N-Ethyl-*N*-Nitrosourea Mutagenesis of a 6- to 11-cM Subregion of the *Fah–Hbb* Interval of Mouse Chromosome 7: Completed Testing of 4557 Gametes and Deletion Mapping and Complementation Analysis of 31 Mutations

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ABSTRACT

An interval of mouse chromosome (Chr) 7 surrounding the albino (*Tyr*; *c*) locus, and corresponding to a long 6- to 11-cM *Tyr* deletion, has been the target of a large-scale mutagenesis screen with the chemical supermutagen *N*-ethyl-*N*-nitrosourea (ENU). A segment of Chr 7, from a mutagenized genome bred from ENU-treated males, was made hemizygous opposite the long deletion for recognition and recovery of new recessive mutations that map within the albino deletion complex. Over 6000 pedigrees were analyzed, and 4557 of these were completely tested for mutations specifying both lethal and gross visible phenotypes. Thirty-one nonclustered mutations were identified and assigned to 10 complementation groups by pairwise *trans*-complementation crosses. Deletion-mapping analyses, using the extensive series of radiation-induced *Tyr* deletions, placed the loci defined by each of these complementation groups into defined intervals of the *Tyr*-region deletion map, which facilitates the identification of each locus on physical and transcription maps of the region. These mutations identified seven new loci and provided new ENU-induced alleles at three previously defined loci. Interestingly, no mutations were recovered that recapitulated three phenotypes defined by analysis of homozygous or partially complementing albino deletions. On the basis of our experience with this screen, we discuss a number of issues (*e.g.*, locus mutability, failure to saturate, number of gametes to screen, allelic series) of concern when application of chemical mutagenesis screens to megabase regions of the mouse genome is considered.

THE interval of mouse chromosome (Chr) 7 surrounding the albino (*Tyr*; *c*) locus has been the subject of numerous genetic and molecular studies, primarily because of the availability of a large number of heritable, well-characterized deletion mutations (Rinchik and Russell 1990; Holdener-Kenny *et al.* 1992). The *Tyr* deletions thus provide a set of reagents with which to facilitate the molecular mapping of this region by fine-structure ordering of loci defined by DNA probes and microsatellite markers (Rinchik and Russell 1990; Holdener-Kenny *et al.* 1992; Rikke *et al.* 1997). Likewise, the ability to associate phenotypes with homozygosity for specific *Tyr* deletions has been a powerful way to correlate some of the genes later cloned from the *Fes–Hbb* region with specific steps in mammalian development (*e.g.*, Gluecksohn-Waelsch 1979; Russell *et al.* 1982; Niswander *et al.* 1988, 1989; Holdener *et al.* 1994). However, correlating cloned genes with their specific biological functions only by analysis of deletion phenotypes suffers from a number of challenges. For example, deletion homozygotes are often deficient for megabases of DNA, making it difficult to identify the specific deleted transcription unit(s) responsible for the phenotype in question. Likewise, the very nature of deletion mutations that extend from a single specific locus such as *Tyr* would, on average, make it difficult to identify late-acting genes mapping far from the specific locus. Indeed, the largest of the distally extending *Tyr* deletions is lethal in preimplantation stages when homozygous (Russell and Raymer 1979; Russell *et al.* 1982), a result not surprising considering how much DNA is deleted in these large deletions (6–11 cM; perhaps anywhere from 6 to 22 Mb). Finally, a danger inherent in studying all deletion phenotypes is that one may actually be examining a contiguous gene syndrome (Schmickel 1986; Rinchik *et al.* 1995), in which the observed phenotype is actually a composite of a number of individual phenotypes, each contributed by the loss of function of different transcription units.

We had previously reported (Rinchik *et al.* 1990; Rinchik 1991) a mutagenesis strategy for the genetic analysis of a subregion of the *Fah–Hbb* interval defined by the long, 6- to 11-cM Del (7)*Tyr*$^{c\text{-}26DVT}$ 1Rl deletion (hereafter abbreviated Del(*c*)^{26DVT}). This strategy employed the mouse germ-cell supermutagen *N*-ethyl-*N*-nitrosourea (ENU; Russell *et al.* 1979), combined with a two-cross hemizygosity screen, to induce and recover presumptive

This manuscript is dedicated to Dr. Liane B. Russell in celebration of her 50th anniversary at the Oak Ridge National Laboratory and in recognition of her remarkable career in mouse genetics.

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point mutations at Chr-7 loci residing within the limits of the Del(c)^{26DVT} deletion. In the initial report describing this long-term experiment (Rinchik *et al.* 1990), 972 mutagenized gametes were completely tested for the presence of new recessive mutations specifying both prenatal and postnatal abnormalities. From these initial 972 gametes, a total of nine nonclustered mutations, representing three general phenotypic groups and six genetic complementation groups, were described. A number of these mutations have since been analyzed further with respect to map position within the *Tyr* (albino) deletion complex (Rinchik and Carpenter 1993; Rinchik *et al.* 1993a; Potter *et al.* 1995), time of death for prenatal lethals (Rinchik *et al.* 1993a), and more exact characterization of the mutant phenotype (Holdener *et al.* 1995a; Potter *et al.* 1997).

This mutagenesis experiment has now been completed, and the present article summarizes the results of the genetic testing of an additional 3585 pedigrees, for a total of 4557 mutagenized gametes. The results of finalized complementation and deletion-mapping studies provide evidence (i) for recovery of a total of 31 nonclustered mutations [which includes the 9 previously reported mutations (Rinchik *et al.* 1990)] representing 10 loci; and (ii) for allelic series of mutations, with differences in severity, at several loci. The implications of the final data for the design and interpretation of large-scale mutagenesis experiments for megabase/centimorgan stretches of the mouse genome are also addressed.

MATERIALS AND METHODS

Mice: All stocks were bred at the Oak Ridge National Laboratory (ORNL) and have been described in detail elsewhere (Rinchik *et al.* 1990, 1993a; Rinchik and Carpenter 1993). For the sake of conformity with much past literature, the *Tyr* (tyrosinase) locus is indicated by its previously used symbol, *c* (albino). Both Del (*c*) deletion chromosomes and *c*-marked chromosomes carrying new ENU-induced mutations were maintained opposite chromosomes 7 marked with the c^{ch} (chinchilla) allele of *c*. Mice of the genotype c^{ch}/c or c^{ch}/Del (*c*) are lighter in color than are c^{ch}/c^{ch} homozygotes. Additional information about mouse stocks, including information on all the mutations reported here, can be found in the ORNL Mutant Mouse Database (http://lsd.ornl.gov/htmouse/mmdmain.htm/).

Mutagenesis: Five groups of BALB/cRl males (204 total males) were given four weekly intraperitoneal injections (100 mg/kg) of ENU as previously described (Rinchik *et al.* 1990). ENU was obtained from either Radian (Austin, TX) or Sigma (St. Louis, MO).

Breeding strategy and mutation detection: The hemizygosity screen used to detect mutations within the interval corresponding to the Del(c)^{26DVT} deletion has been presented in detail previously (see Figure 1 in Rinchik *et al.* 1990). In brief, mutagenized chromosomes 7, genetically marked with the original, viable *c* mutation, were bred from ENU-treated G_0 BALB/c males and were isolated in G_1 females by crossing the G_0 males to (C57BL/10Rl × C3Hf/Rl)F_1 G_0 females. The G_1 females were then mated to males heterozygous for the long Del(c)^{26DVT} deletion. Among the G_2 progeny, albino segregants carry a mutagenized chromosome 7 opposite a deleted chromosome 7 and would thus express any new recessive phenotype due to a new ENU-induced mutation (generically designated *m*) mapping within the limits of the Del(c)^{26DVT} deletion. Albino G_2 animals were therefore inspected for new visible phenotypes (size, gross morphology, nervous behavior, ability to swim, etc.). Equally important was the finding of a lack of the albino class in the G_2 progeny, as this was evidence for the induction of a new lethal mutation. Whenever possible, at least 30 G_2 progeny were scored for each pedigree. We chose a cut-off of less than two albinos in 30 classified offspring as providing evidence that the pedigree in question was segregating a *c*-linked ENU-induced lethal mutation (see Rinchik *et al.* 1990 for details). *c*-marked chromosomes carrying new, ENU-induced, visible, or lethal mutations (*i.e.*, $c\,m$) were recovered from the G_1 female ($+\,+/c\,m$) or from G_2 light-chinchilla progeny ($c^{ch}\,+/c\,m$) and were propagated in breeding stocks for further genetic analysis of mutant phenotypes (Rinchik *et al.* 1990, 1993a; Rinchik and Carpenter 1993).

Complementation and deletion-mapping crosses: Any presumed $c^{ch}\,+/c\,m$ mouse used for complementation or deletion-mapping crosses was progeny-tested by a cross to c^{ch}/Del(c)^{26DVT} (30 progeny classified for each) to ensure that it still carried a $c\,m$ chromosome (see Rinchik *et al.* 1993a); these genetically tested mice were considered "proved" carrriers. For complementation crosses, proved carriers of one mutation ($c^{ch}\,+/c\,m1$) were crossed to proved carriers of another mutation ($c^{ch}\,+/c\,m2$). Absence of the albino class (for lethal mutations) or presence of an abnormal phenotype (for visible mutations) indicated noncomplementation, a result that assigned *m1* and *m2* to the same complementation group (at which time they were considered to be alleles of the same locus until proved otherwise). Deletion-mapping crosses of $c^{ch}\,+/c\,m$ mice to carriers of *c* deletions have been described in detail elsewhere (Rinchik and Carpenter 1993; Rinchik *et al.* 1993a) and involved crossing proved $c^{ch}\,+/c\,m$ carriers to c^{ch}/Del (*c*) heterozygotes. A mutation was considered to be included within the deletion if there were no albino progeny [$c\,m$/Del (*c*)] in 30 offspring of this cross (for the lethal mutations), or if the albino progeny were abnormal (for the visible mutations). Normal albino progeny would be expected to comprise approximately one-quarter of the progeny in complementing combinations in either the deletion-mapping or complementation crosses.

RESULTS

Complete testing of 4557 ENU-mutagenized gametes for recessive mutations mapping within the 6- to 11-cM Del(c)^{26DVT} deletion: Over a period of 6 yr (1986–1992), 204 BALB/cRl (G_0) male mice were treated with ENU and bred to (C57BL/10Rl × C3Hf/Rl) F_1 females to generate 6652 G_1 females ($+/c$). Among these 6652 females, each of 4557 had a G_2 progeny of sufficient size to determine whether the G_1 female carried a new recessive mutation, mapping within the limits of the Del(c)^{26DVT} deletion, that would produce a hemizygous phenotype detectable by the simple screening criteria outlined in materials and methods. Among these 4557 pedigrees (*i.e.*, 4557 tested gametes of the mutagenized G_0 male), 36 new mutations were identified. Five of these 36 mutations were identified as noncomplementing clusters among the first 972 tested gametes

TABLE 1

Independent mutations recovered in the *c*-region hemizygosity screen

Mutation	Hemizygous phenotype[a]	Homozygous phenotype[a]	Complementation group[b]
181SB[c]	PL	PL, NL	*l7Rn1*
375SB[c]	PL	PL	*l7Rn2*
4940SB	PL	PL	*l7Rn2*
6633SB	PL	PL	*l7Rn2*
677SB[c]	PL	PL	*l7Rn3*
1777SB	PL	PL	*l7Rn3*
2292SB	PL	PL	*l7Rn3*
2521SB	Slight R	WT, slight R[d]	*l7Rn3*
4323SB	PL	PL	*l7Rn3*
6105SB	PL	PL	*l7Rn3*
1108SB[c]	PL	PL	*l7Rn4*
4742SB	PL	PL	*l7Rn4*
1989SB[c]	PL	PL, NL, R	*l7Rn5* (*eed*)
2235SB[c]	PL	PL, NL, R	*l7Rn5 (eed*)
3354SB[c]	PL	PL	*l7Rn5* (*eed*)
4234SB[c]	PL	PL, NL	*l7Rn6*
494SB[c]	R	R	*fit1*
764SB[c]	R	R	*fit1*
3452SB	Slight R	~WT	*fit1*
4226SB	Slight R	Slight R	*fit1*
4397SB	R	R	*fit1*
5772SB	JL[e]	JL[e]	*sjds*
5961SB	NL	NL	*Fah*
6287SB	JL	JL	*Fah*
26SB[c]	Shaker	Shaker	*sh1 (Myo7a)*
816SB[c]	Shaker	Shaker	*sh1 (Myo7a*)
3336SB	Shaker	Shaker	*sh1 (Myo7a)*
4494SB	Shaker	Shaker	*sh1 (Myo7a)*
4626SB	Shaker	Shaker	*sh1 (Myo7a)*
5745SB[f]	Shaker	Shaker	*sh1 (Myo7a)*
5824SB[f]	Shaker	Shaker	*sh1 (Myo7a)*

[a] PL, prenatally lethal; NL, neonatally lethal (usually within 24 hr of birth); JL, juvenile lethal (variable time of lethality—anytime from 1 to 5 wk of age, with weaning typically occurring at 3–4 wk of age); R, runting; WT, wild type. The hemizygous phenotype is defined by that exhibited when the mutant chromosome is heterozygous with the Del $(c)^{26DVT}$ deletion. The homozygous phenotype is often variable (*e.g.*, 2521SB and 1989SB). Information on phenotypes can also be found at the ORNL Mutant Mouse Database (http://lsd.ornl.gov/htmouse/mmdmain.htm/).

[b] Determined from the data presented in Figure 1.

[c] Previously reported in Rinchik *et al.* (1990, 1993a); Rinchik and Carpenter (1993).

[d] Most mice homozygous for $l7Rn^{2521SB}$ are externally normal, although ~20% do show signs of a slight runting phenotype (see Table 2).

[e] Death usually occurs suddenly between 2 and 3 wk of age.

[f] Extinct.

(Rinchik *et al.* 1990). Because the independence of these 5 clustered mutations could not be unequivocally ascertained, they were discarded and will not be considered further. The remaining 31 mutations, which include 12 previously reported (Rinchik *et al.* 1990; Rinchik and Carpenter 1993), produce phenotypes falling into the following groups: prenatal or perinatal lethality; a balance defect caused by mutations at the *Myo7a* (formerly, *sh1*) locus; postnatal runting, with variable times of death; and a sudden juvenile death syndrome, in which pups apparently normal at day 12–13 after birth invariably die usually within 1 wk. Each of these mutations was recovered from a light-chinchilla (c^{ch} +/*c m*) sibling and was propagated in a breeding stock. Table 1 lists the 31 independent mutations recovered from the *c*-region screen.

Complementation and deletion-mapping analyses: The 31 ENU-induced mutations were analyzed genetically for map position by crosses to albino deletions of varying lengths and/or for *trans* complementation by a series of pairwise crosses. Altogether, 146 combinations of pairwise crosses were done to categorize the 31 mutations into complementation groups. The results of these crosses are summarized pictorially in Figure 1.

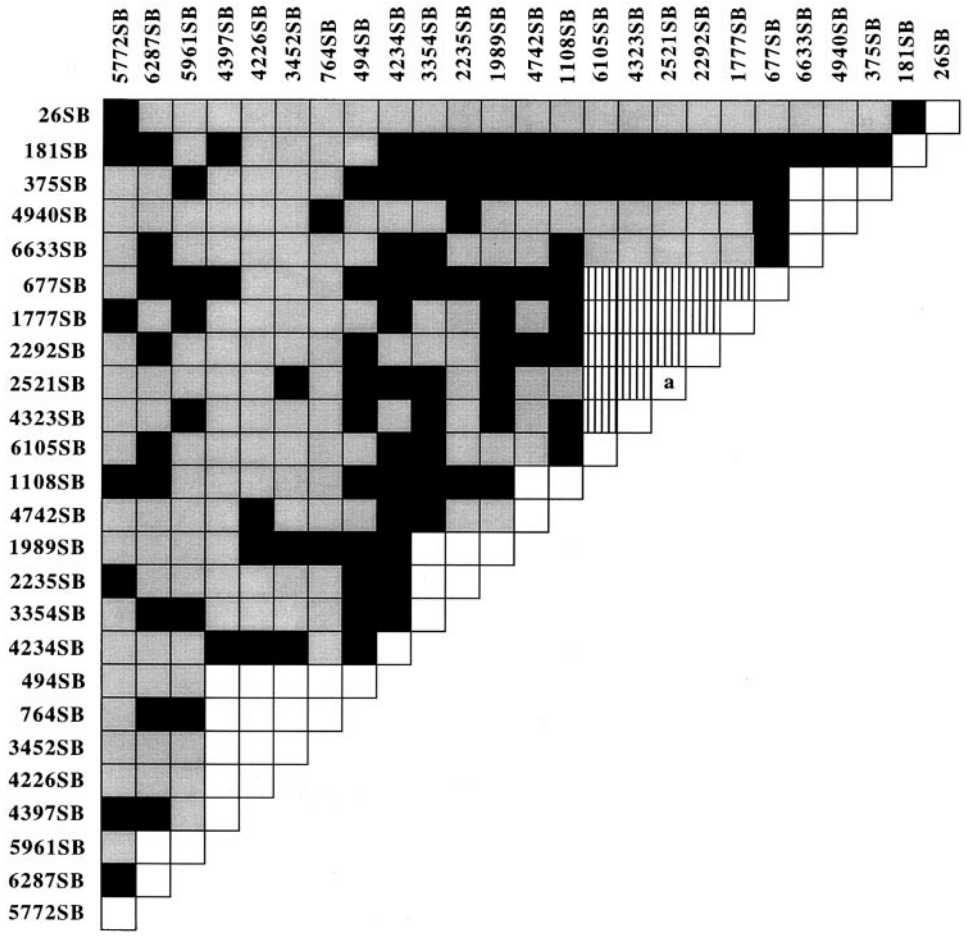

Figure 1.—Summary of pairwise complementation crosses among the ENU-induced *Fah-Hbb*-region mutations. Each of the 140 combinations of intercrosses pictured here represents a cross of c^{ch} + / *c m1* × c^{ch} + / *c m2* mice, where *m1* and *m2* represent independently isolated ENU-induced mutations. Before its use in these complementation tests, each mouse was proved to carry the corresponding ENU-induced mutation by a progeny-testcross to c^{ch} + / Del(*c*)^{26DVT}. Open boxes denote combinations that failed to complement for a specific phenotype; that is, the albino class (*c m1* / *c m2*) was either missing or abnormal. Black boxes denote complementing combinations, in which normal albinos were found. The 25 white boxes along the diagonal are not included in the number of complementation crosses, but simply reflect the outcome of tests performed to ascertain the phenotype of mice homozygous for a given *m*. Stippled boxes denote crosses that were not made. The vertically hatched boxes represent combinations that are presented separately and in more detail in Table 2. Data from reciprocal crosses were pooled for this summary, as there did not appear to be any difference in outcome. The box marked with an "a" denotes the apparently wild-type phenotype observed in the majority of *l7Rn3*^{2521SB}/*l7Rn3*^{2521SB} homozygotes (as opposed to the slight runting phenotype observed in *l7Rn3*^{2521SB} hemizygotes). *sh1*^{26SB} (*Myo7a*^{26SB}), the prototype ENU-induced *sh1* (*Myo7a*) mutation, is included in this grid because it was involved in several intercrosses with other *c*-region ENU-induced mutations; however, the six additional ENU-induced alleles of *sh1* (*Myo7a*) listed in Table 1, each of which fails to complement *sh1*^{26SB} (see text), were not involved in crosses with any other non-*sh1* mutations and therefore were not included in this figure.

We had previously shown the 26SB and 816SB mutations to be alleles of *Myo7a* (*sh1*) (Rinchik *et al.* 1990), and mice carrying each of the remaining five shaker-1-like mutations were crossed to c^{ch} + / *c sh1*^{26SB} mice to test for allelism. Indeed, shaker animals were present in the progeny of each of these crosses (data not shown); thus, seven alleles of *Myo7a* were found among these 4557 tested gametes (Table 1). To simplify Figure 1, only *sh1*^{26SB} is shown in the complementation grid because it is the only *sh1* (*Myo7a*) mutation to be tested with other ENU-induced, non-*sh1* mutations.

Six complementation groups [*l7Rn1*, *l7Rn2*, *l7Rn3*, *l7Rn4* (Rinchik *et al.* 1990) and *l7Rn5* (now *eed*) and *l7Rn6* (Rinchik and Carpenter 1993; Holdener *et al.* 1995a)] had been previously defined by eight prenatally lethal mutations. The map positions of these loci had also been previously ascertained by crosses to albino deletions (Rinchik and Carpenter 1993; Rinchik *et al.* 1993a). Subsets of the crosses shown in Figure 1 assigned each of the remaining seven prenatal-lethal mutations (4940SB, 6633SB, 1777SB, 2292SB, 4323SB, 6105SB, and 4742SB) to an already defined and mapped locus; thus, no new loci were identified by these eight additional prenatally lethal mutations (Table 1). To augment these complementation data, we tested many of the additional prenatal-lethal mutations for map position by crosses to specific albino deletions that should have breakpoints to either side of the mutant locus. In every case, the pairwise complementation cross data were consistent with this additional deletion mapping (data not shown).

Complex complementation results emerged from the pairwise crosses involving *l7Rn3* alleles. Five of the alleles (677SB, 1777SB, 2292SB, 4323SB, and 6105SB) were recovered as prenatally lethal mutations, whereas the sixth, 2521SB, causes a mild runting phenotype when hemizygous with Del(*c*)^{26DVT}; and ~80% of mice have no obvious externally visible runting phenotype when homozygous. The prototype mutation defining *l7Rn3*, *l7Rn3*^{677SB}, as well as some of the other *l7Rn3* alleles,

TABLE 2

Complementation and deletion-mapping analyses of ENU-induced *l7Rn3* alleles

	Males c^{ch} + / c m^a					
Females	677SB	1777SB	2292SB	2521SB	4323SB	6105SB
ENU Mutations						
677SB	6/297[b]	1/40	2/72	4r/31	3/53	0/24
1777SB		4/220	2/67	3/23 (1r)[c]	0/50	1/52
2292SB			1/53	13/74	0/52	9/50 (3r)
2521SB				87/337 (17r)	12/96 (5r)	2/7
4323SB					6/286	3/56 (1r)
6105SB						1/51
Deletions						
26DVT	20/1773[d]	3/837	8/1259	146/759 (143r)	5/948	5/477
11DSD	7/22	ND[e]	ND	ND	ND	8/28
9FR60Hb[f]	10/24	ND	7/28 (lr)	5/40	ND	6/22
3R60L[f]	8/31	ND	5/31	6/36	ND	8/22
14FR60Hb[f]	12/36	ND	8/50	5/14	ND	ND
1FDFoHrc	0/52	ND	0/57	5r/75	ND	1/39
4PB	0/54	ND	0/44	3r/21	ND	0/28

[a] *m* denotes one of the six *l7Rn3* alleles indicated. Each of these proved c^{ch} + / c *m* males was crossed to either a c^{ch} + / c *m* female (carrying the ENU mutations) or to the c^{ch} + / Del(*c*) female deletion-heterozygote indicated.

[b] Number of albinos over total progeny classified at weaning. "r" denotes runted animals. Progeny were classified at 3 wk of age. In complementing combinations, albinos [*c ml*/*c m2* or *c m*/Del(*c*)] should compose 25% of the progeny. Extreme deficiency of albinos or the presence of predominantly runted albinos indicate noncomplementing combinations. In noncomplementing combinations, a small number of normal albinos was presumed to represent recombinants that had lost *m* from the *c m* chromosome. These presumed *c m*/*c* + recombinants were not progeny tested.

[c] The numeral followed by "r" in parentheses denotes the number of visibly runted albinos observed at weaning among the total number of albinos indicated.

[d] The numbers of classified progeny are large for the $Del(c)^{26DVT}$ deletion compared to the other *c* deletions because in the initial years of maintenance for each mutant line, each presumed c^{ch} + / c *m* carrier was progeny tested by a cross to c^{ch} + / $Del(c)^{26DVT}$ and a minimum of 25–30 progeny were collected for each line.

[e] Not done.

[f] The distal breakpoint of $Del(c)^{9FR60Hb}$ is at the *D7Rn6* locus; and $Del(c)^{14FR60Hb}$ and $Del(c)^{3R60L}$ both delete *D7Rn6* (see Rinchik *et al.* 1993b). Thus, the $Del(c)^{14FR60Hb}$ and $Del(c)^{3R60L}$ distal breakpoints define the proximal border of the *l7Rn3* interval because they complement *l7Rn3* mutations (see Figure 2).

failed to complement the slight runting phenotype of 2521SB (Table 2), which suggested that 2521SB is a hypomorphic allele of the *l7Rn3* locus. We determined that 2521SB did indeed map into the *l7Rn3* interval [previously defined by the deletion mapping of $l7Rn3^{677SB}$ (Rinchik *et al.* 1993a)] by observing that *c 2521SB*/Del $(c)^{9FR60Hb}$, *c 2521SB*/Del $(c)^{14FR60Hb}$, and *c 2521SB*/Del $(c)^{3R60L}$ compounds were normal, but that *c 2521SB*/Del $(c)^{1FDFoHrc}$ animals were runted (see Figure 2 for a subset of albino deletions used in the deletion-mapping analyses and Table 2 for a summary of the deletion-mapping data). [The finding of five normal albinos among 40 classified progeny of the c^{ch} + / $Del(c)^{9FR60Hb}$ × c^{ch} + / c $l7Rn3^{2521SB}$ cross is not significantly different from the expected 10 among 40 (*t*-test; $P = 0.07$).] We failed to observe any runted progeny in crosses between $l7Rn3^{2292SB}$ and $l7Rn3^{2521SB}$, and the number of albino progeny did not deviate significantly from the expected number for the cross (Table 2). Moreover, we obtained evidence for complete complementation between $l7Rn3^{6105SB}$ and $l7Rn3^{2521SB}$, by observing that two outwardly normal albinos were recovered in only seven classified progeny in that cross (Table 2).

Additional evidence for a complex pattern of complementation between *l7Rn3* alleles was obtained from crosses between $l7Rn3^{2292SB}$ and $l7Rn3^{6105SB}$. Each of these alleles was recovered as a prenatal-lethal mutation when hemizygous, and each fails to complement $l7Rn3^{677SB}$, $l7Rn3^{1777SB}$, and $l7Rn3^{4323SB}$ for prenatal lethality (Table 2). However, $l7Rn3^{2292SB}$ and $l7Rn3^{6105SB}$ can complement each other for prenatal lethality. The data in Table 2 show that nine albino animals were recovered from a cross of c^{ch} + / c $l7Rn3^{2292SB}$ × c^{ch} + / c $l7Rn3^{6105SB}$. Three of these albinos were runted and six were of normal size, which demonstrated significant intraallelic complementation for lethality. Table 2 also shows deletion-mapping data that confirm that these two alleles map within the *l7Rn3* interval [they both are complemented by $Del(c)^{9FR60Hb}$ and $Del(c)^{3R60L}$, but not by $Del(c)^{1FDFoHrc}$].

One final interesting observation was made involving

TABLE 3

"Maternal effect" in transmission or recovery of a *l7Rn3* allele

Cross		Progeny[a]		
Female	Male	Albino[b]	Total	Albino (%)
c^{ch} + / Del(*c*)^{26DVT}	c^{ch} + / *c l7Rn3*^{2521SB}	158	896	17.6
c^{ch} + / *c l7Rn3*^{2521SB}	c^{ch} + / Del(*c*)^{26DVT}	25	557	4.5

[a] Progeny were classified at weaning.

[b] These numbers represent slightly runted albinos [*c l7Rn3*^{2521SB} / Del(*c*)^{26DVT}] recovered from the indicated cross. *l7Rn3*^{2521SB} is one of the six ENU-induced alleles of *l7Rn3*, and it causes a mild runting phenotype when hemizygous. The difference in the proportion of affected albinos recovered from the reciprocal crosses is highly significant ($P \ll 0.0001$).

mutations at the *l7Rn3* locus. Maintenance of the stock carrying the mild-runting allele *l7Rn3*^{2521SB} provided evidence for a dominant maternal effect in the recovery of runted progeny. Table 3 shows extensive data for reciprocal crosses in which *l7Rn3*^{2521SB} was inherited either from the dam or from the sire. In crosses of proved carriers of *l7Rn3*^{2521SB} with carriers of Del(*c*)^{26DVT}, significantly more runted albino progeny are observed when *l7Rn3*^{2521SB} is inherited from the sire. It is not yet known whether this is a true transmission ratio distortion, or whether fetuses/neonates simply fail to thrive when *l7Rn3*^{2521SB} is inherited from the dam. Analysis of uterine contents of late-gestation fetuses from such reciprocal crosses should address these questions. Because the other *l7Rn3* alleles are lethal when hemizygous, we have not yet been able to determine whether this effect is characteristic of all the alleles or is unique to the *l7Rn3*^{2521SB} allele.

The *fit1* locus had been previously defined by two alleles (494SB and 764SB) that cause a runting syndrome (Rinchik *et al.* 1990), and it had been mapped to a region distal to *eed* by crosses to a number of *c* deletions (Potter *et al.* 1995). Figure 1 and Table 1 demonstrate that three additional alleles of *fit1* were detected among the 4557 tested gametes for a total of five alleles with different severities of effect (see also Potter *et al.* 1997).

Three mutations (5961SB, 6287SB, and 5772SB) represent alleles at two loci not heretofore reported for this experiment. 5961SB and 6287SB, each of which causes postnatal lethality when hemi- or homozygous, map within the limits of the relatively small, proximally extending *c*^{14CoS} deletion, and, in fact, are mutations in the fumarylacetoacetate hydrolase (*Fah*) gene (J. Aponte, D. K. Johnson, D. A. Carpenter and E. M. Rinchik, unpublished results). We have also observed a difference in severity of effect between these two alleles, with *Fah*^{5961SB} hemizygotes dying perinatally and *Fah*^{6287SB} hemizygotes dying in later juvenile/weanling stages, perhaps recapitulating, respectively, the acute and chronic forms of human hereditary tyrosinemia.

The final mutation, 5772SB, causes sudden death of normal-looking juvenile pups commencing ~14 days after birth. Deletion-mapping analyses demonstrated that the locus defined by 5772SB maps distal to the Del(*c*)^{4PB} breakpoint. Crosses of c^{ch} + / *c 5772SB* males to c^{ch} + / Del(*c*) females carrying either the Del(*c*)^{26DVT} or Del(*c*)^{12FR60Hb} deletion yielded albino progeny at birth that were missing by weaning (3 wk), whereas a similar cross to a c^{ch} + / Del(*c*)^{4PB} deletion heterozygote yielded eight normal, healthy albino [*c 5772SB*/ Del(*c*)^{4PB}] progeny at weaning. Similar complementation was observed in crosses to six additional albino deletions, including Del(*c*)^{1FDFoHrc}, whose distal breakpoint is the next deletion breakpoint mapping proximal to the Del(*c*)^{4PB} distal breakpoint (Figure 2). Figure 1 shows that 5772SB complements mutations at *Myo7a* (*sh1*) and at *l7Rn1* [as well as at *Fah*, *l7Rn3*, *l7Rn4*, and *fit1*]. Thus, the 5772SB mutation defines a new locus, provisionally designated *sjds* (sudden juvenile death syndrome), which maps in the vicinity of *l7Rn1* and *Myo7a*.

An evolving mutation map of the *Fah–Hbb* region of mouse chromosome 7: Figure 2 shows a map of the region of mouse Chr 7 corresponding to the Del(*c*)^{26DVT} deletion that incorporates all of the mutation data presented here as well as a number of loci defined by complementation analyses between albino deletions, spontaneous mutations, and selected DNA polymorphisms. The boxed loci represent those that are newly defined by the ENU-induced mutations recovered in this experiment, and the dots above these loci indicate the number of repeat mutations at each locus, on the basis of the complementation data presented in Figure 1.

DISCUSSION

The report provides a summary of a long-term experiment designed to recover ENU-induced mutations that map within a mouse Chr-7 region corresponding to large deletion. The design of this experiment capitalized on the ability of ENU to induce point mutations at high efficiency in spermatogonial stem cells (Russell *et al.* 1979; Hitotsumachi *et al.* 1985) as well as on the availability of a highly genetically defined set of

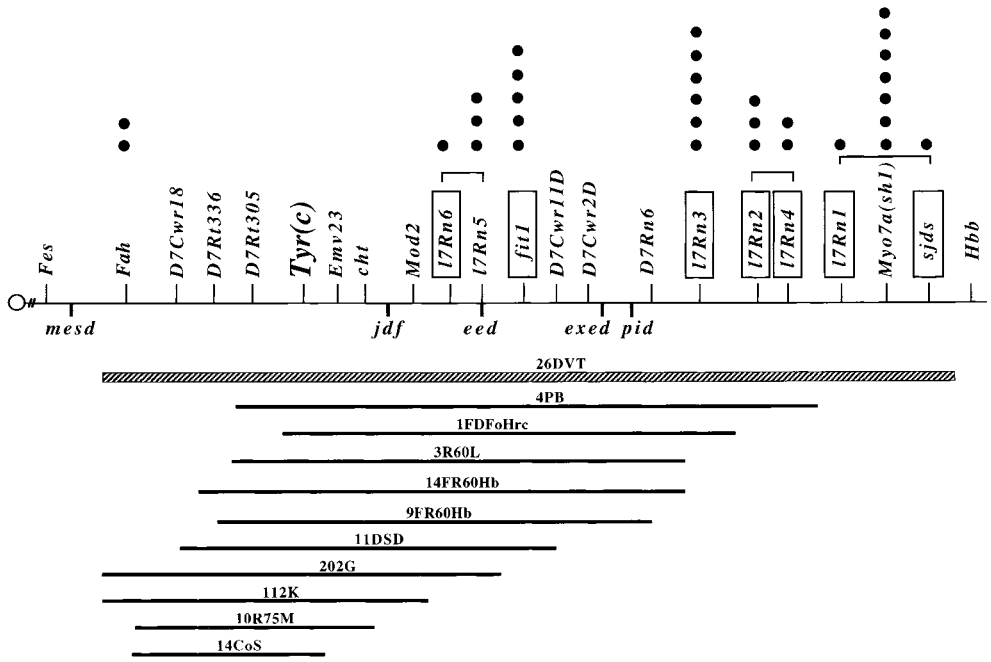

Figure 2.—A summary ENU-mutation/deletion map of the *Fah—Hbb* region of mouse chromosome 7. Chromosome 7 is represented by the thin line, with the centromere represented by the circle on the left. The darker lines below the map represent a subset of a larger number of *Tyr* deletions used to map new ENU-induced mutations; the name of each deletion is indicated above each line. The Del $(c)^{26DVT}$ deletion, used to recognize new mutations initially, is represented by the long hatched box. The black dots above the map denote new ENU-induced mutations, placed into specific complementation groups by the analyses presented in Figure 1. Loci in boxes represent those defined solely by ENU-induced mutations recovered in this experiment. "Loci" (functional units) listed below the chromosome map are those defined by phenotypes of *Tyr* deletions of varying length. The *eed* locus/phenotype, defined by deletions, is identical to that shown by $l7Rn5^{3354SB}$, one of the ENU-induced mutations recovered in this experiment (Rinchik and Carpenter 1993; Holdener *et al.* 1995a; Schumacher *et al.* 1996). A searchable locus list for this and all segments of the mouse genome can be found at http://www.informatics.jax.org/mrktools.html/.

overlapping deletions that encompass the albino (*c*; *Tyr*) locus for fine-structure mapping and placement of mutant loci on a physical map (*e.g.*, Rinchik and Russell 1990; Holdener-Kenny *et al.* 1992; Kelsey *et al.* 1992; Holdener *et al.* 1995b). This experiment was originally implemented to test the logistical efficacy of a strategy to improve functional maps of regions of the mammalian genome by chemical mutagenesis (Rinchik 1991). The choice of a two-generation hemizygosity screen, employing a deletion for recognizing new recessive mutations, was important in this experiment because such a design provided for the screening of a large number of gametes for the same defined length of genome, thus addressing the question of whether "saturation mutagenesis" of a chromosomal region would be feasible in the mouse. In this initial experiment, we wanted to screen a number of gametes large enough to allow the "average locus" to mutate five to seven times to test experimentally how the known skew in locus mutability would affect the outcome and interpretation of a regional-mutagenesis approach. A three-generation protocol, in which mutagenized chromosomes are made homozygous for detection of new recessives, is a powerful method for recovering new mutations, but the required extra generation severely limits the number of gametes one can screen. The "genetic end-game" of this hemizygosity screen—namely, placement of new presumed point mutations on a deletion/physical map—was made possible by the extensive panel of albino deletions and their use in simple, one-cross "pseudodominance" deletion-mapping tests. Thus, we hope that this original large-scale experiment will provide a prototype for correlating transcription maps of megabase regions, forthcoming from the genome project, with functional/mutation maps created by genetic analysis of chemically induced mutant phenotypes.

Variability in locus mutability, chance, and no "saturation mutagenesis": The number of gametes fully tested for new recessive mutations (4557) was large enough to allow for six to seven mutations per locus. This initial assumption was based on the average per-locus mutation frequency ($\sim 1.5 \times 10^{-3}$) for the seven loci scored in the visible specific-locus test at this repeated-dose regimen of ENU (Hitotsumachi *et al.* 1985; Rinchik 1991). The final mutation tally and the distribution of mutations among the loci in this region raise some important issues. Our results of 31 mutations among 10 loci in 4557 gametes yield an average per-locus frequency of 6.8×10^{-4}, slightly less than half the average per-locus frequency found for the specific-locus test. Furthermore, we anticipated a wide variation in locus mutability with ENU, as this phenomenon has been repeatedly observed in other mutagenesis experiments [*e.g.*, in the visible specific locus test several of the seven visible loci are much more mutable than others with ENU (*e.g.*, Hitotsumachi *et al.* 1985; Russell *et al.* 1990)]. Several loci, such as *Myo7a* (*sh1*), *l7Rn3*, and perhaps *fit1*, behaved as "average loci," because seven, six, and five alleles, respectively, were recovered at each. However, loci such as *l7Rn1*, *l7Rn4*, *l7Rn6*, *sjds*, and *Fah*,

were not particularly mutable in this experiment. This relative immutability could be caused by many factors associated with the nature of the gene itself (*e.g.*, small size, encoded-protein structure fairly resistant to amino acid changes, small number of intron-exon junctions, etc.), or caused simply by chance alone (see below). Thus, considerable investment of time and resources would have to be expended to obtain additional alleles at these loci, a situation made even more difficult by the fact that homozygosity for the known mutations at each of these loci is incompatible with viability in adults, thus making it difficult to generate and propagate new alleles even from a simple one-cross specific-locus test using an existing viable allele (Russell 1951).

We believe that the observed variable mutability among the loci at which mutations were detected makes it impossible to speak precisely of "saturation mutagenesis" of this, or probably any other, genomic region in the mouse, and we prefer the more conservative term "high-efficiency regional mutagenesis," with high-efficiency referring solely to the use of a supermutagen such as ENU. The problem of extrapolating from data such as those presented here, or from data in a much more tractable organism such as Drosophila, to estimates of gene density per unit chromosome has been previously discussed (Barrett 1980). The existence of loci (*e.g.*, *l7Rn1*, *sjds*) at which a very rough point estimate of mutation frequency per locus can be set at $\sim 2 \times 10^{-4}$ (*i.e.*, 1/4557, on the basis of the recovery of a single mutation of that complementation group) suggests that there may be other loci in this region, which could be identified by visible mutant phenotypes in a screen for ENU-induced mutations, that were not recovered in this particular experiment. It is interesting to note that a mutation frequency of 1/4557 is not strictly significantly different from one of 7/4557 (Fisher's exact test; $P = 0.07$). This somewhat surprising result makes it even more likely that there are *c*-region loci that simply weren't hit because of chance, in addition to those not hit, or not hit more often, because of real locus-mutability differences.

Thus, it is unlikely that the 10 loci identified by these 31 mutations necessarily represent the true estimate of all detectable ENU-mutable loci possible within the limits of the Del(*c*)^{26DVT} deletion. Compounding this problem is the fact that, for logistical reasons in this initial experiment, we screened for only very obvious phenotypes (*e.g.*, lethality and simple, externally visible, characters and behaviors). More complete screening protocols, such as those for biochemical abnormalities or for more subtle abnormal behaviors or morphologies, would probably increase the number of loci that could be defined in such mutagenesis screens.

The 6- to 11-cM estimated size of the Del(*c*)^{26DVT} deletion suggests that it may be on the order of 10–22 Mb in physical length; the exact physical size of this deletion will be derived from the eventual fruits of the genome program. If one assumes the average gene to be 50–100 kb (a tenuous assumption at best), there could perhaps be 200–400 genes in this region, with only 10 identified by the ENU mutations reported here. Of course, determination of the number of transcription units that actually served as targets for this particular mutagenesis experiment will have to await further genomic analyses. It is likely that regions of the genome will vary widely in their gene density, and perhaps our results provide some insight into the gene density of this particular region of Chr 7. A similar hemizygosity screen is in progress for mutations in the ~4-cM Del(*ru2 p*)^{46DFiOD} deletion encompassing the Chr-7 pink-eyed dilution (*p*) locus (Rinchik *et al.* 1995). This screen has produced 19 mutations detecting eight loci in only 1244 tested gametes (Rinchik *et al.* 1995; E. M. Rinchik and D. A. Carpenter, unpublished data), but even this result is far below the number one might expect from estimates of physical size of the corresponding deletion.

How many gametes to screen: The success and the efficiency of mutagenizing a particular chromosomal segment in the mouse depends, of course, on the efficiency of the mutagen as well as on the power and breadth of the phenotype screening employed and on the number of gametes screened. If phenotype screening were broader than what we employed here (*i.e.*, if screens were able to detect phenotypes in addition to external abnormalities, obvious neurological/behavioral disorders, and lethality), one might expect to detect additional loci within the region being mutagenized and to recover less severe alleles of loci defined by quite detrimental alleles. Clearly, the number of gametes screened has a direct impact both on the number of loci identified and on the number of independent repeat mutations found at each locus. In this context, it is interesting to examine to what extent the output of this particular screen would have been reduced as a result of limiting the number of gametes tested, either for reasons of cost or logistics. For example, had this experiment been terminated after screening only 2000 gametes (which would have made this type of screen perhaps more feasible for the typical mouse facility), 18 of the 31 mutations would not have been detected, including the defining mutations at the *l7Rn6*, *sjds*, and *Fah* loci. Moreover, the rich series of five alleles at *fit1* would have been reduced to two; six alleles of *l7Rn3* would have been reduced to four; and only one allele each of *l7Rn2* and *l7Rn4* would have been recovered. Examples such as these, as well as the variability in locus mutability discussed above, should be taken into consideration if this type of screen is to be applied to a specific chromosomal region.

Failure to recapitulate several deletion phenotypes: Also cogent in this context is that we failed to recover mutations at several "loci" that have been defined by pairwise complementation analyses of the albino deletions themselves. For example, the *jdf*, *exed*, and *pid*

"loci," shown below the chromosome in Figure 2, are defined by the homozygous deletion phenotypes, respectively, of juvenile runting and male infertility, early postimplantation developmental arrest, and preimplantation developmental arrest (Lewis *et al.* 1976, 1978; Russell *et al.* 1982; Niswander *et al.* 1989). We failed to recover any ENU-induced mutations that map to these regions of the deletion complex from our test of 4557 gametes. Thus, these loci could be relatively immutable as discussed above, or, alternatively and importantly, these phenotypes could be a result of a contiguous gene syndrome, in which the resulting deletion phenotype is actually an additive effect of the deletion of individual genes. If this were the case, important developmental phenotypes associated with larger chromosomal aberrations, such as deletions, will not be recapitulated by intragenic ENU-induced mutations. On the other hand, one potential contiguous gene (deletion) phenotype—the *eed* early embryonic arrest phenotype—originally defined by Del(*c*)^{11DSD}/Del(*c*)^{11DSD} deletion homozygotes (Niswander *et al.* 1988, 1989; Faust *et al.* 1995) proved, rather, to be a single-gene defect recapitulated by one of the three *l7Rn5* alleles (*eed*^{3354SB}; Rinchik and Carpenter 1993; Holdener *et al.* 1995a; Schumacher *et al.* 1996). Thus, the etiology of the phenotypes caused by homozygous deletion of specific segments of this chromosomal region that were not recapitulated by ENU-induced mutations must await further study.

Allelic series: One particularly positive aspect of mutagenesis screens employing ENU is the potential for creating allelic series of mutations. For example, the five alleles of the *fit1* locus all differ in their effects on the growth rate of neonates and juveniles, and there are marked differences in hematopoietic parameters among mice carrying different alleles (Potter *et al.* 1997). One allele of *l7Rn3*, *l7Rn3*^{2521SB}, causes only a mild runting syndrome when hemizygous, and, in the majority of animals, produces no abnormal external phenotype when homozygous. By contrast, the prototype *l7Rn3* allele, *l7Rn3*^{677SB}, arrests development shortly after implantation in either the hemi- or homozygous state (Rinchik *et al.* 1993a). Moreover, there is a complex pattern of complementation among *l7Rn3* alleles, which will presumably be informative for the functional characterization of the protein product once this is identified molecularly; and we have observed an interesting dominant maternal effect in the transmission or recovery of the *l7Rn3*^{2521SB} mutation. We have also isolated two alleles of the *Fah* gene that lead to an acute or chronic tyrosinemia, perhaps serving as useful models for the distinct forms of the human disease (J. Aponte, D. K. Johnson, D. A. Carpenter and E. M. Rinchik, unpublished results). Likewise, the three alleles recovered at the *eed* locus may prove useful in dissecting the role of this *Polycomb*-type protein (Schumacher *et al.* 1996) throughout development, because one allele (*eed*^{3354SB}) recapitulates the null phenotype of deletion homozygotes, whereas another allele (*eed*^{1989SB}) is less severe, with homozygous animals surviving well into adulthood (Rinchik and Carpenter 1993; Holdener *et al.* 1995a). Thus, such series of ENU-induced alleles should be useful for further genetic and biochemical analyses of the roles of the normal proteins and the effects of these mutant proteins on mammalian development in both time and space.

Recommendations: Many logistical challenges for designing and implementing large regional mutagenesis screens were encountered during the course of this experiment, and several recommendations can be made for future work. If one is employing a hemizygosity screen such as the one reported here, the use of G_1 females rather than G_1 males allows many individual gametes (represented by the G_1 females) to be tested completely without having to breed prohibitively large "selector" deletion stocks [stocks analogous to Del(*c*)^{26DVT}]. One can rotate deletion-heterozygote males in a 7-wk cycle through large numbers of G_1 females (which are relatively easy to generate). A 7-wk cycle, analogous to that used for large-volume specific-locus tests, allows for the cohabitation of a male deletion-heterozygote and a G_1 female for 1 wk, followed by gestation (3 wk), and then classification and weaning of G_2 progeny in another 3 wk, at which time the G_1 female can be remated if she requires further testing by another cycle of crosses. One also has the possibility of recovering X-linked visible mutations for "free" in the G_2 male progeny, and one can build X-linked marker genes into the G_0 generation mice to create the capability to screen for X-linked lethals in the G_2 males. On the other hand, if G_1 males are employed (not done in this experiment), one can test each male with several different selector deletions, thus increasing the coverage of genome screened.

It is also very important to derive G_1 animals from a large enough number of mutagenized G_0 males to reduce the potential of recovering noncomplementing cluster mutations (*i.e.*, noncomplementing mutations derived from the same mutagenized male). Clusters originate when the testis is extensively depleted of spermatogonia as a result of the cytotoxic action of ENU, and then is repopulated with descendants of a limited number of surviving spermatogonia. Such noncomplementing cluster mutations cannot be verified as independent (as they may derive from the same mutated parent stem cell) and represent wasted resources in the genetic characterization of new mutations. For example, in the pilot study previously reported (Rinchik *et al.* 1990), 1311 G_1 females were produced from only 22 ENU-treated G_0 males (an average of 60 G_1 females per G_0 male), and five noncomplementing clusters were identified. In the remainder of the experiment reported here, 5341 G_1 females were produced from 182 treated G_0 males, for an average of 29 G_1 females per male, and no noncomplementing clusters were recovered. How-

ever, these numbers should serve only as guides, and not as absolutes, as the number of offspring per male varied considerably, the above numbers being only averages.

We also do acknowledge a potential confounding problem in interpreting genetic complementation analyses of recessive mutations induced in the type of regional screen described here. By the very design of the experiment, all new mutations are closely linked, making *trans* complementation testing (*i.e.*, observing the phenotype of *c m1*/*c m2* double heterozygotes) feasible and informative, while at the same time making the *cis*-control (*c m1 m2*/+ + +) highly impractical to perform. Indeed, this is a consideration for complementation analyses in short regions in the mouse, because the double-mutant chromosomes required for *cis*-tests would be prohibitively costly to detect, recover, and maintain for each combination of two mutations. Thus, when compared to other organisms of experimental genetics, the mouse will rarely have the *cis*-complementation control performed for closely linked genes. Consequently, for *trans*-combinations that display the mutant phenotype, one cannot formally rule out an unlikely, but still possible, hypothesis that mutations in two closely linked genes are interacting to produce the mutant phenotype in double heterozygotes. Paying special attention to phenotype characterization in complementation tests, as well as to fine-deletion-mapping of mutations, can help diminish this unlikely hypothesis further. For example, in the overall experiment described here, we have eight separate cases where a repeat mutation, found by *trans*-analysis to be in the same complementation group as a deletion-mapped prototype mutation, was itself also deletion mapped; in all eight cases, the repeat mutation mapped to the same fine deletion interval as the prototype, thereby indicating probable allelism (data not shown).

We have shown that a large deletion in the mouse can be used to screen efficiently for recessive ENU-induced mutations, and that this series of mutagenesis, deletion-mapping, and complementation experiments has significantly refined the functional map of the albino deletion complex. Although it is unlikely that saturation mutagenesis can be achieved without a prohibitive investment in mouse numbers and costs, such a regional-mutagenesis approach has provided new mutation resources, complete with variable allelic series at several loci, with which to study gene function in this segment of Chr 7. If one can glean from the results of a genomic analysis that a particular target region is very gene-rich, hemizygosity screening for ENU-induced mutations could be an important, phenotype-driven method to complement other methods for determining gene function. The advent of powerful methods to create deletions (Ramirez-Solis *et al.* 1995) and particularly high-resolution deletion complexes (You *et al.* 1997; Kushi *et al.* 1998; Thomas *et al.* 1998) in embryonic stem cells should be important in applying the mutagenesis and mapping strategies reported here to the additional regions of the mouse genome.

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