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N-Ethyl-*N*-nitrosourea-induced prenatally lethal mutations define at least two complementation groups within the embryonic ectoderm development (*eed*) locus in mouse Chromosome 7

Eugene M. Rinchik, Donald A. Carpenter

Biology Division, Oak Ridge National Laboratory, Post Office Box 2009, Oak Ridge, Tennessee 37831-8077, USA

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Abstract. Two loci [*l(7)5Rn* and *l(7)6Rn*] defined by *N*-ethyl-*N*-nitrosourea (ENU)-induced, prenatally lethal mutations were mapped by means of *trans* complementation crosses to mice carrying lethal deletions of the albino (*c*) locus in Chromosome (Chr) 7. Both loci were found to map to the subregion of the *Mod-2–sh-1* interval that contains the *eed* (embryonic ectoderm development) locus. *eed* has been defined by the inability of embryos homozygous for certain *c* deletions to develop beyond the early stages of gastrulation. Evidence for at least two loci necessary for normal prenatal development, rather than one locus, that map within the *eed* interval came from the observation that two prenatally lethal mutations, 3354SB [$l(7)5Rn^{3354SB}$] and 4234SB [$l(7)6Rn^{4234SB}$], could complement each other in *trans*, but could not each be complemented individually by *c* deletions known to include the *eed* locus. A somewhat leaky allele of *l(7)5Rn* [$l(7)5Rn^{1989SB}$] was also recovered, in which hemizygotes are often stillborn and homozygotes exhibit variable fitness and survival. The mapping of the loci defined by these mutations is likely to be useful for genetic, molecular, and phenotypic characterization of the *eed* region, and mutations at either locus (or both loci) may contribute to the *eed* phenotype.

Introduction

The embryonic ectoderm development (*eed*) locus within the *Fes–Hbb* region of mouse Chr 7 is defined by an abnormality in peri-implantation development that is exhibited by embryos homozygous for certain radiation-induced deletions of the albino (*c*) locus (Niswander et al. 1988, 1989; Sharan et al. 1991, 1992). The eed phenotype was originally characterized by the failure of embryos homozygous for the c^{11DSD} deletion to develop beyond the early stages of gastrulation. Normal development is observed in these embryos up to day 8.5, by which time extraembryonic ectodermal structures, as well as parietal and visceral endoderm, have formed. However, at day 8.5, development of the embryonic ectoderm is clearly abnormal, having arrested at the stage of formation of the primitive streak and of production of mesoderm. By day 9.5, no structures normally derived from the embryonic ectoderm can be found (Niswander et al. 1988, 1989). Moreover, embryonic stem (ES) cell lines cannot be derived from mutant blastocysts, implying that the *eed* gene(s) is required for normal viability of cells derived from the inner cell mass (Niswander et al. 1988).

Recently, embryos homozygous for the c^{202G} deletion, a member of the original Di group of *c* deletions (Russell et al. 1982), were found to exhibit a phenotype identical to that found in embryos homozygous for c^{11DSD} (Sharan et al. 1992). c^{202G} does not extend as far as *D7Cwr11D*, the locus defined by the distal breakpoint of the c^{11DSD} deletion (Sharan et al. 1992). Mice homozygous for either the c^{3H} or c^{112K} deletions, which both delete the segment from *Fah* to *Mod-2*, progress completely through development but die shortly after birth (Gluecksohn-Waelsch 1979; Russell et al. 1982). This neonatal lethality is probably due, at least in part, to a deficiency of fumarylacetoacetate hydrolase (Klebig et al. 1992; Ruppert et al. 1992). Thus, all available genetic mapping data indicate that at least a segment of the earlier-acting, postimplantation-lethal *eed* gene(s) must lie between *Mod-2* and the distal breakpoint of the c^{202G} deletion (Sharan et al. 1992; Fig. 1).

For the past several years, we have been using the presumptive point-mutation inducer, *N*-ethyl-*N*-

Correspondence to: E.M. Rinchik

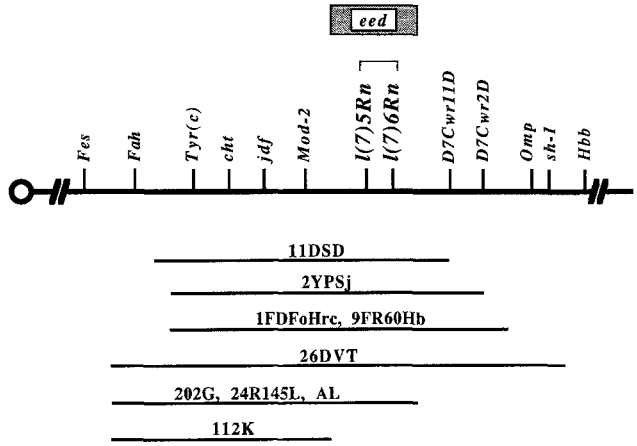

Fig. 1. Deletion mapping of two loci defined by ENU-induced prenatally lethal mutations within the *eed* region of mouse Chr 7. A map of the *Fes–Hbb* region of Chr 7 incorporating the deletion-mapping and complementation data is presented in Tables 1 and 2. The centromere is indicated by the **circle** at the left. The loci *l(7)5Rn* and *l(7)6Rn* are defined by ENU-induced prenatally lethal mutations described in the text. Not all loci known to map to the *Fes–Hbb* region are shown. Below the map are depicted the extents of a subset of *c*-locus mutations that are most relevant for the mapping of these loci. No correlation with physical distance is implied. The **bracket** above *l(7)5Rn* and *l(7)6Rn* indicates that these two loci cannot yet be ordered with respect to each other. The **shaded box** represents the embryonic ectoderm development (*eed*) interval, which is currently defined as the region of non-overlap between the distal breakpoints of the c^{3H} (or c^{112K}) and c^{202G} deletions (Sharan et al. 1992). See Rinchik and associates (1992) for locus references. Recombination frequency estimates (in cM) for the *Tyr(c)–Mod-2* interval range from 0.49 ± 0.49 to 4.08 ± 2.83 (Davisson et al. 1989); *Tyr(c)–Hbb*, 3.46 ± 0.64 to 6.19 ± 0.65 (Davisson et al. 1989; Johnson et al. 1989); *Mod-2–Hbb*, 2.21 ± 1.09 to 6.44 ± 1.22 (Davisson et al. 1989). In general, the male recombination frequencies reported for each of these marker combinations are at the low end of each range (Davisson et al. 1989).

nitrosourea (ENU), to generate a fine-structure mutation map of the *Fes–Hbb* region. Both lethal and viable ENU-induced mutations are being detected by a protocol that produces heterozygotes carrying a mutagenized Chr 7 marked with the original *c* mutation opposite a Chr 7 carrying a large (6–11 cM) *c* deletion (Rinchik et al. 1990). As part of the characterization of this 6- to 11-cM region, each newly recovered mutation is being mapped by means of *trans* complementation crosses to mice carrying *c* deletions of varying size. This report describes the deletion mapping of four prenatally lethal mutations to the *eed* interval and provides evidence that this interval, which is defined by deletion breakpoints, harbors at least two loci necessary for normal prenatal development.

Materials and methods

The origin of ENU-induced *c*-region mutations has been described in detail elsewhere (Rinchik et al. 1990). The four mutations reported here arose in spermatogonial stem cells of BALB/cR1 (*c*/*c*) Generation-0 (G_0) males that had been treated with a total (but fractionated) dose of 400 mg/kg of ENU. The mutations were recovered by a two-cross breeding strategy in which mutagenized chromosomes marked by *c* were made heterozygous in G_2 progeny opposite a chromosome carrying the *Df(c Mod-2 sh-1)*^{26DVT} deletion (abbreviated c^{26DVT}; previously abbreviated c^{Fp} in Rinchik et al. 1990). These particular lethal (*l*) mutations were identified in pedigrees that produced fewer-than-expected or no G_2 albino progeny [c/c^{26DVT}]. Although we typically tried to raise 30 G_2 progeny from the G_1 female in each pedigree (Rinchik et al. 1990), this was not always possible (e.g., the G_1 female became sterile or died before 30 progeny were raised). Therefore, c^{ch} +/*c l* light-chinchilla G_2 siblings in pedigrees that were suggestive of segregating a *c*-linked *l* were routinely crossed to c^{ch} +/c^{26DVT} mice to test for heritability of any putative *l*. Thereafter, the mutant *c l* chromosomes, recovered from the c^{ch} +/*c l* light-chinchilla G_2 siblings, were maintained by crossing c^{ch} +/*c l* mice to mice of the inbred strain 47BS-c^{ch}/c^{ch}, which exhibit a darker, full chinchilla color. The *c* mutation serves as the marker for all *l*s, and because it is potentially separable from *l* by crossing over in c^{ch} +/*c l* carriers, c^{ch} +/*c l* mice in each generation were crossed to c^{ch} +/c^{26DVT} mice to progeny-test for the presence of *l*. Males or females that produced no albinos in 30 progeny of this testcross were considered to be proved c^{ch} +/*c l* carriers.

Lethal albino deletions [*Df(c)*] were maintained by crossing light-chinchilla heterozygotes [c^{ch}/*Df(c)*] to mice of the noninbred, chinchilla stock 2A-c^{ch}/c^{ch}. New ENU-induced lethal mutations were mapped with respect to breakpoints of lethal albino deletions by crossing proved carrier males (c^{ch} +/*c l*) with females heterozygous for lethal albino deletions [c^{ch}/*Df(c)*].

Results and discussion

An important step in the analysis of new lethal mutations generated by ENU mutagenesis of the 6- to 11-cM region spanned by the c^{26DVT} deletion (Rinchik et al. 1990) is the mapping of any new mutation by *trans* complementation analysis with albino (*c*) deletions of varying length. Table 1 summarizes the results obtained when proved carrier males [e.g., c^{ch} +/*c l*] of four prenatally lethal mutations (1989SB, 2235SB, 3354SB, and 4234SB) were crossed to females heterozygous for *c* deletions [c^{ch}/*Df(c)*]. Albinos [*c l*/*Df(c)*] are expected to comprise approximately 25 percent of the offspring, and crosses that produced zero or one albino offspring per 30 classified progeny were considered to be non-complementing combinations (Rinchik et al. 1990). The data in Table 1 indicate that none of the four mutations could be complemented by c^{11DSD}, the prototypic *eed* deletion, or by any tested deletion that extends into the region presumed to contain the *eed* locus (Niswander et al. 1989; Sharan et al. 1991, 1992). Of particular note are the data observed with the three Di-group deletions c^{202G}, $c^{24R145L}$, and c^{AL}. These three mutations all include *Mod-2* (Russell et al. 1982), but not the *D7Cwr11D* locus, which is the site of the distal breakpoint of the c^{11DSD} deletion [Sharan et al. 1992 (for c^{202G}), and E.M. Rinchik and M.D. Potter, unpublished data (for $c^{24R145L}$, c^{AL})]. Because embryos homozygous for c^{202G} exhibits the eed phenotype (Sharan et al. 1992), and because c^{112K} deletes the *Mod-2* locus (Russell et al. 1982) but complements each of these new ENU-induced mutations for prenatal lethality (Table 1), the loci defined by the ENU-induced mutations must map to the *eed* interval. Thus, at least a segment of the corresponding gene(s) must map to the distal region of non-overlap between the c^{112K} and c^{202G} deletions on the genetic and physical maps of the *Fes–Hbb* region (Fig. 1).

Table 1. Deletion mapping of prenatally lethal, ENU-induced *c*-region mutations.[a]

Albino deletion [*Df(c)*]		ENU-induced mutation (*l*)			
Allele	Complementation-group designation[b]	1989SB	2235SB	3354SB	4234SB
c^{11DSD}	Bem1	1/77 (1)[c,d]	0/48	0/42	0/48 (2)
c^{2YPSj}	Bem3	0/41	0/42	0/36	0/39
$c^{1FDFoHrc}$	Bp1	0/36	0/33	ND[e]	0/53 (2)
$c^{9FR60Hb}$	Bp3	0/51	ND	ND	0/45
c^{202G}	Di1	0/40	0/63	0/30	0/67
$c^{24R145L}$	Di2	0/65 (2)	0/43	ND	1/51[d]
c^{AL}	Di4	2/108[d]	0/41	0/44	0/85 (5)
c^{112K}	E1	19/80	7/28	11/28	7/27
c^{3DENb}	—[f]	0/46 (1)	0/33	0/55	1/73 (1)[d]
c^{26DVT} [g]	Fp1	2/487[d]	1/542[d]	2/790[d]	1/492[d]

[a] Proved carrier males (c^{ch} +/ *c l*) were crossed to females heterozygous for albino deletions [c^{ch}/*Df(c)*].
[b] Complementation group designations are based on Russell and co-workers (1982) and Niswander and colleagues (1989).
[c] Number of normal albino progeny/total number of progeny, classified at 3 weeks of age. In complementing combinations, albinos should comprise 25% of the progeny. Lack of albinos indicates non-complementing combinations, which are indicated by an underline. Crosses were inspected daily for new births (and the presence of albinos at birth). The numeral in parentheses indicates the number of albinos in that combination that were found dead within 24 h after birth; these early deaths are not included in the ratio of albino/classified progeny.
[d] Normal albinos found in non-complementing combinations were presumed to be recombinants that lost *l* from the *c l* chromosome. These normal albinos represent one-fourth of the total number of recombinant progeny possible and are the only recombinant class readily detectable. These presumed *c* +/*Df(c)* recombinants were not progeny tested.
[e] ND, not done.
[f] c^{3DENb} is a relatively new *c* deletion that has not yet been assigned to a complementation group, but its distal breakpoint lies proximal to the c^{202G} distal breakpoint. (M.D. Potter and E.M. Rinchik, unpublished data).
[g] Complementation data for this deletion were obtained from progeny testcrosses of male carriers of ENU-induced mutations to female deletion heterozygotes (as part of normal maintenance of the lethal SB stocks). In contrast to the other crosses in the table, these were not checked daily for new progeny. The frequencies of normal albino progeny in these particular crosses give the following estimates of the *male* meiotic recombination frequency (in cM) between *c* and the respective *l*: 1.6 ± 0.6 (1989SB); 0.7 ± 0.4 (2235SB); 1.0 ± 0.4 (3354SB); 0.8 ± 0.4 (4234SB). These recombination frequencies are consistent with other recombination data obtained for markers within this region (see Fig. 1 legend).

The number of complementation groups defined by the four ENU-induced lethal mutations was determined by pairwise crosses between proved carriers of each mutation. The data in Table 2 indicate that two complementation groups are defined by the four mutations. 4234SB is able to complement each of the other three mutations, as shown by the presence of normal albino progeny (*c 4234SB/c l*) in crosses of appropriate heterozygotes. Table 2 also presents the control cross, in which 4234SB homozygotes are shown to die prenatally [only two (presumably recombinant) albinos were recovered in 218 progeny of a cross of 4234SB heterozygotes]. Thus, the 4234SB mutation defines a complementation group, *l(7)6Rn*, that is distinct from any of the other mutations. On the other hand, pairwise crosses among 1989SB, 2235SB, and 3354SB indicated non-complementation in each case (Table 2). Therefore, these three mutations belong to a single complementation group and thus presumably represent alleles of a single locus, *l(7)5Rn*. Because the four mutations gave an identical pattern of complementation with the *c* deletions (Table 1), it is impossible at this time to order *l(7)5Rn* and *l(7)6Rn* within the *eed* interval (Fig. 1).

The data in Table 2 also provide evidence for variation in the severity of the lethal phenotype among members of the *l(7)5Rn* complementation group. For example, mice homozygous for $l(7)5Rn^{1989SB}$ were often observed in the progeny of intercrosses of het-

Table 2. Complementation analyses of ENU-induced, prenatally lethal mutations in the cross: c^{ch} +/*c l-1* × c^{ch} +/*c l-2*.

Allele[a]		Number of progeny[b]			
l-1	*l-2*	*c/c*	c^{ch}/c	c^{ch}/c^{ch}	Total
1989SB	1989SB	54 (51)	263	142	459
1989SB	2235SB	4 (3)	42	34	80
2235SB	1989SB	2 (2)	15	6	23
1989SB	3354SB	0	21	22	43
3354SB	1989SB	0	30	20	50
2235SB	2235SB	1	84	32	117
2235SB	3354SB	0	37	15	52
3354SB	2235SB	0	33	17	50
3354SB	3354SB	3	243	111	357
4234SB	4234SB	2	134	82	218
4234SB	1989SB	14	20	5	39
1989SB	4234SB	6	12	5	23
2235SB	4234SB	2	3	2	7
3354SB	4234SB	11	25	12	48

[a] In each case, the *l-1* allele is contributed by the female.
[b] Progeny were classified at 3 weeks of age. In complementing combinations, albinos (*c l-1/c l-2*) should comprise 25% of the progeny. Lack of albinos or the presence of albinos with abnormal phenotypes indicate non-complementing combinations; these combinations are indicated by an underline. The parentheses indicate the number of animals included in the total that were visibly small (anywhere from 30% to 80% of the size of their normal littermates). In noncomplementing (underlined) combinations, a small number of normal albinos was presumed to represent recombinants that had lost *l* from the *c l* chromosome (i.e., *c l*/*c* +). These presumed recombinants were not progeny tested. The c^{ch}/c class includes primarily c^{ch} +/*c l-1* and c^{ch} +/*c l-2* progeny, and the c^{ch}/c^{ch} class includes primarily c^{ch} +/c^{ch} + progeny, although all other combinations of recombinant progeny are possible.

erozygotes. However, these homozygotes were variably reduced in size (anywhere from 30% to ~80% the size of their normal siblings). They also exhibited variability in fitness and survival; some died before weaning, but others could live well past weaning, and some were even fertile. On the other hand, no visibly abnormal albino neonates were ever observed in intercrosses of $l(7)5Rn^{3354SB}$ heterozygotes in 357 classified progeny. [The three normal albinos that were observed in these 357 progeny probably represent recombinant progeny (c l/c +) that are heterozygous for $l(7)5Rn^{3354SB}$].

Evidence for leakiness of the $l(7)5Rn^{1989SB}$ mutation in the hemizygous state can also be obtained from the deletion-mapping data presented in Table 1. Several crosses produced albino progeny that were either stillborn or were found dead within 24 h after birth. Again, this hemizygous leakiness was never observed in deletion-mapping crosses involving $l(7)5Rn^{3354SB}$, but the number of progeny scored was not as large as in the $l(7)5Rn^{3354SB}$ intercrosses [207 (Table 1, 3354SB column total) versus 357 (Table 2)]. The difference in temporal lethality between the $l(7)5Rn^{1989SB}$ and $l(7)5Rn^{3354SB}$ mutations, and the degree of homozygous versus hemizygous leakiness of $l(7)5Rn^{1989SB}$, indicate that these mutations should be useful reagents for studying the organismal function of the *l(7)5Rn* gene product during prenatal, neonatal, and juvenile development.

Evidence for hemizygous leakiness of the $l(7)6Rn^{4234SB}$ mutation can also be found in the deletion-mapping data presented in Table 1; stillborn albinos (or albinos that died within 24 h after birth) were observed in four combinations. These results appear to conflict with the observation that neonatal death was never observed in $l(7)6Rn^{4234SB}$ intercrosses, which could produce $l(7)6Rn^{4234SB}/l(7)6Rn^{4234SB}$ homozygotes (Table 2). However, these intercrosses were not routinely checked daily for births (as was the case for the deletion-mapping experiments); therefore, stillborn albinos could conceivably have been missed. In this context, it will be interesting to compare the time of death of fetuses either homozygous or hemizygous for $l(7)6Rn^{4234SB}$ by examining the uterine contents of timed pregnant females from both types of crosses.

The eed phenotype is currently defined by the failure of embryos homozygous for the c^{11DSD} (Niswander et al. 1988, 1989) or c^{202G} (Sharan et al. 1992) deletions to develop beyond the early stages of gastrulation. Because the *eed* locus has been defined by the phenotype of deletion homozygotes, it has been difficult to ascertain whether the observed failure in embryonic ectoderm development is due to the inactivity of one gene or more than one gene. Because both the *l(7)5Rn* and *l(7)6Rn* loci map within the c^{202G} deletion, mutations at either locus, or at both loci, could conceivably contribute to the eed defect. The mutations at *l(7)5Rn* and *l(7)6Rn* are likely to be intragenic, on the basis of previous genetic and molecular analyses of mutations induced by ENU in stem-cell spermatogonia (Popp et al. 1983; Russell, Rinchik 1987; Russell et al. 1990; Peters et al. 1990; Zdarsky et al. 1990; Brannan et al. 1992). Thus, these mutations should be useful reagents both for dissecting the *eed* phenotype and for aiding in the isolation and verification of candidate genes within the *eed* region that are responsible for this developmental defect of the early postimplantation embryo.

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