Complementation Analyses for 45 Mutations Encompassing the *pink-eyed dilution* (*p*) Locus of the Mouse

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ABSTRACT

The homozygous and heterozygous phenotypes are described and characterized for 45 new *pink-eyed dilution* (*p*) locus mutations, most of them radiation-induced, that affect survival at various stages of mouse development. Cytogenetically detectable aberrations were found in three of the new *p* mutations (large deletion, inversion, translocation), with band 7C involved in each case. The complementation map developed from the study of 810 types of compound heterozygotes identifies five functional units: jls and jlm (two distinct juvenile-fitness functions, the latter associated with neuromuscular defects), pl-1 and pl-2 (associated with early-postimplantation and preimplantation death, respectively), and nl [neonatal lethality associated with cleft palate (the frequency of rare "escapers" from this defect varied with the genotype)]. Orientation of these units relative to genetic markers is as follows: centromere, *Gas-2*, pl-1, jls, jlm *p*, nl (equatable to *cp1* = *Gabrb3*); pl-2 probably resides in the *c*-deletion complex. pl-1 does not mask preimplantation lethals between *Gas2* and *p;* and no genes affecting survival are located between *p* and *cp1*. The alleles specifying mottling or darker pigment (generically, p^m and p^x, respectively) probably do not represent deletions of *p*-coding sequences but could be small rearrangements involving proximal regulatory elements.

THE mouse specific-locus test (W. L. RUSSELL 1951) has over a period of decades generated numerous radiation- or chemically induced, as well as spontaneous, mutations involving each of seven marker loci. Many of these mutations have been propagated in breeding stocks, and sets of mutations involving a locus have been entered into large-scale pair-wise complementation analyses. Using large sets of Oak Ridge mutations, such analyses have been completed for the *dilute* (*d*) and *short-ear* (*se*) loci in chromosome *9* (RUSSELL 1971), the *albino* (*c*) locus in chromosome *7* (RUSSELL *et al.* 1982), the *brown* (*b*) locus in chromosome *4* (RINCHIK 1994), and the *agouti* (*a*) locus in chromosome *2* (L. B. RUSSELL, unpublished results). Complexes of deletions that overlap at the marker locus have been characterized by these analyses, and pseudo-dominance tests with loci that flank the specific-locus markers revealed that complexes could range up to 11 cM, corresponding to physical lengths ranging to perhaps 20 megabases (RINCHIK and RUSSELL 1990). The complementation analyses further identified "functional units" defined by specific deletional phenotypes, each of which could represent either a single gene or several genes, and located them within intervals bounded by individual deletion breakpoints.

Initially, such functional units identify factors required for crudely defined developmental features, such as survival to different stages (preimplantation, postimplantation, birth, *etc.*), but as the sophistication of phenotype analyses have increased, and particularly as molecular characterization of the regions has progressed, the functional maps have been considerably refined at the same time as physical maps are being developed (*e.g.,* RINCHIK *et al.* 1986, 1994; JENKINS *et al.* 1989; JOHNSON *et al.* 1989; NISWANDER *et al.* 1989; KLEBIG *et al.* 1992a; NICHOLLS *et al.* 1993; RINCHIK 1994). Additionally, recent saturation-mutagenesis studies, utilizing the powerful point-mutation inducer *N*-ethyl-*N*-nitrosourea (ENU), have refined the functional maps of genomic regions associated with some of the deletion complexes, have identified single-gene components of certain complex phenotypes, and have provided information on the density of "essential" genes per unit length of genome (RINCHIK *et al.* 1990, 1993a; RINCHIK and CARPENTER 1993).

Not only have the deletion complexes identified loci of considerable developmental interest (RINCHIK *et al.* 1990, 1994; MERCER *et al.* 1991; KINGSLEY *et al.* 1992; KLEBIG *et al.* 1992b; RUPPERT *et al.* 1992; CULIAT *et al.* 1993; RINCHIK 1994; AVRAHAM *et al.* 1995), but they are providing the genetic and physical reagents for positional cloning of functionally significant genes within the region, both newly identified and previously known ones. Several of these genes have major significance for understanding human genetic disorders (*e.g.,* congenital cleft palate) (CULIAT *et al.* 1994). Reciprocally, certain genes that have already been cloned in humans can be mapped into the mouse deletion complexes and

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thus become accessible to experimental studies that cannot be performed in humans, *e.g.*, studies involving manipulations of embryos.

Genetic studies involving 11 radiation-induced Harwell *pink-eyed-dilution* (*p*)-locus mutations were reported by LYON *et al.* (1992). Here we present the results of large-scale complementation and deletion analyses of 45, mostly radiation-induced, Oak Ridge mutations encompassing the *p* locus, as well as cytogenetic and time-of-death studies for subsets of these. A number of these mutations, several of them involving regions of conserved mouse/human synteny, have already been utilized in molecular studies. The region immediately encompassing and somewhat distal to *p* is homologous to human *15q11-q13,* whereas the proximal end of the deletion-complex region is homologous to human *11p15.* The human homologue of the *p* locus, *P* (GARDNER *et al.* 1992), corresponding to the *D15S12* locus, probably encodes an integral membrane-transport protein whose absence is associated with tyrosinase-positive oculocutaneous albinism (RINCHIK *et al.* 1993b; SPRITZ 1994; LEE *et al.* 1995). At least three other human sequences from the *15q11-q13* region map (in the same order) to the mouse *p*-deletion-complex region, but one sequence from this region in humans (*D15S9h-1/Znf127*) maps outside the mouse deletion complex (NICHOLLS *et al.* 1993). A gene, *cp1,* whose deletion results in cleft palate, has been mapped near the distal end of the *p* complex (CULIAT *et al.* 1993). It has been suggested that this gene very probably corresponds to the locus encoding the $\beta 3$ subunit of the Type-A γ-aminobutyric acid ($GABA_A$) receptor (CULIAT *et al.* 1993, 1994). One of the largest *p* deletions has been found to include the *Myod1 (myogenic differentiation factor-1), Kcnc1 (subunit of voltage-gated potassium channels), Tph (tryptophan hydroxylase), Saa1 (serum amyloid-A multigene family members), Ldh1 (lactate dehydrogenase A), and Ldh3 (lactate dehydrogenase C)* loci (MENDEL 1987; STUBBS *et al.* 1994). The *Myod1* locus, mapping within this deletion, was shown earlier to be located 3–4 cM proximal to *p* (SCRABLE *et al.* 1990).

The genetic and complementation analyses reported here have revealed the pigmentation, neuro-motor behavior, viability, and fitness of homozygotes for each *p* mutation and of various combinations of mutations. As a result, we have been able to define several functional units within the *p*-deletion complex and to generate a complementation map for 45 mutations that could be oriented with respect to chromosome 7 markers. Three of the mutations were shown to involve gross chromosome rearrangements; most of the remainder are probably deletions of various lengths, except for a few that may be other types of small rearrangements. In the accompanying paper (JOHNSON *et al.* 1995), these mutations are characterized in greater detail by utilizing a number of molecular probes.

MATERIALS AND METHODS

Mice used in these studies came from stocks propagating independent *p*-locus (*pink-eyed dilution*) mutations that had arisen over the course of decades in specific-locus mutagenesis experiments conducted at Oak Ridge National Laboratory. In such experiments, a test stock homozygous for seven visible markers, including *p,* is crossed to mutagenized (101/Rl × C3H/Rl)F_1 mates, which are homozygous wild-type for each of the markers. The mutagen, dose, dose-rate, and germ-cell stage exposed are listed in Table 1 for each mutation. Of the 45 mutations, 39 originated in experiments using external radiation sources (X rays, gamma rays, or neutrons), three originated in experiments with internal radiation emitters (tritiated water or ^{239}plutonium), two came from chemical mutagenesis studies with methylnitrosourea, and one occurred in an unexposed control. The original *p* mutation of the mouse fancy, which is carried in the tester stock used in the mutagenesis experiments, was also used in several of the intercrosses.

New presumed *p*-locus mutations in the mutagenesis experiments were recovered as $p^*/+$ p c^{ch} (where p^* designates any new *p*-locus mutation, and c^{ch}, *chinchilla,* is an allele at the *albino* locus, 14 cM distal to *p*). After allelism with *p* was confirmed, the primary mutant was crossed to $+$ $c^{ch}/+$ c^{ch}, and at least six wild-type offspring collected, each of which became the founder of a line. On average, 86% of the founders are expected to be $p^*/+$ c^{ch} (and 14% $p/+$ c^{ch}). Each founder was again crossed to $+$ $c^{ch}/+$ c^{ch}, and those of his/her offspring tested to carry p^* (or *p* in ~14% of the lines) were backcrossed to the founder. Lines that failed to yield progeny that were of the *pink-eyed-dilution* or an intermediate phenotype were assumed to carry a prenatally lethal mutation involving *p,* and such a mutation was subsequently propagated from one of these lines. In the present study, all mutations used, except for the standard *p* allele, were homozygous lethal, most of them prenatally, but a few neonatally or postnatally (see below).

The *p* locus has been mapped to position 28 in chromosome 7 (BRILLIANT *et al.* 1994). Nearby chromosome 7 genes used by us for preliminary deletion mapping were *ruby eye 2* (*ru2*), a coat- and eye-color gene that is an anchor locus at location 25, *lactate dehydrogenase 1* (*Ldh1*) at 24, and *twister* (*twt*), a neurological mutant whose position has been established with less certainty than the above (integrated to within ~5 cM, rather than ~1 cM) at 28 (BRILLIANT *et al.* 1994).

Of the 1035 combinations of two different mutations that are possible for 45 mutations, 810 (almost 80%) were made. Several mating pairs were required for each of these combinations to accumulate the required numbers of offspring. The preferred cross was $+$ c^{ch}/p^1 $+$ × $+$ c^{ch}/p^2 $+$ (where p^1 and p^2 represent any two different *p*-locus mutations), although c^{ch} was not carried by all parents. Except in a few crosses where offspring were examined in late fetal stages (see below), mated pairs were permitted to breed continuously, and each breeding pen was checked at least at weekly intervals but often more frequently when a birth was imminent; therefore, the first observation of offspring (alive or dead) ranged from late fetal stages to day 6 postnatally. A random sampling of ~100 birth records shows that the initial check was made on the day of birth in 53.8% of cases, on days 1 or 2 in 25.6 % of cases, and on day 3 in 12.8% of cases; only 7.7% of the litters were first checked between days 4–6, inclusive. Before the eruption of hair, offspring were classified on the basis of eye pigment.

Survival status and phenotypes were again recorded when offspring were weaned at 25–31 days of age, at which time all litters without living p^1/p^2 offspring were discarded. If p^1/p^2 segregants were present, each member of the litter was weighed. Each weanling p^1/p^2 was matched with at least one

TABLE 1

Origin of *p*-locus mutations

Mutation[a]	Germ-cell stage[b]	Mutagen[c]	Dose[d]	Dose rate (R/min)	Interval for fractions
		A. Prenatally lethal *pink-eyed dilution* mutants			
2DFiOD	Spermatogonia	X rays	500 + 100	90	24 h
23DFiOD	Spermatogonia	X rays	500 + 100	90	24 h
46DFiOD	Spermatogonia	X rays	500 + 100	90	24 h
41DTD	Spermatogonia	X rays	500 + 500	90	24 h
45DTD	Spermatogonia	X rays	500 + 500	90	24 h
47DTD	Spermatogonia	X rays	500 + 500	90	24 h
3DTR	Spermatogonia	X rays	500 + 500	90	2 h
15DThW$_b$	Spermatogonia	X rays	6 × 50	90	1 wk
19DVT	Spermatogonia	X rays	5 × 200	90	1 wk
25DVT	Spermatogonia	X rays	5 × 200	90	1 wk
26FAT$_w$	Oocytes	X rays	200	90	
2FBC$_f$Fo$_b$	Oocytes	Caffeine[e] + X	200 + 200	90	
83FBFo	Oocytes	X rays	400	0.8	
8FDFoD	Oocytes	X rays	200 + 200	90	24 h
3FR60L$_g$	Oocytes	Neutrons	60	0.16	
132G	PG	X rays	300	90	
2HATh	PG	X rays	300	900	
58HATh	Spermatogonia	X rays	300	900	
8OK	Spermatogonia	X rays	600	90	
1OZ$_b$	Oocytes	X rays	400	90	
55PB	Spermatogonia	X rays	1000	90	
30PU$_b$	Spermatogonia	^{239}Pu citrate	10 μCi/kg		
4R250H	Spermatogonia	Neutrons	250	88	
8R250M	Spermatogonia	Neutrons	250	0.8	
12R30L$_b$	Spermatogonia	Neutrons	30	0.16	
3R30M	Spermatogonia	Neutrons	30	0.80	
3RD300H	Spermatogonia	Neutrons	100 + 200	88.0	24 h
226THO-I	Spermatogonia	^3H_2O	0.5 m Ci/g		
15ThP	Spermatogonia	X rays	300	90	
24Z$_b$	Oocytes	X rays	400	90	
1DTTM$_b$	Spermatogonia	X rays	4 × 500	90	4 wk
1MNURf	PG	MNU	75 mg/kg		
2MNURf	PG	MNU	75 mg/kg		
		B. Other *p*-locus mutations (see Table 2)			
18CoS	Spermatogonia	Gamma rays	600	48	
39DSD	Spermatogonia	X rays	600	90	
12DTR	Spermatogonia	X rays	500 + 500	90	2 h
9DTW	Spermatogonia	X rays	5 × 200	90	1 wk
17FATw$_b$	Oocytes	X rays	200	90	
7FR60Lb	Oocytes	Neutrons	60	0.16	
116G	PG	X rays	300	90	
39K	Spermatogonia	X rays	600	90	
48PB	—	(Control)	—	—	
12R250M	Spermatogonia	Neutrons	250	0.8	
4THO-II	PG	^3H_2O	0.5 m Ci/g		
103G	PG	X rays	300	90	
Original *p*	—	—	—	—	

[a] Mutations in all tables are designated by their superscript symbols.
[b] PG, postspermatogonial stages.
[c] X, X rays; MNU, methyl nitrosourea; ^3H_2O, tritiated water.
[d] All radiation doses in roentgens (R) unless otherwise indicated.
[e] 200 and 100 mg/kg caffeine before and after first fraction of X rays, respectively.

TABLE 2

Mutations with phenotypes other than classical pink-eye and/or not prenatally lethal

Pigment type	Stage of lethality		
	Prenatal	Neonatal	Juvenile[a]
Pink-eyed	See Table 1A	*7FR60L$_b$* *116G* *39K* *4THO-II*	*9DTW*
Dark pink-eyed	*17FATw$_b$* *48PB*		*12DTR* *12R250M* *103G*
Mottled			*18CoS* *39DSD*

[a] Animals homozygous for the juvenile-lethal mutations exhibit neuromuscular abnormalities.

+/? littermate (+/? designates +/+, +/p^1, or +/p^2), like-sexed if possible, for a long-term survival study, in which animals, housed four to six per cage, were observed at weekly, or more frequent, intervals until they died or reached 365 days of age. At sacrifice, spleens and livers were saved for DNA preparations used for molecular analyses of the region encompassing the *p* locus (CULIAT *et al.* 1993, 1994; NICHOLLS *et al.* 1993; RINCHIK *et al.* 1993b; JOHNSON *et al.* 1995).

RESULTS

Homozygotes and heterozygotes: *Phenotypes:* In combination with the standard *p* allele, 38 of the 45 independent mutations produce hair and eye pigmentation indistinguishable from that seen in *p/p*. Of these 38, 33 mutations are prenatally lethal in homozygotes (generically designated p^{pl}), four ($p^{7FR60Lb}$, p^{116G}, p^{39K}, and $p^{4THO-II}$) are neonatally lethal (generically designated p^{nl}), and one (p^{9DTW}) is a juvenile lethal (p^{jl}). Five mutations, designated p^x, when combined with the standard *p* allele, produce pigmentation intermediate between that characteristic of *p/p* and wild-type. Of these five, two ($p^{17FATwb}$ and p^{48PB}) are prenatally lethal (generically, p^{xpl}), and three (p^{12DTR}, $p^{12R250M}$, and p^{103G}) are juvenile lethal (generically, p^{xjl}), *i.e.*, exhibiting increased mortality at various preweaning ages as well as reduced long-term survival. Finally, two mutations (p^{18CoS} and p^{39DSD}) produce a mottled phenotype in combination with *p* (namely, diluted-pigment areas on wild-type background). Homozygotes for these mutations are smaller than their littermates and survive less well both pre- and postweaning; they have been classified as juvenile lethals, with the generic designation p^{mjl}. These various types of mutations are summarized in Table 2.

For two of the four p^{nl} mutations ($p^{7FR60Lb}$ and $p^{4THO-II}$), homozygotes occasionally survive past birth but with different frequencies. About 1.2% of $p^{7FR60Lb}/p^{7FR60Lb}$ mice survived up to 3 weeks and none beyond; in the case of $p^{4THO-II}/p^{4THO-II}$, however, 8.5% died between 8 and 22 days, and 9.9% survived past 26 days and were classified as small and "nervous." A breakdown of these data, derived 1974–1976 (shortly after the mutation had occurred), indicates that the high incidence of survivors is assignable to one of nine mated pairs; for the other eight pairs, the incidences of survival for 8–22 days and >26 days were 1.7 and 3.3%, respectively, for a total of 5%. This corresponds closely to the more recent findings of CULIAT *et al.* (1993), who discovered that neonatal death of $p^{4THO-II}$ homozygotes was associated with cleft palate, and who reported ~5% escapers among presumed homozygotes, with half of these dying at ~17 days, and the other half surviving well past 28 days. The high yield of escapers seen in the progeny of one of the early females may have been the result of suppressing modifiers that have since been lost from the stock.

Animals heterozygous for certain of the *dark-pink-eyed* (p^x) mutations (p^{48PB}/p, $p^{12R250M}/p$) have eyes that are externally indistinguishable from those of *p/p* at birth but change to a dark ruby color by weaning age. Other p^x heterozygotes (p^{17FATw}/p, p^{12DTR}/p) as newborns have less-than-fully-pigmented eyes that darken with age, and still others (p^{103G}/p) have virtually fully pigmented eyes from birth on. Among the three p^x-type mutations that are not prenatally lethal, homozygotes for two ($p^{12R250M}/p^{12R250M}$ and p^{103G}/p^{103G}) appear to have fully pigmented eyes from birth on, whereas p^{12DTR} homozygotes are born with less-than-fully-pigmented eyes that have darkened by weaning age. Eumelanin in the fur of p^x/p mice is intermediate in color between that of *p/p* and wild-type animals (*p* does not dilute phaeomelanin). In mice homozygous for p^{xjl} mutations, the fur is generally no darker than in p^{xjl}/p heterozygotes.

No karyotypic anomalies are detectable in the mottled mutations p^{18CoS} and p^{39DSD} (RUSSELL 1965). While homozygotes are poorly viable (and male-sterile in limited tests), heterozygotes are fully viable and fertile, producing normal litter sizes. Neither of the mutations affects recombination frequencies in the *p-c* interval. Crosses of $p^m/p \times p^m/p$ have yielded three wild-type exceptions among about 3500 offspring; two of these were tested to be +/*p* (the third was not tested).

Mice homozygous for any of the juvenile-lethal mutations of the p^{jl}, p^{xjl}, and p^{mjl} types are characterized by neuromuscular anomalies. When picked up by the tail, these animals exhibit a twitching of the hindlimbs, soon followed by a severe tremor of the forelimbs. Weanlings are distinctly smaller than their like-sexed wild-type littermates. In limited fertility tests of surviving homozygotes (two to four males and three to eight females tested per stock), males have been invariably sterile, but one p^{18CoS}/p^{18CoS} female produced one litter.

Time of death of prenatally-lethal mutations: In an effort to determine the approximate developmental stage at which death occurs in mice homozygous for prenatally lethal (p^{pl}) mutations that enter into prenatally-lethal compounds, we analyzed uterine contents in pregnancies involving each of three such mutations, p^{2MNURf},

TABLE 3

Results of uterine dissections on E14.5

Mutation	Experimental matings[a] No. of c.l.	Implants per c.l. (%)	Resorbing moles/c.l. (%)	Control matings[b] No. of c.l.	Implants per c.l. (%)	Resorbing moles/c.l. (%)	*P* value[c]
2MNUR$_f$	80	80.0	27.5	70	80.0	11.4	0.01
48PB	69	81.2	24.6	73	76.7	2.7	0.0002
2DFiOD	102	63.7	10.8	98	74.5	9.2	0.10

[a] In the case of the p^{2MNURf} and p^{2DFiOD} mutations, matings were of the type $p^{pl}/p^{x} \times p^{pl}/p^{x}$, where p^{x} was the fully viable allele p^{7R75M} segregating within each of the mutant stocks; all embryos had some eye pigment, as expected if p^{2MNURf} and p^{2DFiOD} are early lethals. In the case of the p^{48PB} mutation, matings were of the type $p^{xl}/p \times p^{xl}/p$, where p is the original *pink-eye* mutation segregating within the mutant stock; none of the embryos had an eye pigment; however, eye pigment is not diagnostic in this cross (see text).

[b] In the case of the p^{2MNURf} and p^{2DFiOD} mutations, matings were of the type $p^{pl}/p^{x} \times p^{x}/p^{x}$. In the case of the p^{48PB} mutation, matings were of the type $p^{xl}/p \times p/p$.

[c] For *2MNURf* and *48PB*, experimental-control comparison of resorption moles; for *2DFiOD*, experimental-control comparison of total implants.

p^{48PB}, and p^{2DFiOD}. In each stock, females heterozygous for the lethal were randomized into two groups, with one group ("experimental") mated to males of the same genotype, and the other group ("control") to males homozygous for a viable *p* allele segregating within the same stock. The uterine environments for the experimental and control matings were thus comparable, and the expectation of p^{pl}/p^{pl} conceptuses was 25 and 0%, respectively. In the case of the p^{2MNURf} and p^{2DFiOD} mutations, the females were of the type p^{pl}/p^{x}, where p^{x} was the fully viable allele p^{7R75M}. All embryos, unless there should be p^{pl}/p^{pl} survivors, are expected to have eye pigment, and this was in fact found. In the case of the p^{48PB} mutation, the females were p^{xl}/p, where *p* was the standard *pink-eye* mutation. Because the eyes of p^{48PB}/p animals do not develop pigment until after birth, pigment is expected to be absent both in the heterozygous and *p/p* segregants; pigmented eyes were not found in any of the fetuses.

Uteri were dissected 14 days after observation of a vaginal plug [embryonic day (E) 14.5]. Living fetuses were examined as to eye color, and other implantation sites were classified as to probable time of death; in addition, corpora lutea (c.l.) were counted in the ovaries. The experimental and control matings in each stock were compared with regard to two ratios: total implants/c.l. and resorption moles/c.l. (Table 3). In the cases of p^{2MNURf} and p^{48PB}, there was no reduction in total implants but a clear increase in resorption moles. However, results for p^{2DFiOD} indicate preimplantation death of homozygotes, since total implants are reduced while the frequency of resorption moles appears unaffected.

Cytogenetic analyses: By the use of high-resolution Giemsa banding, a cytologically detectable deletion was found associated with $p^{46DFiOD}$, which encompasses, at a minimum, the *ru2-p* segment (see below). A portion of band 7C (and possibly also of band 7B5) has been deleted (Figure 1).

The p^{2DFiOD} mutation was found completely to complement all other lethals (see below), yet had clearly been prenatally lethal in homozygotes that were generated shortly after the mutation arose. Because of the possibility that p^{2DFiOD} could be the result of two independent point mutations (one at *p* and the other at an essential locus elsewhere) that might have become separated by recombination before the initiation of complementation tests, ~200 additional offspring from $+/p^{2DFiOD} \times +/p^{2DFiOD}$ matings were generated; none was pink-eyed. Cytogenetic analysis revealed that this mutation is an inversion, *[In(7)4Rl]*, with breakpoints within 7C and 7E1 (Figure 1).

Heterozygotes in the stock propagating the prenatally lethal p^{15ThP} mutation were found to be semisterile (*i.e.*, their littersize was reduced). Cytogenetic analysis showed this mutation to be a reciprocal translocation, *T(7;9)23Rl*, with breakpoints in 7C and 9A5.2 (Figure 1).

Deletion mapping with standard markers: Animals heterozygous for each one of the lethal *p*-locus mutations listed in Table 1 were crossed to *ru2/ru2* (*ruby-2*), and at least 30 offspring (actual range, 30–82) were classified from each cross. Only one of the *p*-lethal mutations, $p^{46DFiOD}$, yielded ruby-eyed in addition to wild-type progeny. (Crosses with *ru2* were also made for over 30 *p*-viable mutations not further described in this paper; all produced only wild-type progeny.) For 35 of the mutations listed in Table 1, kidney and liver homogenates of heterozygotes were tested for LDH (lactate dehydrogenase) activity; only one mutation, $p^{46DFiOD}$, exhibited reduced activity (MENDEL 1987). Similar results were obtained for 30 *p*-lethal mutations tested with a probe for *Ldh1;* again $p^{46DFiOD}$ was the only mutation in which this locus was found to be deleted (JOHNSON 1990; JOHNSON *et al.* 1995). Analyses of offspring of a cross to *M. spretus* indicated that the serum amyloid A (*Saa*) gene complex is also missing in $p^{46DFiOD}$ (MENDEL 1987; JOHNSON *et al.* 1995), whereas sequences corre-

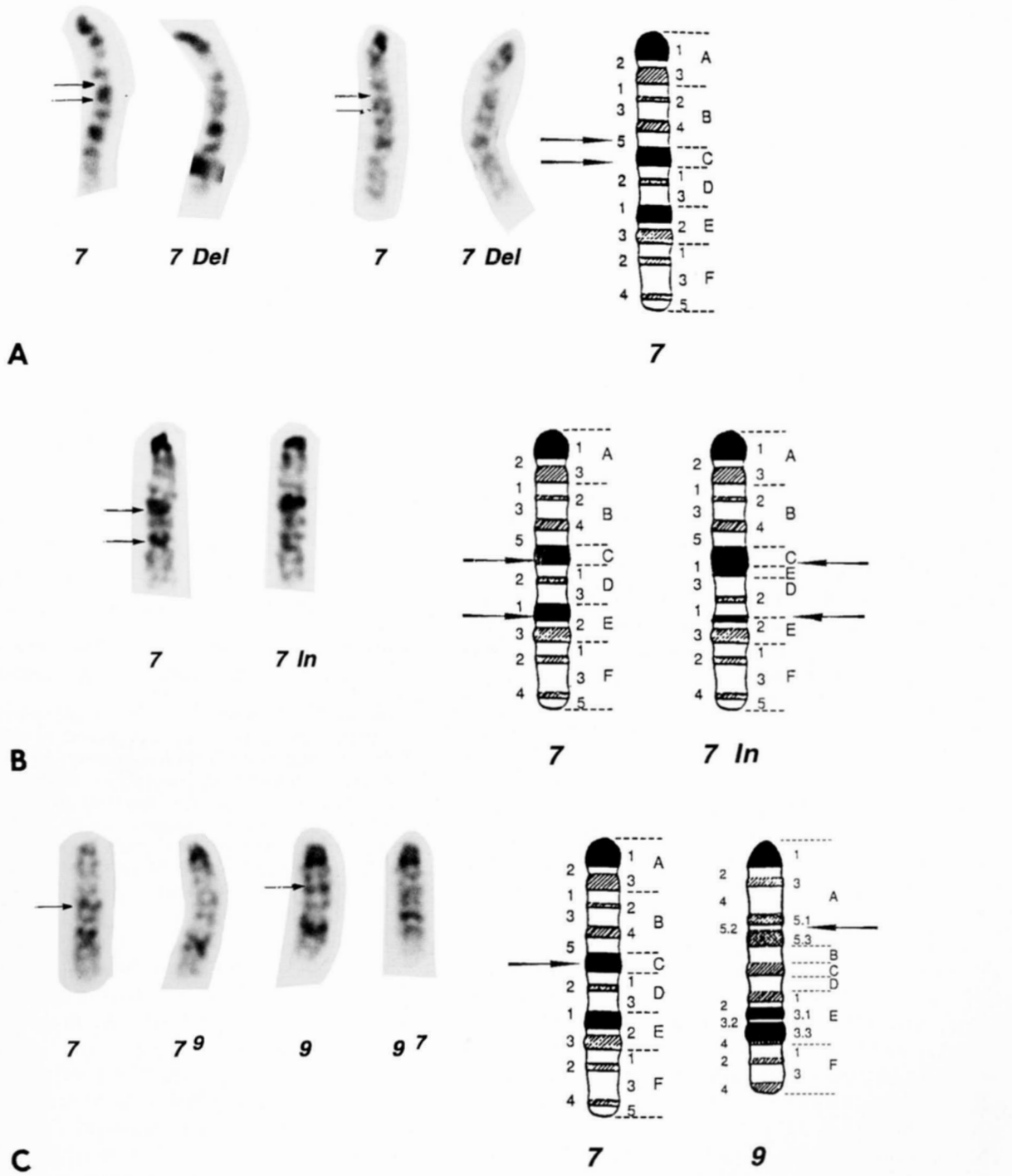


FIGURE 1.—Three *p*-locus mutations that are cytogenetically detectable chromosomal rearrangements. In each case, G-banded metaphase chromosomes are shown on the left and ideograms on the right. Breakpoints are indicated by arrows. (A) $p^{46DFiOD}$ deletes portions of bands 7C and possibly also of 7B5. (B) p^{2DFiOD} is an inversion, designated *In(7)4Rl,* with breakpoints within bands 7C and 7E1. (C) p^{15ThP} is a reciprocal translocation, designated *T(7;9)23Rl,* with breakpoints in 7C and 9A5.2.

sponding to the gene *luteinizing hormone b* (*Lhb*) are not (MENDEL 1987).

Heterozygotes from 26 of the *p*-lethal stocks were crossed to *twt/twt* (*twister*) homozygotes, and none yielded twister progeny among at least 17 offspring (range, 17–66; $P < 0.00001$) for 22 of the stocks, and at least 10 offspring ($P < 0.001$) for four. Among *p*-locus mutations that clearly do not delete *twt* is one deleted for *ru2* (namely, $p^{46DFiOD}$) and seven that involve the neonatal functional unit nl (see below) and the sequence *Gabrb3,* as well as, in one case (p^{30PUb}), also the more distal sequence E6-AP (JOHNSON *et al.* 1995), indicating that *twt* is not located within the present limits of the *p*-region deletion complex.

Compound heterozygotes: *Categorization of mutant combinations:* Any cross of the type $+/p^1 \times +/p^2$ (where p^1 and p^2 represent any two different *p*-locus mutations) in which no p^1/p^2 offspring were observed among a minimum of 35 classified offspring was assumed to identify a prenatally lethal (pl) combination of *p* mutations. There were ~400 such combinations, identified by crosses that yielded a total of nearly 22,000 progeny, an average of 54.8 classified offspring per cross.

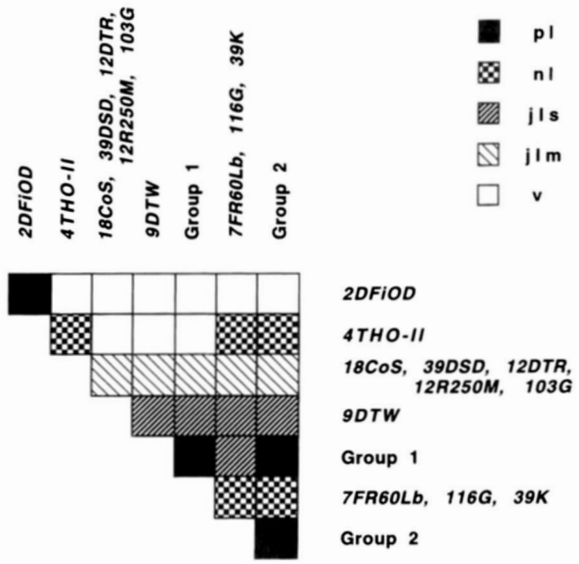
Compound heterozygotes that survived to or past birth were classified on the basis of subsequent survival history (see below) into the following categories: nl, neonatal lethal; jls, severe juvenile lethal; jlm, mild juvenile lethal; v, viable. The classification process was an iterative one; while individual combinations did not always fit clearly into one or another category, the overall pattern that eventually emerged for the total array of combinations involving a given mutation was generally unambiguous. Long-term survivals, as well as weaning weights, also fitted the overall patterns. The combinations found to fit into the various categories are summarized in Figure 2.

Pigmentation phenotype of compound heterozygotes: All surviving compound heterozygotes involving only p^{pl}, p^{nl} and/or p^{jl} mutations had a pigmentation phenotype similar to *p/p*. Combinations of p^m with any other mutations resembled p^m/p or p^m/p^m in appearance (spots of reduced pigment intensity on a full-color background). Combinations of p^x mutations with any mutation (except with p^m mutations) had pigmentation intermediate between *p/p* and wild-type. While not all possible p^{x1}/p^{x2} combinations were made, those observed indicated that eyes were fully pigmented at birth, even in cases where either p^{x1}/p or p^{x2}/p had less than fully pigmented eyes at birth.

Survival before weaning of compound heterozygotes: For compound heterozygotes that survived to or past birth, the incidence of death observed before weaning was computed for both p^1/p^2 and wild-type progeny for two separate intervals: by the time of the first observation of a litter (days 0–6) and between days 7 and weaning. These results, in addition to overall survival to weaning, are summarized in Table 4. In addition, the loss of p^1/p^2 offspring before the time of the first observation was estimated from ratios (see below).

For nonpink-eyed progeny, overall survival to weaning was consistently between 89 and 95% (overall mean for 368 crosses, 91.8%) with about half of the overall deficit due to death before day 7. Very similar percentages (89–94%) were seen for p^1/p^2 animals in the 79 v combinations. At the other end of the spectrum, p^1/p^2 progeny in the 54 nl combinations survived to weaning with a frequency of only 0–7.4%, and the great preponderance of deaths occurred before day 7.

It has been shown by CULIAT *et al.* (1993) that neonatal death of $p^{4THO\text{-}II}/p^{4THO\text{-}II}$ and of certain $p^{4THO\text{-}II}$ compound heterozygotes is associated with the presence of



Group 1: *46DFiOD, 41DTD, 3DTR, 15DThWb, 25DVT, 26Fatw, 2FBCfFob, 83FBFo, 3FR60Lg, 58HATh, 8OK, 10Zb, 4R250H, 12R30Lb, 3R30M, 3RD300H, 15ThP, 24Zb, 1MNURf, 2MNURf, 17FATWb, 48PB*

Group 2: *23DFiOD, 45DTD, 47DTD, 19DVT, 8FDFoD, 132G, 2HATh, 55PB, 30PUb, 8R250M, 226THO-I, 1DTTMb*

FIGURE 2.—Summary of survivals characterizing *p*-locus-mutation homozygotes and compound heterozygotes. The different *p* alleles are indicated by their superscripts. □, normal survival, behavior, and weight (*i.e.*, complete complementation, v combinations); ■, prenatal death (pl combinations); checkerboard shading denotes neonatal death (nl combinations) with rare escapers (see text); ▨, juvenile-lethality with the "severe juvenile-lethal" (jls) pattern; ▧, juvenile-lethality with the "mild juvenile-lethal" (jlm) pattern (see text and Figure 4).

a cleft palate, as was the case for p^{cp}/p^{cp} described earlier (PHILLIPS 1973). This condition is presumed to cause aspiration of suckled milk into the lungs. Uterine dissections were performed to determine the phenotype of nine of the numerous types of combinations that our breeding data had indicated to be neonatally lethal. In each of 16 pregnancies examined (most of them on E17.5), one parent was heterozygous for $p^{4THO\text{-}II}$. Of 107 fetuses observed, 19 were pink-eyed, and, among these, 17 had a cleft palate, and two appeared to have a normal palate (one of these was observed on E15.5). Of 88 nonpink-eyed fetuses, 87 had a normal palate and one a possibly mildly cleft one (this fetus was seen on E15.5 when palate closure is not always complete).

The uterine contents were also examined for three $p^{2HATh}/+$ females impregnated by $p^{116G}/+$ males. This cross had originally failed to yield any pink-eyed offspring among 144 classified progeny, and the combination was tentatively classified pl. The uteri, examined 1 or 2 days before expected delivery, were found to contain 29 living and apparently healthy fetuses; of these,

TABLE 4

Survival to weaning of *p* combinations that survive past birth

Phenotype of combination[a]	Involving mutation	No. of combinations[b]	No. offspring classified by day 6		Dead by day 6 (%)		Died day 7 to weaning (%)		Surviving to weaning (%)	
			+/?[c]	$p^1/p^{2\,d}$	+/?	p^1/p^2	+/?	p^1/p^2	+/?	p^1/p^2
v	*2DFiOD*	44	1595	537	2.8	2.4	4.1	3.9	93.1	93.7
	4THO-II	26	1128	371	3.8	3.8	5.9	4.3	90.2	91.9
	Standard *p*	10	156	167	10.3	5.4	5.1	5.4	84.6	89.2
	All v[e]	79	2839	1052	3.6	3.4	4.9	4.2	91.5	92.4
jlm	*18CoS*	34	1371	314	5.3	0	4.6	21.3	90.2	78.7
	39DSD	22	761	191	5.7	0	1.4	26.7	92.9	73.3
	12DTR	33	1345	357	3.8	0.6	2.5	14.0	93.7	85.4
	12R250M	27	1121	351	3.7	6.6	1.8	25.1	94.5	68.4
	103G	27	1096	267	6.1	0	4.9	22.8	89.0	77.2
	All jlm[e]	138	5398	1408	4.6	1.8	3.3	20.2	92.1	78.0
jls	*9DTW*	36	1394	285	4.9	23.9	4.7	28.8	90.5	47.4
	7FR60L$_b$	21	845	209	2.8	27.8	2.5	41.6	94.7	30.6
	116G	22	878	204	4.3	35.8	5.2	27.0	90.4	37.3
	39K	21	628	154	4.8	20.8	1.1	33.1	94.1	46.1
	All jls[e]	97	3640	839	4.3	27.4	3.5	32.5	92.2	40.0
nl	*7FR60L$_b$*	15	642	56	4.4	96.4	6.2	3.6	89.4	0
	116G	15	959	73	6.5	90.4	3.2	5.5	90.3	4.1
	39K	15	531	43	7.3	97.7	2.3	2.3	90.4	0
	4THO-II	15	789	94	6.0	70.2	3.9	22.3	90.1	7.4
	All nl[e]	54	2630	229	6.2	87.3	4.0	9.6	89.7	3.1
Total		368	14,507[f]	3,528[f]	4.7		3.5		91.8	

[a] v, viable; jlm, mild juvenile lethal; jls, severe juvenile lethat; nl, neonatal lethal.

[b] Each combination resulted from a different cross of type $+/p^1 \times +/p^2$ (usually several matings for each cross). In combinations involving standard *p*, the parent was *p/p*.

[c] +/? designates offspring classified as either wild type or c^{ch}/c^{ch} on the basis of hair and/or eye pigment. (Either type may be $+/p$ or $+/+$).

[d] p^1 and p^2 symbolize any two different *p*-locus mutations. The p^1/p^2 category includes offspring classified on the basis of hair and/or eye pigment as $p^1 + {}^c/p^2$-, p^1c^{ch}/p^2c^{ch} or p^1/p^2 (*c*-locus constitution unknown).

[e] Adjusted for combinations that appear in more than one line, *e.g.*, *2DFiOD* × *4THO-II* is included in the combinations involving *2DFiOD* also in the combinations involving *4THO-II* but is counted only once in the summation.

[f] In addition, 125 animals (0.7% of all observed) were not classified by hair or eye pigment (dead by day 6).

six (21%) were pink-eyed and had a cleft palate, and the combination was reclassified as nl. As the $p^{2HATh}/+ \times p^{116G}/+$ cross continued to breed, it eventually yielded one pink-eyed offspring (dead by day 1) among 166 classified progeny.

Of 229 p^1/p^2 animals from nl combinations, 87.3% were dead by the time of first observation (and numerous others had presumably been cannibalized before observation). Of these 229 mice, respectively 9.6 and 3.1% died between days 7 and weaning age and between weaning and 1 year of age (see long-term survival, below). These escapers from the cleft-palate phenotype were of greatly reduced size (see below) and "nervous." It is of interest that in compound heterozygotes involving $p^{4THO-II}$, which within the nl category had the highest overall survival to weaning, the deaths tended to occur later during the preweaning interval than in other nl combinations (Table 4).

The 138 jlm and the 97 jls combinations differ from each other with respect to overall survival to weaning: 78.0% (range 68–85%) and 40.0% (range 31–47%), respectively. Further, about half of the observed preweaning death in the jls combinations occurs before day 7, whereas most of the observed death in jlm combinations occurs after day 6. Surviving animals of either type exhibit the neuromuscular symptoms typically seen in p^{jl} and p^{xjl} homozygotes (see above).

Long-term survival of compound heterozygotes: Table 5 summarizes the long-term outcome for animals that survived to weaning, and Figure 3 shows the survival curves. Survival of p^1/p^2 offspring from combinations classified as viable (v) was equivalent to that of the nonpink-eyed (wild-type and c^{ch}/c^{ch}) offspring of all combinations. As was seen for preweaning survival, there is a clear difference between jlm and jls combinations. Because almost one-quarter of the former, but only 5% of the latter, survive to 365 days, the underestimation of the mean that results from truncation of the data at 365 days is

TABLE 5

Long-term survival of *p* combinations that survive past weaning

Genotype	Phenotype of combination[a]	No. observed	Survival (days)		Surviving to 365 days (%)[c]
			Mean[b]	Median	
+/ and c^{ch}/c^{ch}	All[d]	2,377	350.6	>365	93.2
p^1/p^2	v[e]	739	344.3	>365	91.6 (91.2, 91.9)
p^1/p^2	jlm	856	230.6	248	21.4 (16.8, 27.0)
p^1/p^2	jls	316	129.7	117	5.2 (2.4, 11.7)
p^1/p^2	nl	7[f]	117.4	32	[f]

[a] See Table 4 (footnote *a*) for definition of symbols.

[b] All surviving animals were killed at 365 days, and their lifespan recorded as 365. The means, therefore, are underestimates of the true average lifespans.

[c] Range for different groups of combinations, within overall combination type, is shown in parentheses, *e.g.*, for jlm, these separate groups involve mutations p^{18Cos}, p^{39DSD}, p^{12DTR}, $p^{12R250M}$, and p^{103G}.

[d] Because weanlings from pl (prenatally lethal) combinations did not include p^1/p^2 animals, no wild-type or *chinchilla* sibs from such combinations are included in the long-term survival data.

[e] Data include 12 $p^{46DFiOD}/p^{4THO-II}$ animals, which did not survive as long as the average of other combinations. Mean survival for this genotype was 297.2 days, and only eight of the 12 survived to 365 days.

[f] All seven came from combinations in which one parent contributed $p^{4THO-II}$. Survival ages were 25, 29, 31, 32, 123, 215, and >365 days.

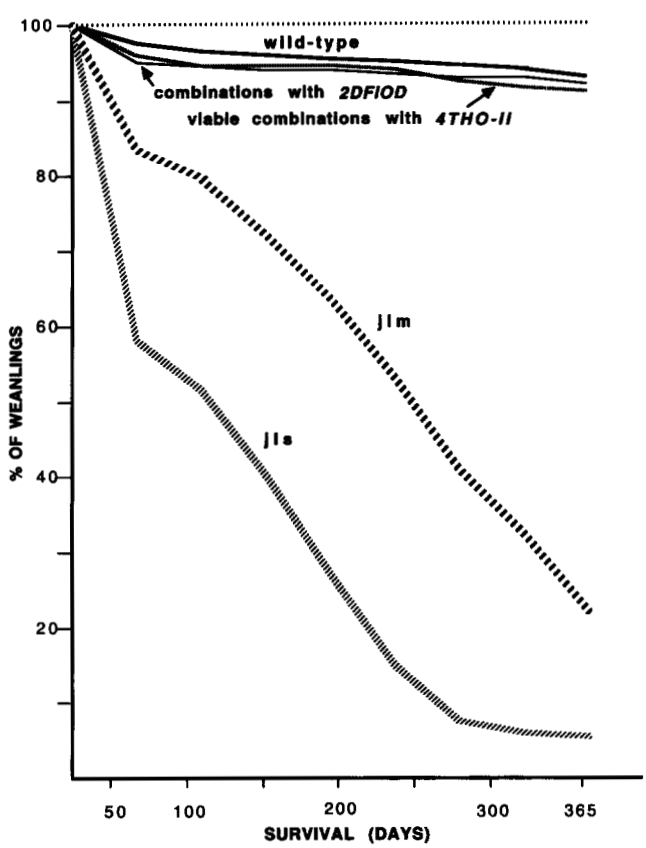


FIGURE 3.—Survival between weaning and 1 year of age for certain types of compound heterozygotes involving lethal *p*-locus mutations and for their wild-type (+/*p** and +/+) littermates. Combinations of any *p*-lethal mutation with p^{2DFiOD} exhibit normal long-term survival, as do combinations of those *p*-lethal mutations that complement $p^{4THO-II}$ (see Figure 2). The jlm and jls combinations have different long-term survival patterns.

clearly a factor for jlm but is negligible for jls combinations; thus, the real difference between the two classes of combinations is actually larger than indicated by the means calculated from the truncated life spans. The survival curves for jlm and jls combinations have a similar downward slope in their relatively straight midportions, but the curve for jls combinations begins to flatten after day 238; therefore, survival to day 237 was chosen for computation of survival patterns (see below). Animals that exhibited the neuromuscular syndrome at weaning also did so during their subsequent, shortened life span.

Regarding nl combinations, it is significant that of the seven p^1/p^2 escapers that survived to weaning (see above and Table 5), only four died immediately postweaning (by day 32) and three lived for extended periods of time, namely 123, 215, and 365 days, respectively. Rare cases of similar long-term survival have been reported for $p^{4THO-II}/p^{4THO-II}$ (CULIAT *et al.* 1993) and for p^{cp}/p^{cp} (PHILLIPS 1973).

Survival patterns of compound heterozygotes: The survival patterns of various types of combinations are summarized in Table 6. The numbers found either alive or dead at the time of the first observation (days 0–6), at weaning, and on day 237 (see above) are based on actual counts. Because dead offspring are often rapidly devoured by their mothers, an estimate had to be made for the number of p^1/p^2s born in the case of combinations that are not regularly prenatally lethal. This base number was calculated from the nonpink-eyed animals (alive plus dead) counted at the time of first observation (days 0–6), making the assumptions that the likelihood of survival to birth is equal for all segregants, and that disappearance of nonpink-eyed segregants between birth and first observation is negligible for the purpose

TABLE 6

Nonprenatally-lethal p^1/p^2 conceptions surviving to various ages

	p^1/p^2				+/?
Phenotype of combination	v	jlm	jls	nl	All
Estimated base number[a]	894	1799	1213	877	14,507
Surviving proportion (%)[b]					
Past birth, minimum[c]	99.0	78.2	69.2	26.1	100.0[f]
To first observation[d]	96.0	76.9	50.2	3.3	95.4
To weaning	92.1	61.0	27.7	0.8	91.6
To day 237[e]	86.1	32.4	4.1	0.1	87.1

[a] Number expected at birth if all segregants have equivalent probabilities of prenatal survival. On the assumption that p^1/p^2s constitute 25% of all conceptuses, the base number is calculated as one-third of +/ offspring within same type of combination at first observation (see Table 4). Death of non-p^1/p^2 offspring between birth and first observation is assumed to be negligible and is set at zero for purposes of deriving survival patterns.

[b] Percentage of base number.

[c] Based on total number of p^1/p^2 (alive and dead) classified at time of first observation (days 0–6).

[d] Based on number of p^1/p^2 alive at time of first observation (days 0–6).

[e] Calculated from results shown in Figure 3 (see text).

[f] Because the base number for p^1/p^2 combinations is estimated relative to the number of +/? classified (see footnote *a*), the percentage of +/? surviving past birth must here be taken arbitrarily as 100%.

of deriving the overall survival patterns. Assuming p^1/p^2s constitute 25% of all conceptuses, the base number of p^1/p^2 animals was calculated as one-third of the number of nonpink-eyed mice (alive plus dead) observed within the same class of combinations.

Survival "past birth" is calculated from dead plus living p^1/p^2 animals counted at first observation and, because of cannibalism, obviously represents a minimum. On the other hand, "survival to first observation" is based on only living p^1/p^2s. The p^1/p^2 deficits calculable from these two classifications therefore represent offspring dying very early and somewhat later, respectively, during the birth-to-first-observation interval. The first one of the deficits could theoretically also include deaths occurring before birth; however, uterine dissection results for several of the combinations (see above), as well as results reported by CULIAT *et al.* (1993, 1994), indicate that the great bulk of the deficits are probably due to death after birth.

The distribution of deaths is shown graphically in Figure 4 for the different classes of combinations. The pattern for viable (v) p^1/p^2 combinations is virtually indistinguishable from that for the nonpink-eyed segregants from all combinations. The pattern for each of the other classes obviously differs from the wild-type pattern, and the various classes (jlm, jls, and nl) clearly differ amongst themselves.

Weaning weights: Weaning-weight comparisons were made only among like-sexed littermates. Further, to avoid any bias that might result from the major interlitter variation in weaning weights, equal numbers of entries for pink-eyed (p^1/p^2) and nonpink-eyed (+/?) offspring were made for each litter (+/? designates mice that might be +/+ or +/p*). For example, if a litter included one p^1/p^2 and three +/? males, as well as one p^1/p^2 female, the weight of the p^1/p^2 male was matched with the average weight of the three +/? males, and the weight of the p^1/p^2 female, having no like-sexed match, was ignored. For the sum of the entries for combinations involving a specific mutation, the ratio of pink-eyed to nonpink-eyed weaning weight was then computed separately for each sex. The results are shown in Table 7.

In v combinations, p^1/p^2s weigh roughly the same as do +/? littermates. It is noteworthy, however, that p^1/p^2 weaning weights were lower in the $p^{4THO\text{-}II}/+ \times p^{46DFiOD}/+$ cross than in other crosses involving $p^{4THO\text{-}II}/+$. Offspring of this cross also had somewhat reduced long-term survival (see Table 5, footnote *e*), although their preweaning survival was typical of the class. There is considerable consistency among combinations within the jlm and within the jls classes, and no overlap between these two classes. Because of very poor survival, few weaning-weight matches were available for the nl class; whereas weight reduction appeared greater than for the jls class, it was not dramatically so. Overall, it is of interest that, in 13 of the 14 comparisons for various groups, female p^1/p^2s weighed slightly more than did males, relative to like-sexed +/? littermates.

DISCUSSION

The homozygous and heterozygous phenotypes are described and characterized for 45 new *p*-locus mutations and for 810 compound heterozygotes generated in pairwise combinations of these mutations. Some of these combinations, involving 20 of the mutations, have already been utilized in molecular studies that, by use of deletion mapping, have localized several DNA sequences in the region surrounding the locus (CULIAT

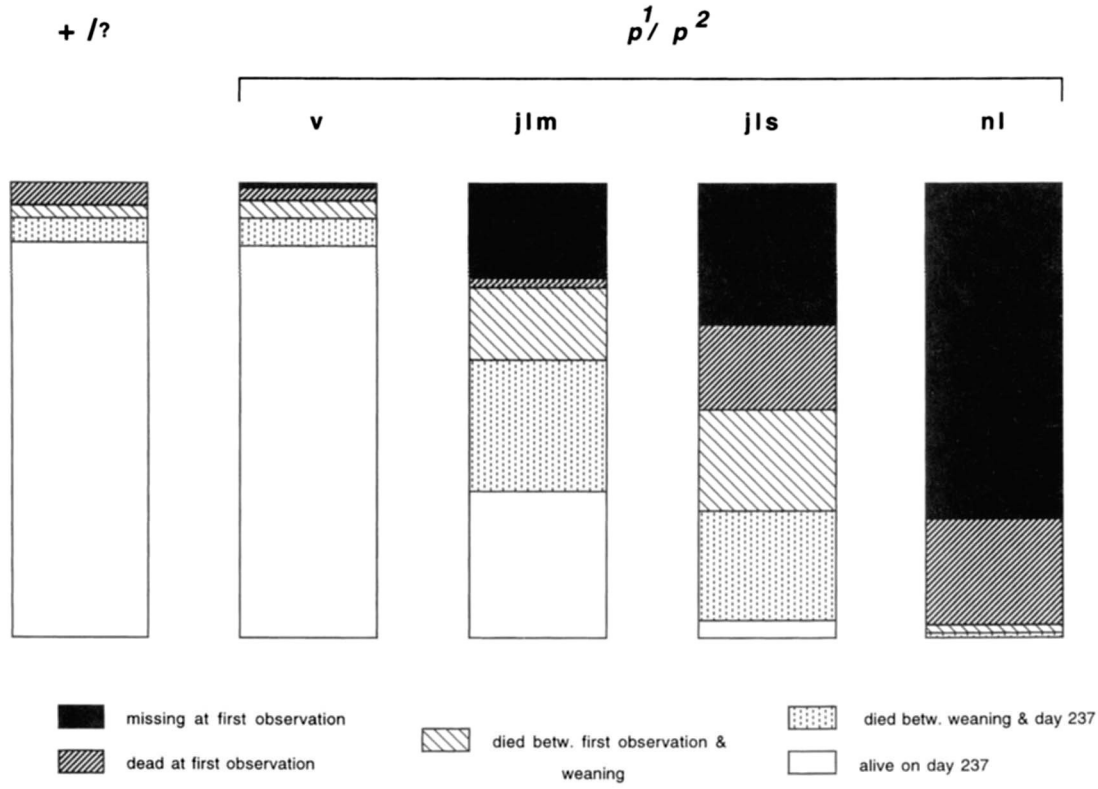


FIGURE 4.—Distribution of deaths for v, jlm, jls, and nl combinations of lethal *p*-locus mutations, and for their wild-type (+/p^* and +/+) littermates.

et al. 1993, 1994; NICHOLLS *et al.* 1993; RINCHIK *et al.* 1993b; JOHNSON *et al.* 1995).

The alleles characterized have considerably expanded each known phenotypic category of *p*-locus mutations, as well as defining new categories. Thus, 33 prenatally lethal mutations have been added to the three known previously (LYON *et al.* 1992). The two previously reported alleles that specify intermediate pigmentation, p^d and p^{bs}, are respectively viable and of "reduced" viability (LYON *et al.* 1992). Five new intermediate-pigmentation (generically, p^x) alleles are described here, two of which (p^{48PB} and $p^{17FATwb}$) are prenatal lethals, and three of which (p^{12DTR}, $p^{12R250M}$, p^{103G}) are "mild juvenile lethals." These new alleles also specify an array of eye pigmentations at birth, ranging from unpigmented ($p^{12R250M}/p$, p^{48PB}/p) through less-than-fully pigmented (p^{17FATw}/p, p^{12DTR}/p) to apparently fully pigmented (p^{103G}/p).

A new class of *p*-locus alleles (generically, p^m) is represented by two mutations (p^{18CoS} and p^{39DSD}) that specify a mottled phenotype (RUSSELL 1964, 1965). Rare wild-type exceptions that have been observed in $p^m/p \times p^m/p$ crosses (see RESULTS) might represent reverse mutations (*e.g.*, resulting from recombinational excision of a retrotransposon) or recombination between $+^p$ and a "mottling" allele at a closely linked locus. If the latter were the explanation, this factor must be proximal to *p*, as indicated by linked markers that were present in one of the crosses. Complementation mapping (see below) also localizes the mild-juvenile-lethal function associated with the p^m mutations proximal to *p*. Because p^m alleles, in combination with known deletions of *p* and flanking regions, produce a mottled (rather than *p/p*-like) phenotype, it is unlikely that these mutations represent deletions of *p* coding sequences, although they could be deletions of *p* regulatory sequences 5′ and proximal to *p* coding sequences. The p^m alleles could also be result of inserted sequences (IAP, VL30, etc.) (BULTMAN *et al.* 1994; MICHAUD *et al.* 1994), although their radiation origin renders this hypothesis less likely.

Cytogenetic analyses performed on three of the p^{pl} mutations, a deletion, an inversion and a translocation, provide consistent evidence that *p* is located in band 7C. Because the prenatally lethal inversion p^{2DFiOD} fully complements all other lethal mutations involving the *p* locus, we concluded that the p^{2DFiOD} lethality, which occurs before implantation, is associated with the other breakpoint. The latter appears to be located in 7E, within which lies the *c* locus (RUSSELL and CACHEIRO 1977). Tests currently under way indicate that the lethality associated with p^{2DFiOD} is not complemented by the *c*-locus deletion c^{26DVT} (RUSSELL *et al.* 1982).

Of the 45 new mutations analyzed, 44 were induced (all but two of these by radiation). The single spontaneous mutation, p^{48PB}, produces intermediate pigmentation but is prenatally lethal, *i.e.*, it is a pleiotropic mutation. Among 6005 offspring of $p^{48PB}/p \times p^{48PB}/p$ crosses, one was wild-type and one was mosaic for wild-type and

TABLE 7

Weaning weights of p^1/p^2 combinations

Phenotype of combination	Involving mutation	Males		Females		Both sexes	
		No. of $+/?\text{-}p^1/p^2$ matches	Weight ratio (%)[a]	No. of $+/?\text{-}p^1/p^2$ matches	Weight ratio (%)[a]	No. of $+/?\text{-}p^1/p^2$ matches	Weight ratio (%)[a]
v	*2DFiOD*	43	98.1	44	98.6		
	4THO-II[b]	48	95.7	43	94.9		
	All v		96.9		96.8	178	96.8
v?	*4THO-II* × *46DFiOD*	8	79.1	4	85.4	12	81.2
jlm	*18CoS*	24	56.9	20	62.8		
	39DSD	15	59.0	22	64.8		
	12DTR	30	59.0	35	61.7		
	12R250M	26	54.5	33	57.7		
	103G	38	56.8	43	61.2		
	All jlm		57.1		61.3	295	59.3
jls	*9DTW*	29	48.7	42	51.8		
	7FR60L$_b$	8	41.3	10	46.0		
	116G	27	45.5	32	47.2		
	39K	27	46.4	31	49.6		
	All jls		46.4		49.4	215	48.0
nl	*4THO-II*	3	37.8	4	43.8	7	41.0

[a] Ratio of average p^1/p^2 to average matched +/ weights (expressed as %); see text. Combination values (*i.e.*, all v, all jlm, all jls) are weighted averages.
[b] Except *4THO-II* × *46DFiOD*.

p^{48PB}/p, possibly indicating that this mutation may not be a simple deletion.

We had shown in earlier analyses of mutations involving the *c, d,* and/or *se* loci that larger lesions were more frequently induced in oocytes and postspermatogonial stages than in stem-cell spermatogonia (RUSSELL *et al.* 1991). A similar relation is found for the 45 *p* lethals in this study; 38.5% of the prenatal and neonatal lethals, but only 16.7% of the juvenile lethals, were induced in oocytes and postspermatogonial stages ($P < 0.05$ in a chi-square test with Yates correction). The difference becomes considerably greater when the large number of nonlethal induced *p*-locus mutations (which are not addressed in the present paper) are included in the comparison. While no exhaustive tabulation has yet been made, a random sampling indicates that virtually all of these were induced in spermatogonial stem cells.

Complementation map: Only one of the mutations, $p^{46DFiOD}$, was found to delete standard loci in the region, namely, *Ldh1, Myod1,* and *ru2*. Whereas the orientation relative to the centromere has not yet been determined for six loci within the 500-kb region that encompasses *Ldh1* and *Myod1* (STUBBS *et al.* 1994), this region is probably proximal to *ru2* (JOHNSON 1990), which, in turn, is proximal to *p*. The remainder of the map (Figure 5) had to be based on the functional information here analyzed, as well as on data obtained with molecular probes (CULIAT *et al.* 1993, 1994; NICHOLLS *et al.* 1993; JOHNSON *et al.* 1995). Because $p^{4THO-II}$, a neonatally lethal mutation, fully complements (except for the pigment phenotype) a large number of prenatally lethal mutations, pl and nl functional units can be localized on opposite sides of *p*. The functional units pl-1 and nl must lie proximal and distal to *p*, respectively, because $p^{46DFiOD}$ is among the prenatally lethal mutations that are complemented by $p^{4THO-II}$. [The order centromere, pl, *p*, nl had also been deduced on the basis of a subset of the mutations here analyzed (NICHOLLS *et al.* 1993); however, LYON *et al.* (1992) were unable to orient their map with respect to the centromere.]

All of the various functional parameters studied provide consistent evidence for two types of juvenile lethality, mild and severe, designated jlm and jls, respectively. Because juvenile-lethal mutations p^{9DTW}, p^{12DTR}, $p^{12R250M}$, p^{103G}, p^{18CoS}, and p^{39DSD} fully complement $p^{4THO-II}$, jls and jlm functional units must lie proximal to *p*. Because combinations between juvenile-lethal and prenatally-lethal mutations are juvenile lethal, jls and jlm must lie between pl-1 and *p*, with jlm closer to *p* than jls. It is noteworthy that combinations involving either one of these units exhibit the neuromuscular phenotype described for the homozygotes (twitching of the hindlimbs and severe tremor of forelimbs when animals are suspended by the tail). On the basis of the evidence presented, this neuromuscular function is inseparable from the jlm function on the map. Recent findings on

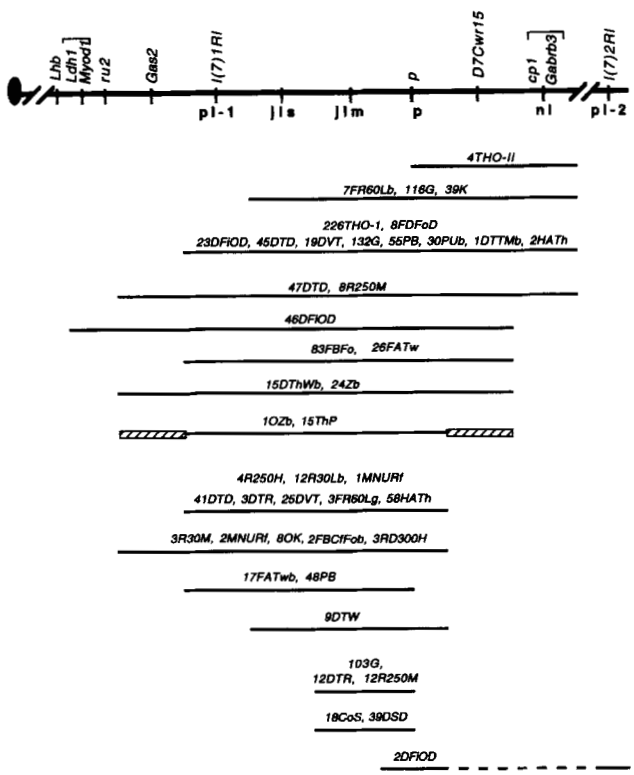


FIGURE 5.—Complementation map of the region surrounding the *p* locus, derived from intercrosses of lethal *p*-locus mutations. The centromere is at the left of the chromosome 7 segment shown in the top line. Selected genes and DNA sequences are shown above the line in italics, and functional units, deduced from the complementation tests, are below the line in bold type (see text and Figure 2 legend for symbols). Each functional unit could represent either a single gene or several genes. No correlation with any physical distance is implied by the spacing of symbols. Symbols for individual alleles are indicated by their superscripts above the lines that show the genetic extent of presumed deletions. Alleles shown above the same line could not be distinguished on the basis of complementation data, but several of these alleles are distinguishable on the basis of molecular data (see the companion paper, JOHNSON *et al.* 1995). A diagonally striped narrow box at the end of a line denotes uncertainty due to absence of information. The inversion p^{2DFiOD} is depicted with a broken line between the presumed rearrangement locations. The functions designated by jlm (mild juvenile lethality) include neuromuscular anomalies, and those designated by nl (neonatal lethality) include nervous behavior in the rare escapers. The functional unit p includes the standard pigment phenotype (resembling that of *p/p*) as well as the various intermediate pigment phenotypes associated with p^x or p^m alleles (see text). However the deletion lines for the p^x or p^m alleles are depicted as extending through only part of the p unit to represent our hypothesis that only regulatory sequences are affected by these mutations. Comapping of the *Gabrb3* locus and the nl functional unit is based on results of CULIAT *et al.* (1993, 1994) (in the case of p^{8FDFoD} and $p^{226THO-I}$, the extent of the deletion line shown in Figure 5 is based on functional data only; the allele p^{39K} was not tested with the *Gabrb3* probe).

ENU-induced point mutations mapping to this region indicate that the complex phenotype (which also includes male sterility) can result from deletion of a single highly pleiotropic gene (RINCHIK *et al.* 1995).

Because p^{2DFiOD}, a preimplantation lethal, fully complements all other *p*-locus lethals, the pl-2 unit is not associated with the *p*-locus region. This mutation has also been found not to be deleted for any *p*-region probes (JOHNSON *et al.* 1995). Our finding that p^{2DFiOD} is an inversion leads us to conclude that the lethal is associated with the distal breakpoint, and results of genetic tests strongly suggest that this breakpoint lies within the *c*-deletion complex. (On the other hand, p^{15ThP}, associated with a reciprocal *T(7;9)* translocation, does not complement other prenatally-lethal *p*-locus mutations, and there is therefore no *a priori* reason to assign the lethality to the chromosome *9* breakpoint.) Thus, despite the very large number of prenatally lethal mutations analyzed (33), only one prenatal-lethal factor, pl-1, has been identified within the *p* complex. Similarly, LYON *et al.* (1992) identified only one pl factor in complementation studies that involved three prenatally-lethal Harwell *p* mutations. It is, of course, possible that this factor could mask the existence of more than one gene needed for prenatal survival; tentatively, a locus associated with the pl-1 function has been designated *l(7)1Rl.* Time-of-death analysis of two of the mutations presumed to delete pl-1, including one that also deletes *Gas2 (growth-arrest-2)* (see below), indicates postimplantation lethality; therefore, there cannot be any preimplantation lethals between *Gas2* and *p.*

The map thus developed on the basis of complementation data and pseudodominance tests places and orients six functional units relative to several standard markers. Two of the functional units (pl-1 and pl-2) identify factors necessary for prenatal survival (postimplantation and preimplantation, respectively), one (nl) for neonatal survival, and two (jlm and jls) for juvenile (*i.e.*, young-adult) survival. The gene associated with pl-2 is tentatively designated *l(7)2Rl* and may correspond to the preimplantation-survival function earlier identified within the *c*-deletion complex (RUSSELL *et al.* 1982).

Independent studies, confirmed by the limited uterine-dissection results for nl compound heterozygotes reported in this paper, have shown that the failure to survive at birth is associated with cleft palate, and that deletion of the $\beta 3$ subunit of the type-A γ-aminobutyric-acid neurotransmitter receptor probably leads to abnormal development of the palatal shelves (CULIAT *et al.* 1993, 1994). The nl function may therefore be equated with the *cleft-palate-1* (*cp1*) gene, which has been localized to an interval bounded distally by the *Gabrb3* locus and proximally by the distal breakpoint of p^{83FBFo} (which is distal to *Gabra5*) (CULIAT *et al.* 1994). The $p^{4THO-II}$ mutation is somewhat leaky, and it is of interest that the rare homozygotes that escape the cleft-palate phenotype exhibit nervous behavior. Because complementing combinations involving $p^{4THO-II}$ are neuromuscularly normal (at least on behavioral observation), the jlm-associated neuromuscular function is apparently totally distinct from the "nervousness" exhibited by $p^{4THO-II}/$

$p^{4THO-II}$ escapers. A similar conclusion was drawn by LYON *et al.* (1992) for the distinctness of the nervousness associated with p^{cp} on the one hand and p^{bs} and p^{25H} on the other. Future detailed neurological studies may uncover fundamental differences between these phenotypes. Complementing compound heterozygotes that are homozygously deleted for the *Gabrg3* and/or *Gabra5* loci (γ3 and α5 subunits, respectively, of the type-A γ-aminobutyric-acid neurotransmitter receptor), which are located between *p* and the *Gabrb3* locus (NAKATSU *et al.* 1993), do not exhibit any overt neurological disturbances or other obvious phenotypes (CULIAT *et al.* 1994).

The extensive data obtained for the various compound heterozygotes that delete the nl unit provide a survival profile for animals that escape neonatal death. Overall, ~3% were found to survive to weaning, at which time their weight is ~40% that of like-sexed littermates. Surprisingly, one of these escapers survived to at least 365 days, and another lived ~7 months. For $p^{4THO-II}$ homozygotes, CULIAT *et al.* (1993) had also noted survival of two animals to at least 9 months. nl-deleting combinations that involved $p^{4THO-II}$ exhibited a consistently better survival at each preweaning stage than did those involving $p^{7FR60Lb}$, p^{116G}, or p^{39K}. This difference could perhaps be due to an additive effect of deletion of jl units in the latter group of combinations.

The complete complementation with regard to short- or long-term survival and body size between $p^{4THO-II}$ and a large number of deletions that do not involve nl indicates that no genes affecting overt fitness are located within the interval between *p* and the distal breakpoint of p^{83FBF0}. This interval includes the *Gabrg3* and *Gabra5* loci, which specify two subunits of the $GABA_A$ receptor (CULIAT *et al.* 1994), as well as the *D7Cwr15* sequence (JOHNSON 1990; JOHNSON *et al.* 1995).

Heterozygosity for the null condition at the *p* gene, represented by numerous p^{pl}/p and p^{nl}/p combinations, produces a pigmentation phenotype superficially resembling that of mice homozygous for the original *p* allele, *p/p*. Because $p^{4THO-II}$ breaks within *p*, rather than deleting it completely (NICHOLLS *et al.* 1993), complete ablation of the *p* locus is not achieved by any combination of mutations studied here that fully complement for viability detriments. Such ablation is, however, achieved by several combinations that complement for prenatal and neonatal mortality (*e.g.*, $p^{7FR60Lb}/p^{25DVT}$, $p^{7FR60Lb}/p^{3DTR}$ and others). Whereas, superficially, such animals resemble *p/p*, independent evidence (RUSSELL 1983) indicates that *p* may not be a null but a hypomorph.

Because the heterozygous or homozygous null condition at *p* (p^{del}/p or p^{del}/p^{del}) specifies a pigment phenotype at least as light as that of *p/p*, mutations that produce a darker or mottled pigmentation (generically, p^xs and p^ms, respectively) may be presumed not to have deleted the coding regions of the *p* locus. The p^x-type mutations, including the previously described p^d and p^{bs}, (LYON *et al.* 1992) are of a variety of phenotypes with regard to time and degree of pigment development. All of the p^x-type mutations (except p^d), as well as the two p^ms, also produce effects on fitness (survival, size, neuromuscular well-being) or are prenatally lethal. They can all be fitted into a linear map extending from somewhere within (or upstream from) the *p* locus proximally through jlm (p^{103G}, p^{12DTR}, $p^{12R250M}$, p^{18CoS}, p^{39DSD}) or through jlm, jls, and pl-1 ($p^{17FATwb}$ and p^{48PB}). This functional linearity does not, however, prove that these mutations are simple deletions. In fact, the finding of rare "revertants" within the 48PB stock, as well as in intercrosses among p^m heterozygotes (p^{18CoS}/p, p^{39DSD}/p), may indicate the existence of other types of small rearrangements, or mutations in closely linked regulatory sequences, like enhancers or large promoters. A p^x or p^m phenotype is evidently not a prerequisite for a juvenile-lethal *p*-locus mutation, as shown by p^{9DTW} (Table 2 and Figure 5), and by p^{6H} and p^{25H} (LYON *et al.* 1992).

Until complementation maps are anchored by molecular information, the postulated positions of functional units are dictated only by the objective of finding an order that yields the smallest number of deviations from linear fit; thus, alternative configurations are always theoretically possible. It is, therefore, gratifying that the considerable molecular information that has recently become available for the region surrounding the *p* locus is consistent with the order developed on the basis of the functional evidence. This information (NICHOLLS *et al.* 1993; CULIAT *et al.* 1994; JOHNSON *et al.* 1995) further permits refinement of the map to include both the functional and structural information (Figure 5).

Deletion mapping with a *MYOD1* cDNA clone indicated that the *Myod1* locus resides within the limits of the $p^{46DFiOD}$ deletion (SCRABLE *et al.* 1990). It had been shown earlier (MENDEL 1987) that $p^{46DFiOD}$ does not delete *Lhb*. Therefore, the proximal margin of the *p*-deletion complex may be placed between *Lhb* and the 500-kb region encompassing *Ldh1* and *Myod1* (STUBBS *et al.* 1994). The distal margin of the deletion complex is not marked by any standard loci. It should be noted, however, that of 16 deletions extending through the nl functional unit, seven were not tested with *twt* and two only partially so (the position of *twt*, like that of *p*, is currently assigned to 28 in chromosome 7, but with considerably less certainty, *i.e.*, integrated to within ~5 cM, rather than ~1 cM) (BRILLIANT *et al.* 1994). None of 12 *p*-locus mutations that extend distally through *Gabrb3*, however, deletes *Znf127 (D15S9h-1)* or *Snrpn* sequences (NICHOLLS *et al.* 1993; JOHNSON *et al.* 1995), indicating that the distal margin of the complex lies proximal to these DNA markers, which themselves are only ~1 cM distal to *p*.

Deletion mapping with the mouse microdissection clone *D7Cwr15* was carried out for 38 *p*-locus mutations (JOHNSON 1990; JOHNSON *et al.* 1995), and this clone

was found to be deleted in 19 of these; many, but not all of these extend to or through the nl functional unit. This indicates that *D7Cwr15* is located between *p* and nl. Additional data, obtained with *Gabrg3, Gabra5,* and *Gabrb3* probes (CULIAT *et al.* 1993, 1994; JOHNSON *et al.* 1995), indicate that *D7Cwr15* lies between *Gabrg3* and *Gabra5*. Because results for *Gabrg3* and *Gabra5* are described in the accompanying paper (JOHNSON *et al.* 1995), they are not depicted in Figure 5.

Tests with a probe for *Gas2 (growth arrest 2)* were carried out for almost all of the *p*-locus mutations (JOHNSON *et al.* 1995), and the results are included in Figure 5 because they are informative with regard to the placement of pl-1 and the conclusion that there is no preimplantation lethal between *Gas2* and *p*.

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