efficiency, we used the average number of colonies formed from 1,000 to 1×10⁶ ENU-treated cells to calculate the percentage of cells that survived the ENU treatment.

Selection of Hprt-mutant clones. As residual wild-type Hprt activity in mutant cells will interfere with selection, it is necessary to sub-culture the mutagenized cells for a period of time requiring one to two passages. If the sub-culturing is done in large scale, many Hprt mutants will be siblings derived from the same parental mutant, resulting in a skewed estimation of the mutation frequency. The 96-well sub-culturing procedure ensures clonality during cell passages and is critical for an accurate measurement of the mutation frequency. We sub-cultured the mutagenized CT129/Sv cells for at least 9 d after ENU exposure with one to two passages to allow depletion of wild-type Hprt protein. At the end of the sub-culturing, we added 6-thioguanine (10 µM; 6-TG) to the medium to select for Hprt mutants. We selected one million non-mutagenized cells simultaneously to assess the spontaneous mutation rate at the Hprt locus. The 6-TG-containing growth medium was renewed every other day during the selection and 6-TG-resistant (6-TGr) colonies were scored after 7–9 d. Spontaneous 6-TGr colonies were never observed in the number of non-mutagenized cells plated in all experiments. We calculated the mutation frequency at the Hprt locus by dividing the number of 6-TGr clones by the total number of clones screened. When cells were plated by limiting dilution, the average number of colonies formed in three randomly chosen 96-well plates was used to deduce the total number of colonies screened.

Selection of HSV-TK mutants. We selected mutagenized IR4-B2 cells 4 d after ENU treatment with FIAU (0.2 µM) for HSV-tk mutants. We selected one million non-mutagenized cells simultaneously to assess the spontaneous mutation rate, which is typically 2–3 FIAU-resistant (FIAUr) colonies per one million non-mutagenized cells plated. The total number of colonies screened was obtained by multiplying the number of cells plated by the overall ENU survival percentage. We calculated the mutation frequency at the HSV-tk locus by dividing the number of FIAUr colonies by the total number of colonies screened.

Sequence analysis of the ENU-induced mutations at the Hprt locus. We isolated total RNA from ES cells using guanidine-isothiocyanate lysis buffer (Qiagen). Briefly, cells were lysed in 96-well cell-culture dishes.
lysat was transferred to a 96-well RNAse plate and the manufacture’s instructions were followed to isolate total RNA. First-strand cDNA synthesis and PCR was performed in a single tube using the Access RT–PCR System (Promega). Primers were designed (F-5′-CCCTCCCTGCGAGCGTCTT-3′, R-5′-GAAACCTGGTAAATTTACGTGGG-3′) to cover the entire ORF of the Hprt cDNA. PCR products were purified with the Qiagen 96-well PCR clean-up kit (Qiagen).

We used 30–40 ng of the PCR products and 5 pmol of either forward or reverse primer for sequencing (ABI Big Dye terminator cycle sequencing). We used 2 µl of the sequencing reaction in a final reaction volume of 10 µl. Cycle sequencing was performed as described by the manufacturer. The products were run through a 96-well Centrisep column (Princeton Separations), dried under vacuum and loaded on an ABI 377 sequencing gel. Sequence analyses were performed using the Sequencer, Phred and Phrap programs.

Generation of chimaeric mice. We injected Hprt mutant ES cells into C57BL/6 blastocysts according to standard methods.

Agt (encoding Agt) transcript and activity analysis. Total RNA from CT129/Sv ES cells and CD1 primary fibroblasts was isolated using Tri Reagent (Molecular Research) and reversed transcribed using Moloney murine leukemia virus reverse transcriptase (Gibco, BRL). RT–PCR of mouse Agt transcripts was performed using the forward primer 5′-GCATGGAGGAGGAGGAGT-3′ and the reverse primer 5′-AGTTGGGTGTCGTCGACTTCACTGC-3′. As an internal control for RNA integrity and equal loading, we amplified Gapd (encoding glyceraldehyde-3-phosphate dehydrogenase) mRNA in the same tube using the forward primer 5′-CCCTGGAGGCTGGG-3′ and the reverse primer 5′-CAGTCTGGGATGGAC-3′. We carried out 25 cycles of amplification for all samples under the following conditions: 94 °C for 45 s, 56 °C for 1 min and 72 °C for 1 min.

The Agt assay was performed as described22. Briefly, cells were lysed and Agt activity in the lysates was measured as removal of the [3H]methyl group from the O6 position of guanine in calf thymus DNA alkylated with [3H]N-methylaminosaurosine. The Agt activity was normalized against the DNA content in different cell extracts. Mouse primary fibroblast lystate was included as a positive control for the assay.

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