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 spermatogonia. 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 $Df(c\ Mod\text{-}2\ sh\text{-}1)^{26DVT}$ deletion were recovered. They represented three phenotypic groups: prenatal lethality (six mutations); a fitness/runting syndrome (three mutations, provisionally designated as *fit* variants); and a neurological/balance-defect abnormality (four mutations). Complementation analysis provided evidence for a true repeat mutation at the *sh-1* (shaker-1) locus (for the neurological mutations) and another at the here defined *fit-1* (fitness-1) 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 1.5×10^{-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 $Df(c\ Mod\text{-}2\ sh\text{-}1)^{26DVT}$ mutation (abbreviated c^{Fp1}) 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^{Fp1}, $Df(c\ Mod\text{-}2\ sh\text{-}1)^{26DVT}$; cM, centimorgan.
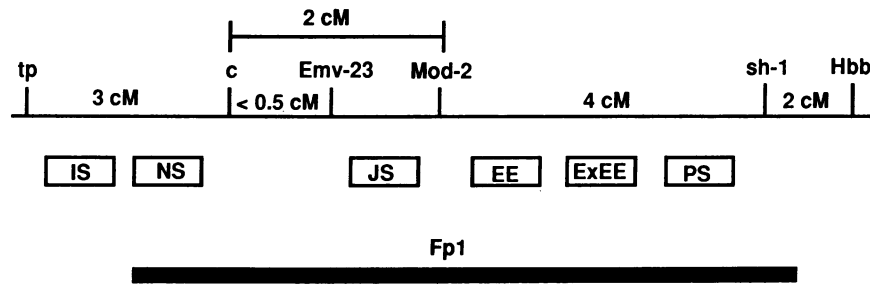*To whom reprint requests should be addressed.

FIG. 1.   Genetic and functional map of the region of mouse chromosome 7 surrounding the albino (*c*) locus. This map has been modified and abbreviated from reported maps (16, 17). Marker loci, included on the standard genetic map (2), are as follows (starting proximally): *tp*, taupe; *c*, albino; *Emv-23*, endogenous ecotropic murine leukemia provirus-23 (18); *Mod-2*, malic enzyme (mitochondrial form); *sh-1*, shaker-1; and *Hbb*, globin (β chain). (Descriptions of these loci are given in ref. 19.) Distances are given in cM. Boxes below the map represent chromosomal subregions ("functional units")—defined by complementation analysis of independent, radiation-induced lethal mutations (16)—necessary for the following functions: IS, implantation survival (16, 20); PS, preimplantation survival (16, 20, 21); EE, embryonic ectoderm formation (17, 22); ExEE, extraembryonic ectoderm formation (17, 22); NS, regulation of certain liver enzymes and serum proteins (for review, see ref. 23); JS, juvenile fitness (16, 24), male fertility (24), and pregnancy completion (24). The dark line below the subregions indicates the presumed extent of the 6- to 11-cM $Df(c\ Mod-2\ sh-1)^{26DVT}$ mutation (abbreviated Fp1) (16).

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 c^{Fp1} deletion, as well as additional lethals generated from the hemizygosity screen reported below, were maintained opposite a c^{ch} (chinchilla)-marked chromosome 7. Mice of the genotype c^{ch}/c or c^{ch}/c^{Fp1} are a light-chinchilla color, as opposed to the full, darker, chinchilla (c^{ch}/c^{ch}) phenotype. [Breeding data from the c^{ch}/c^{Fp1} stock provide evidence for a slight transmission ratio distortion observed at weaning (0.55 for c^{ch}; 0.45 for c^{Fp1}), probably reflecting a slight, detrimental heterozygous effect of c^{Fp1}.] 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 four weekly intraperitoneal injections of EtNU (100 mg/kg each week) for a total fractionated dose of 400 mg/kg (10).
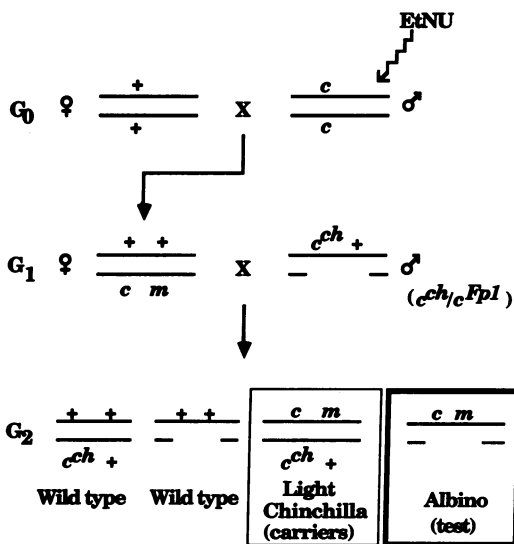

FIG. 2.   Breeding protocol used to generate and detect EtNU-induced recessive mutations within the region corresponding to the 6- to 11-cM c^{Fp1} deletion. The heavily outlined box highlights the G₂ albino class, which is the test class for new mutations in this screening protocol. The lightly outlined box highlights the G₂ light-chinchilla carrier class, from which new induced mutations can be recovered and propagated. *m* is a mutation induced by EtNU.

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^{Fp1} deletion. Male BALB/cRl (*c/c*) mice (in the G₀ generation) were treated with EtNU and, after fertility returned, they were mated to highly fecund (C57BL/10Rl × C3Hf/Rl)F₁ females. All offspring (G₁) from this cross carried a mutagenized paternal genome, including a chromosome 7 marked with the albino (*c*) mutation. G₁ females were crossed to c^{ch}/c^{Fp1} males, which are heterozygous for the long "tester" deletion. Only the G₂ albino (c/c^{Fp1}) animals would be expected to express newly induced recessive mutations that mapped within the segment corresponding to that deleted in the Fp1 deletion. Whenever possible, at least 30 G₂ progeny were produced and were examined at birth for the absence of the albino c/c^{Fp1} class, as this would be evidence that the G₁ female carried a newly induced, prenatally lethal mutation causing embryonic or fetal death when hemizygous. If G₂ 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, G₂ 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 G₂ progeny are "stored" heterozygously in the light-chinchilla (c^{ch} +/*c m*) G₂ carrier siblings, as well as in the founding G₁ female, for propagation in breeding stocks.

Any G₁ 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^{ch} +/*c l*) progeny were crossed to c^{ch} +/c^{Fp1} mice to verify transmission. The finding of a single albino pup in the G₂ 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^{Fp1} Region. A total of 1311 G₁ females, generated from EtNU-treated *c/c* males, were crossed to c^{ch}/c^{Fp1} 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 c^{Fp1} deletion

G₁ female*	Albinos/total in G₂ progeny†		Phenotype‡
	Born	Weaned	
181	0/29	0/21	Lethal
208	0/25	0/25	Lethal
375	0/25	0/21	Lethal
677	1/56	0/51	Lethal
1049	0/25	0/19	Lethal
1108	2/47§	1/44§	Lethal
494	12/33	10/31¶	Runting
531	4/17	4/17‖	Runting
764	4/13	0/9**	Runting
26	6/19	2/9††	Balance
716	7/47	3/41††	Balance
816	13/46	11/43††	Balance
824	6/24	3/19††	Balance

*Mutant designations are derived by adding the suffix SB to the identification number of the G₁ female.

†Albinos are expected to be 25% of the G₂ progeny. Often, a G₁ female became sterile or died before 30 progeny could be obtained; therefore, G₂ carriers of presumed lethals were tested (see below).

‡G₂ carriers (c^{ch} +/c m) of a presumed new mutation were crossed to c^{ch} +/c^{Fp1}. A minimum of 30 G₃ 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 G₃ 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 slightly runted compared with littermates. However, in 93 classified progeny from the cross of c^{ch} +/c 1108SB G₂ carriers with c^{ch} +/c^{Fp1}, no albinos were detected.

¶The sizes of all albinos were 50–70% that of nonalbino siblings.

‖The sizes of three albinos were 50–70% that of nonalbino 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 c^{Fp1} 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-1* (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 c^{ch} +/c m 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 *Sl* (steel; chromosome 10); 1132SB, an allele of *Ta* (tabby; X chromosome); 595SB, a mutation associated with a neurologic abnormality manifested by a slight "tipsy" 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 c^{Fp1} deletion detectable by EtNU mutagenesis.

Because the phenotype associated with the induced neurological mutation 26SB is strikingly similar to that of the standard shaker-1 (*sh-1*) mutation, and because it was possible to detect additional *sh-1* mutations with our hemizy-

gosity-screening protocol (c^{Fp1} is deleted for *sh-1*), we made the cross c^{ch} *sh-1*/c + × c^{ch} +/c *26SB* to test for allelism. If 26SB were another allele of *sh-1*, we should expect, in the absence of recombination, 50% of the light-chinchilla class (namely, c^{ch} *sh-1*/c 26SB) to manifest the *sh-1* 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-1* locus and was designated $sh\text{-}1^{26SB}$.

Within each phenotypic group of induced mutations (lethals, fitness, and neurological, "shaker-like"), proved carriers of one mutation (c^{ch} +/c *m1*) were crossed to proved carriers of another mutation (c^{ch} +/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 c^{ch} +/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)-1Rn, 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,

Table 2. Complementation analysis of EtNU-induced c-region mutations from cross: c^{ch} +/c *m1* × c^{ch} +/c *m2*

Allele		Albino (*c m1*/*c m2*)		
m1	*m2*	Total progeny, no.*	Phenotype*	
Lethal mutations				
181SB	375SB	9/33†	9/9	Normal
375SB	677SB	16/46	16/16	Normal
677SB	181SB	20/88†	20/20	Normal
1108SB	677SB‡	8/25	8/8	Normal
1049SB	677SB‡	1/47	1/1	Normal
375SB	1108SB	18/59†	18/18	Normal
181SB	1108SB	7/40	7/7	Normal
181SB	181SB	31/236§	26/31	Runted¶‖
181SB	208SB‡	8/50†	8/8	Runted‖
Runting mutations				
494SB	764SB	24/92†	24/24	Runted
531SB	764SB‡	5/18	5/5	Runted
Balance mutations				
26SB	816SB	22/84	20/22	Shaker¶
716SB	26SB‡	8/25	8/8	Shaker
824SB	816SB‡	4/14	4/4	Shaker
824SB	716SB	5/17	4/5	Shaker¶
824SB	26SB	8/18	8/8	Shaker

*Progeny were classified at weaning. In the case of full complementation, normal albinos should comprise 25% of the progeny. Complementation is indicated by expected frequency of albinos *and* their normal phenotype. All other combinations are noncomplementing.

†Data were pooled from reciprocal crosses.

‡Cross between members of a cluster (i.e., the mutations arose from the same G₀ male).

§Fifty-nine albinos would be expected from this cross if there were no associated lethal/semilethal phenotype.

¶Normal albinos were presumed to be recombinants that lost *m* from the *c m* chromosome.

‖These albinos were severely runted and were always dead by weaning.

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208SB may represent another isolate of a single mutation that arose in a spermatogonial stem cell. The same situation is true for 1049SB, a lethal mutation clustered with 677SB; for 531SB, a *fit* mutation clustered with 764SB, an independent *fit* allele (see below); for the balance-defect mutation 716SB, clustered with the *sh-1* mutation 26SB; and for the balance-defect mutation 824SB, clustered with 816SB (Tables 2 and 3).

On the other hand, the summaries in Tables 2 and 3 show cases of noncomplementation not associated with clustering. Thus, 816SB fails to complement *sh-1²⁶ˢᴮ*, and the 764SB *fit* mutation fails to complement 494SB, the prototype *fit* mutation. In these cases, the noncomplementing mutations each arose from a different mutagen-treated male, and, therefore, represent true repeat mutations at the *sh-1* locus and the here defined *fit-1* locus.

DISCUSSION

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 natures 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 *t* region of chromosome 17 because the use of the *c^{Fpl}* 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 albinos in 30 G_2 offspring as the cut-off for defining a new lethal mutation because a binomial distribution with $P = 0.225$ (where P

Table 3. Complementation groups identified by EtNU-induced *c*-region mutations

Mutation	Group*	Phenotype	Clustered†	Repeat mutation‡
181SB	*l(7)-1Rn*	Lethal	No	—§
208SB	—	Lethal	Yes (181SB)	CR
375SB	*l(7)-2Rn*	Lethal	No	—
677SB	*l(7)-3Rn*	Lethal	No	—
1049SB	—	Lethal	Yes (677SB)	CR
1108SB	*l(7)-4Rn*	Lethal	Yes (677SB)	—
494SB	*fit-1*	Runting	No	—
764SB	*fit-1*	Runting	No	R (494SB)
531SB	—	Runting	Yes (764SB)	CR
26SB	*sh-1*	Balance	No	—
716SB	—	Balance	Yes (26SB)	CR
816SB	*sh-1*	Balance	No	R (26SB)
824SB	—	Balance	Yes (816SB)	CR

*Complementation groups (and presumably genetic loci) are defined by the data presented in Table 2.
†A mutation is clustered with another if it arose from the same mutagen-treated G_0 male. The mutant designation in parentheses indicates the first mutation identified in that cluster. Only clusters belonging to the same phenotypic group are indicated.
‡Based on data from Table 2. CR, cluster repeat (i.e., not an unequivocal repeat mutation); R, true repeat [the mutant designation in parentheses indicates the prototypic mutation (and corresponding locus) involved in the repeat]. All cluster repeats were discarded.
§—, Not applicable.

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 G_2 albinos, 77 false positives would be encountered, necessitating a much larger (and unproductive) effort in genetic testing of G_2 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 G_2 offspring. Crossing over in a + +/*c l* G_1 female can lead to the production of recombinant *c* +/*c^{Fpl}* and + *l*/*c^{Fpl}* 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 G_2 albinos (*c* +/*c^{Fpl}*) in 30 offspring. Hence, of 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, there always exists 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-1* and two independent *fit-1* 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/cRl (*c/c*) strain was mutagenized. Moreover, if they were pre-existing, the G_0 BALB/c male would have had to be heterozygous for each mutation and would have transmitted it to one-half of his G_1 daughters. Evidence for this was not observed in the G_2 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-1* and *fit-1* 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 *c^{Fpl}* 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 c^{Fp1} 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 $l(7)$-$1Rn^{181SB}$ mutation. Homozygotes exhibit a variable, somewhat leaky, "semilethal" phenotype (Table 2); however, crosses of heterozygotes [c^{ch} +/c $l(7)$-$1Rn^{181SB}$] with deletion carriers [c^{ch} +/c^{Fp1}] have never produced a noncrossover albino segregant manifesting a similar, extremely runted phenotype. This result implies that in the hemizygous state [c $l(7)$-$1Rn^{181SB}$/c^{Fp1}], the 181SB 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 181SB mutation, when combined with hemizygosity for other genes within the c-deletion complex (when in combination with c^{Fp1}), 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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