A strategy for fine-structure functional analysis of a 6- to 11-centimorgan region of mouse chromosome 7 by high-efficiency mutagenesis

(saturation mutagenesis/albino-deletion complex/N-ethyl-N-nitrosourea/hemizygosity screening/recessive lethals)

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ABSTRACT A refined functional map of a 6- to 11-centimorgan region surrounding the albino (c) locus in mouse chromosome 7 is being generated by N-ethyl-N-nitrosourea (EtNU) “saturation” mutagenesis of stem-cell spermatagonia. In the first phase of an experiment that will eventually test at least 3000 gametes, we screened 972 mutagenized gametes for the induction of both lethal and visible mutations with a two-cross breeding protocol. Thirteen mutations mapping within the limits of a segment corresponding to the cytologically visible Df(c Mod-2 sh-J)6DVT deletion were recovered. They represented three phenotypic groups: prenatal lethality (six mutations); a fitness/runting syndrome (three mutations, provisionally designated as fiu variants); and a neurological/balance-defect abnormality (four mutations). Complementation analysis provided evidence for a true repeat mutation at the sh-I (shaker-I) locus (for the neurological mutations) and another at the here defined fiu-I (fitness-I) locus. In addition, four complementation groups were defined by induced lethal mutations; the two other lethal mutations were each part of a cluster. The recovery of the repeat mutations suggests that the EtNU-induced mutation rate, estimated from specific-locus tests, should make it possible to achieve saturation mutagenesis of a chromosomal region. This experiment is providing basic logistical and statistical information on which to base strategies for expanding the functional map of larger segments of the mouse genome by experimental mutagenesis. It is also yielding additional mutations useful in dissecting the functional and molecular complexity of this segment of chromosome 7.

Heritable mutations constitute an important resource for studying the genetic control of organismal development. The current maps of the human (1) and mouse (2) genomes represent a collection of genetic loci defined either by physical polymorphisms in DNA sequence or by functional polymorphisms recognized by perturbations of normal development. However, these maps represent only a small fraction of the total number of loci that must be defined and characterized before one can achieve a significant understanding of the organization and functional complexity of the mammalian genome. One strategy for expanding genomic linkage and functional maps in lower eukaryotes has incorporated the concept of “saturation” mutagenesis to estimate the number of loci that map to a specific genomic segment or that are associated with a specific phenotype (3-8).

Until recently, such experiments could not be attempted in mammalian systems because high-efficiency germ-line mutagenesis, the cornerstone of any saturation-mutagenesis study, was not feasible. However, the discovery of a supermutagen for mouse spermatogonia—namely, N-ethyl-N-nitrosourea (EtNU) (9, 10), which can induce a mean per-locus mutation frequency as high as $1.5 \times 10^{-3}$ (10), opens the way to producing germ-line mutations with high efficiency in any region of the genome. The genetic lesions induced by EtNU are currently thought to be mostly small, intragenic changes (11-13). Indeed, EtNU mutagenesis has recently been employed in the first phase of a saturation-mutagenesis experiment (14, 15) involving the proximal segment of chromosome 17.

To address the question of whether the generation of a detailed fine-structure functional map of a segment of the mouse genome is logistically feasible, we have applied EtNU mutagenesis to a 6- to 11-centimorgan (cM) genomic segment surrounding the albino (c) locus in mouse chromosome 7. Fig. 1 depicts the current proposed functional map of this region, as well as the presumed extent of a long deletion mutation recovered in a radiation-mutagenesis experiment (16, 25). “Functional units,” associated with mutant developmental phenotypes (see Fig. 1), are currently of unknown physical size and complexity and are defined primarily by overlap among many additional deletion mutations available for the region (16, 17). For example, a single lethal functional unit may include many loci, any of which, when mutated, may lead to prenatal death. Moreover, the early preimplantation or implantation death of embryos homozygous for any one of several deletion mutations associated with this region may be masking the expression of later-acting genes contained within a given deletion.

We describe here the initial results from a series of experiments designed to exploit one of the long, cytologically visible c deletions: (i) to detect, by EtNU mutagenesis, the number of distinct genetic loci that are mutable to specific, biologically significant, visible and lethal phenotypes within a defined 6- to 11-cM length of chromosome 7; (ii) to provide fundamental genetic, logistical, and statistical information on which to base strategies for subsequent large-scale expansions of the functional maps of larger segments of the mouse genome; and (iii) to provide the foundation for a fine-structure functional map, based on a series of heritable intragenic mutations with characteristic phenotypes, that can subsequently be correlated with detailed molecular/physical maps currently being developed.

MATERIALS AND METHODS

Mice. All stocks were bred at the Biology Division of Oak Ridge National Laboratory. The recessive-lethal Df(c Mod-2 sh-J)6DVT mutation (abbreviated $c^{F11}$) is a cytologically visible deletion of metaphase band 7E (26). The deletion is hypothesized to be 6-11 cM in genetic length and includes the marker loci c (albino; tyrosinase), Mod-2 (mitochondrial

Abbreviations: EtNU, N-ethyl-N-nitrosourea; $c^{F11}$, Df(c Mod-2 sh-J)6DVT; cM, centimorgan.

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malic enzyme), and sh-1 (shaker-1), as well as other developmentally important genomic subregions defined by complementation analyses of lethal c-locus mutations (Fig. 1) (16, 17). The cFP deletion, as well as additional lethals generated from the hemizygosity screen reported below, were maintained opposite a c<sup>c</sup> (chinchilla)-marked chromosome 7. Mice of the genotype c<sup>c</sup>/c or c<sup>c</sup>/c<sup>Fpl</sup> are a light-chinchilla color, as opposed to the full, darker, chinchilla (c<sup>c</sup>/c<sup>c</sup>) phenotype. [Breeding data from the c<sup>c</sup>/c<sup>Fpl</sup> stock provide evidence for a slight transmission ratio distortion observed at weaning (0.55 for c<sup>c</sup>; 0.45 for c<sup>Fpl</sup>), probably reflecting a slight, detrimental heterozygous effect of c<sup>Fpl</sup>.] The induced mutations are generically denoted as m; in certain places in the text, lethal mutations, a subset of all m mutations, are denoted as l.

**Mutagenesis.** EtNU was obtained from Radian (Austin, TX) and dissolved in phosphate buffer at 3 mg/ml as described (9, 10). Two groups of 12- to 16-week-old inbred BALB/cRl males (6 in one group and 16 in another) were given weekly intraperitoneal injections of EtNU (100 mg/kg each week) for a total fractionated dose of 400 mg/kg (10).

**Breeding Strategy for Hemizygosity Screening.** Fig. 2 depicts the genetic crosses that were used to identify new mutations that map to the region of chromosome 7 corresponding to the DNA missing in the 6- to 11-cM c<sup>Fpl</sup> deletion. Male BALB/cRl (c/c) mice (in the G<sub>0</sub> generation) were treated with EtNU and, after fertility returned, they were mated to highly fecund (C57BL/10R1 × C3Hf/RJ)F<sub>1</sub> females. All offspring (G<sub>1</sub>) from this cross carried a mutagenized paternal genome, including a chromosome 7 marked with the albino (c) mutation. G<sub>1</sub> females were crossed to c<sup>c</sup>/c<sup>Fpl</sup> males, which are heterozygous for the long "tester" deletion. Only the G<sub>2</sub> albino (c<sup>c</sup>/c<sup>Fpl</sup>) animals would be expected to express newly induced recessive mutations that mapped within the segment corresponding to that deleted in the Fpl deletion. Whenever possible, at least 30 G<sub>2</sub> progeny were produced and were examined at birth for the absence of the albino c<sup>c</sup>/c<sup>Fpl</sup> class, as this would be evidence that the G<sub>1</sub> female carried a newly induced, prenatally lethal mutation causing embryonic or fetal death when hemizygous. If G<sub>2</sub> albino progeny were born, they were observed at weaning for visible differences in body size/weight, hair quality, obvious skeletal abnormalities, inability to swim normally, other balance problems, or abnormal nervous activity. (Because of space limitations, G<sub>2</sub> animals were not kept after weaning, so late-onset mutant phenotypes were not scored.) All induced viable or lethal mutations detected by examination of the albino G<sub>2</sub> progeny were "stored" heterozygously in the light-chinchilla (c<sup>c</sup>/c<sup>c</sup>) G<sub>2</sub> carrier siblings, as well as in the founding G<sub>1</sub> female, for propagation in breeding stocks. Any G<sub>1</sub> female that yielded either 0 or 1 albino pup in 30 or more offspring was considered to carry a lethal mutation (l), and her light-chinchilla (c<sup>c</sup>/c<sup>c</sup>) mice were crossed to c<sup>c</sup>/c<sup>Fpl</sup> mice to verify transmission. The finding of a single albino pup in the G<sub>2</sub> progeny could represent either a "leaky" lethal mutation or, more likely, the result of crossing-over between c and l (see Discussion). (Chromosomal inversions, which aid in "removing" the meiotic products of crossing-over between two linked markers, were not used in this breeding protocol.)

**RESULTS**

Detection of Visible and Lethal Mutations Within the c<sup>Fpl</sup> Region. A total of 1311 G<sub>1</sub> females, generated from EtNU-treated c/c males, were crossed to c<sup>c</sup>/c<sup>Fpl</sup> males. Of these 1311 females, 972 (representing 972 mutagenized gametes) were completely tested for both lethal and visible recessive mutations and an additional 90 were tested for visible mutations only (these 90 pedigrees had at least one albino in <30
Table 1. Mutants recovered from hemizygosity screen employing the cFP1 deletion

<table>
<thead>
<tr>
<th>G2 female*</th>
<th>Born</th>
<th>Weaned</th>
<th>Phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td>181</td>
<td>0/29</td>
<td>0/21</td>
<td>Lethal</td>
</tr>
<tr>
<td>208</td>
<td>0/25</td>
<td>0/25</td>
<td>Lethal</td>
</tr>
<tr>
<td>375</td>
<td>0/25</td>
<td>0/21</td>
<td>Lethal</td>
</tr>
<tr>
<td>677</td>
<td>1/56</td>
<td>0/51</td>
<td>Lethal</td>
</tr>
<tr>
<td>1049</td>
<td>0/25</td>
<td>0/19</td>
<td>Lethal</td>
</tr>
<tr>
<td>1108</td>
<td>2/47§</td>
<td>1/44†</td>
<td>Lethal</td>
</tr>
<tr>
<td>494</td>
<td>12/33</td>
<td>10/31†</td>
<td>Runting</td>
</tr>
<tr>
<td>531</td>
<td>4/17</td>
<td>4/17†</td>
<td>Runting</td>
</tr>
<tr>
<td>764</td>
<td>4/13</td>
<td>0/9†</td>
<td>Runting</td>
</tr>
<tr>
<td>26</td>
<td>6/19</td>
<td>2/9†</td>
<td>Balance</td>
</tr>
<tr>
<td>716</td>
<td>7/47</td>
<td>3/41†</td>
<td>Balance</td>
</tr>
<tr>
<td>816</td>
<td>13/46</td>
<td>11/43†</td>
<td>Balance</td>
</tr>
<tr>
<td>824</td>
<td>6/24</td>
<td>3/19†</td>
<td>Balance</td>
</tr>
</tbody>
</table>

*Mutant designations are derived by adding the suffix SB to the identification number of the G2 female.
†Albinos are expected to be 25% of the G2 progeny. Often, a G2 female became sterile or died before 30 progeny could be obtained; therefore, G2 carriers of presumed lethals were tested (see below). §If G2 carriers (cFP1 + /c m2) of a presumed new mutation were crossed to cFP1+/+/cFP1, a minimum of 30 G2 progeny from each carrier were raised. Finding 0 or 1 albino per 30 offspring proved transmission of a lethal; for the runting and balance mutations, finding 0 G2 albinos (and no other class) that displayed the mutant phenotype proved transmission. Each mutation listed was proved transmissible by these criteria and was set up in a breeding stock.
‡These albinos were runted and compared with littermates. However, in 93 classified progeny from the cross of cFP1+/+/c m2 1108SB G2 carriers with cFP1+/+, no albinos were detected.
§The sizes of all albinos were 50–70% that of nonalbinos siblings.
¶The sizes of three albinos were 50–70% that of nonalbinos siblings.
**Albinos were noted to be small at birth but were missing by weaning.
††Severe defect in balance noted in all albinos and only in albinos.

G2 progeny). From the completely tested pedigrees, 13 new mutations mapping within the limits of the cFP1 deletion were identified (Table 1). These mutations could be grouped into three general phenotypic classes: (i) prenatal lethal; (ii) juvenile semi-lethal [provisionally designated fitness (fit) mutations], in which hemizygotes manifested a runting syndrome and died at variable times after birth; and (iii) a neurological syndrome resembling the defects in balance associated with mutations at the sh-l (shaker-1) locus. Table 1 shows the data obtained from each mutant pedigree; in each case, the existence of an induced visible or lethal mutation was verified by testing for the transmission of that mutation from cFP1+/c m2 to G2 light-chinchilla siblings.

In addition to the recessives listed in Table 1, four heritable, dominant variants were identified in G1 progeny. These mutations include: 1403SB, an allele of Slf (stem; chromosome 10); 1132SB, an allele of Tu (tabby; X chromosome); 595SB, a mutation associated with a neurologic abnormality manifested by a slight "tippy" gait; and 1298SB, a mutation that results in a kinked tail.

Complementation Analysis. Complementation analysis of newly recovered mutations provides a means for determining whether two independent mutations that specify similar phenotypes affect the same function. This analysis is, therefore, necessary for estimating the number of genetic loci within the limits of the cFP1 deletion detectable by EtNU mutagenesis.

Because the phenotype associated with the induced neurologic mutation 26SB is strikingly similar to that of the standard shaker-1 (sh-l) mutation, and because it was possible to detect additional sh-l mutations with our hemizygosity-screening protocol (cFP1 is deleted for sh-l), we made the cross cFP1 sh-l/+ × cFP1 +/+ 26SB to test for allelism. If 26SB were another allele of sh-l, we should expect, in the absence of recombination, 50% of the light-chinchilla class (namely, cFP1 sh-l/+ 26SB) to manifest the sh-l balance defect. Of 100 progeny generated from this cross, 28 were albino, 28 were light-chinchilla, 19 were light-chinchilla shakers, and 25 were chinchilla. Consequently, 26SB appears to be an EtNU-induced mutation at the sh-l locus and was designated sh-l26SB.

Within each phenotypic group of induced mutations (lethals, fitness, and neurological, "shaker-like"), proved carriers of one mutation (cFP1 +/c m2) were crossed to proved carriers of another mutation (cFP1 +/c m2). In crosses involving visible phenotypes, albino segregants (c m1/c m2) were examined at weaning for the corresponding mutant phenotype. In crosses involving lethals, the lack of the albino class (expected to be 25% of the progeny) would indicate noncomplementation. (Crossing-over between c and a specific lethal (l), in either cFP1 +/c l parent, could give rise to an occasional albino segregant, even in noncomplementing combinations, but the frequency of such segregants would be much less than 25%).

Table 2 summarizes these complementation data. Four complementation groups (and, presumably, four loci) [l(7)-IRn, l(7)-2Rn, l(7)-3Rn, and l(7)-4Rn] were defined by lethal mutations 181SB, 375SB, 677SB, and 1108SB, respectively. The lethal 208SB did fail to complement another lethal (181SB); however, 208SB arose from the same mutagen-treated G0 sire that gave rise to 181SB, and, therefore, it represents a noncomplementing "cluster repeat." Thus, 181SB and 208SB may not be independent isolates. Rather,
Experimental germ-line mutagenesis with EtNU, which produces primarily small, and, most likely, intragenic, lesions at high efficiency (9–13), can aid in refining the functional map of a chromosomal region corresponding to a large deletion in chromosome 7. Furthermore, incorporation of the saturation criteria into this experiment may provide an estimate of the number and nature of genes within larger segments of the mouse genome. The breeding protocol used here differs from that used in the work of Shedlovsky et al. (14, 15) for EtNU mutagenesis of the r region of chromosome 17 because the use of the cFp1 deletion obviates the need to render mutagenized chromosomes homozygous and makes it possible logistically to screen, with one fewer generation, a larger number of gametes for mutations within a specific (rather than variable) length of genome. Our results, therefore, should complement their work by providing additional data both on the relative mutability of loci and on the ability to approach saturation mutagenesis utilizing EtNU as a mutagen.

For this breeding protocol, we chose >1 albino in 30 G2 offspring as the cut-off for defining a new lethal mutation because a binomial distribution with \( P = 0.225 \) (where \( P \) equals the probability of detecting an albino offspring) predicts that, over a screen of 3500 pedigrees, just 14 pedigrees will be false positives, i.e., nonlethal pedigrees showing a lethal phenotype—the lack of the albino test class—by chance alone. However, if the cut-off were \( >2 \) G2 albinos, 77 false positives would be encountered, necessitating a much larger (and unproductive) effort in genetic testing of G2 carriers presumed to carry new lethals. On the other hand, it is possible to miss a new lethal \( (l) \) using a cut-off of \( >1 \) albinos per 30 G2 offspring. Crossing over in a \( +/c\ ) G1 female can lead to the production of recombinant \( +/cFp1 \) and \( +/cFp1\ ) zygotes. For example, for a locus 6 cM from \( c \), the probability is \( =0.1 \) that a pedigree carrying an \( l \) would be missed because of the finding of two G2 albinos (\( +/cFp1 \)) in 30 offspring. Hence, if all pedigrees segregating an \( l \) (not total pedigrees), one in ten will be classified as wild type and discarded. [These calculations are based on a binomial distribution, with \( N = 30 \) and the probability \( P \) of producing two albino offspring being 0.0174 at 6 cM.]

Missing a lethal pedigree due to crossing over should not be a serious problem; however, because this experiment will eventually screen 3000–4000 gametes. Moreover, because the average mutation rate is assumed to be 1/655 per locus per gamete, there should thus be approximately five chances to identify each locus in the region. A few repeat lethals may be missed due to rare crossing-over, and one might expect to find fewer repeat mutations at greater distances from \( c \). Of course, not all loci are equally mutable with EtNU (10), so missing these repeats will probably be only one component of the variability in locus mutability that we are likely to observe. For example, in the specific-locus experiments on which the above estimate of the average mutation rate is based, four to six times as many \( p \)-locus as \( s \)-locus mutations were observed (10). Thus, if there is always an uncertainty as to whether repeat mutations for all loci within a region can be recovered. This is true even when the target number of gametes to be screened (a number best estimated from the average mutation rate per locus) is judged sufficient.

The mutations detected in our initial screen of 972 completely tested EtNU-mutagenized gametes have provided data that address some of these questions. We detected two independent \( sh-I \) and two independent \( fit-I \) mutations in 1062 pedigrees that produced at least one albino test-class offspring. Therefore, the mutation frequency (induced by EtNU treatment of spermatogonia) of 1/655 per locus per gamete, assumed at the outset of the experiment, may be a reasonable estimate. [It is highly likely that these mutations are EtNU-induced and were not pre-existing in the stocks used for the experiment because the inbred BALB/cRI (c/c) strain was mutagenized. Moreover, if they were pre-existing, the G0 BALB/c male would have had to be heterozygous for each mutation and would have transmitted it to one-half of his G1 daughters. Evidence for this was not observed in the G2 progeny.] Therefore, based on the original (and now seemingly reasonable) estimate of mutation frequency, a screen of 3000–4000 mutagenized gametes (our goal in this particular experiment) should allow, on average, the detection of five or six visible and/or lethal mutations per locus and should provide information on relative locus mutability within this region. Moreover, as the number of pedigrees analyzed increases, we can determine whether variants at the \( sh-I \) and \( fit-I \) loci are the only detectable visible mutations that map within this region of chromosome 7. No repeat mutations, however, have yet been detected for the genes identified by the four lethal mutations in 972 tested gametes.

Interestingly, our initial results suggest that we may define significantly fewer EtNU-induced lethal complementation groups within the \( cFp1 \) region than have been detected within the proximal \((T-H-2)\) region of chromosome 17 (15). However, our finding of repeat mutations at c-linked loci, coupled...
with a comparable frequency of recovery of heritable dominant mutations, suggests that this difference in recovery of lethals probably reflects either a real difference in lethal-gene density between the two regions or, more simply, that the homozygosity-screening protocol and chromosomal markers used by Shedlovsky et al. (14, 15), while not allowing as many gametes to be screened, do allow a larger segment of the genome to be covered.

Clusters of mutations comprised a nontrivial hindrance to efficient mutational analysis of the cFP1 region (Tables 2 and 3). In fact, the 972 pedigrees that were completely tested very probably furnish an overestimate of the actual number of independent gametes screened because the 959 “wild-type” pedigrees that were discarded almost certainly contained clusters. Based on the three non-complementing cluster mutations found among the seven visible (fit-1 and sh-1) mutations that were detected by screening 1062 gametes, we can estimate (grossly) that perhaps only 600 (4/7 of 1062) independent gametes were actually screened. The frequency of clustered mutations can be lowered by increasing the number of mutagenized males. Indeed, we recommend, as a general rule, that as many males as possible be treated for any EtNU mutagenesis experiment in which spermatogonial stem-cell killing (and its resultant higher probability of clustering) becomes significant.

The mutations generated by this initial experiment should be useful genetic tools for continuing the analysis of this chromosomal region. More complete information on the biologic effects of these mutations, as well as on their map positions within the albino-deletion complex, will be presented elsewhere. It is worth noting here, however, an apparent difference in the homozygous vs. hemizygous phenotype of the (k7)-1Rn1815B mutation. Homozygotes exhibit a variable, somewhat leaky, “semilethal” phenotype (Table 2); however, crosses of heterozygotes [c9h+/c (k7)-1Rn1815B] with deletion carriers [c9h+/cFP1] have never produced a noncrossover albino segregant manifesting a similar, extremely runted phenotype. This result implies that in the hemizygous state [c (k7)-1Rn1815B/cFP1], the 1815B mutation is always lethal before birth. Consequently, the lethal phenotype may be related to a gene-dosage effect; two doses of a mutant product may, in about 50% of individuals, be compatible with the completion of gestation and early neonatal/juvenile life. Alternatively, perhaps the effect of the 1815B mutation, when combined with hemizygosity for other genes within the c-deletion complex (when in combination with c9p), causes consistent prenatal death.

Radiation-induced deletion mutations (and especially complexes of deletion mutations) can be important tools for the initial molecular access to, and the subsequent analysis of, large chromosomal regions (27–29). It will be possible to map rapidly region-specific, EtNU-induced, presumably intragenic mutations with respect to breakpoints associated with members of the entire c-deletion complex. This deletion mapping will allow each new mutation to be placed within the genetic, functional, and emerging molecular maps of the region (29). As molecular maps, and particularly transcription maps, become more detailed and more complex (even for small regions of DNA), the availability of single-gene mutations that specify particular phenotypes should be important tools for correlating DNA sequences with biologically significant functions.

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