Molecular Genetics of the *Brown* (*b*)-Locus Region of Mouse Chromosome *4*. II. Complementation Analyses of Lethal *Brown* Deletions

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ABSTRACT

Numerous new mutations at the *brown* (*b*) locus in mouse chromosome *4* have been recovered over the years in germ-cell mutagenesis experiments performed at the Oak Ridge National Laboratory. A large series of radiation- and chemical-induced *b* mutations known to be chromosomal deletions, and also known to be prenatally lethal when homozygous, were analyzed by pairwise complementation crosses as well as by pseudodominance tests involving flanking loci defined by externally visible phenotypes. These crosses were designed to determine the extent of each deletion on the genetic and phenotype map of the chromosomal region surrounding the *b* locus; the crosses also provided basic data that assigned deletions to complementation groups and defined four new loci associated with aberrancies in normal development. Specifically, the pseudodominance tests identified deletions that include the proximally mapping *whirler* (*wi*) and the distally mapping *depilated* (*dep*) genes, thereby bracketing these loci defined by visible developmental abnormalities with landmarks (deletion breakpoints) that are easily identified on the physical map. Furthermore, the complementation crosses, which were supplemented with additional crosses that allowed determination of the gross time of lethality of selected deletions, defined four new loci required for normal development. Homozygous deletion of one of these loci (*b-associated fitness, baf*) results in a runting syndrome evident during postnatal development; deletion of one locus [*l(4)2Rn*] causes death in the late gestation/neonatal period; and deletion of either of two loci [*l(4)1Rn* or *l(4)3Rn*] results in embryonic death, most likely in pre-, peri- or postimplantation stages. The placement of these new functionally defined loci on the evolving molecular map of the *b* region should be useful for continuing the analysis of the roles played in development by genes in this segment of chromosome *4*.

MUTATIONS at the *brown* (*b*) locus in mouse chromosome *4* result in the synthesis of brown, rather than black, pigment within melanocytes (reviewed in SILVERS 1979). Specific-locus mutation-rate experiments performed at the Oak Ridge National Laboratory and elsewhere have generated many agent-induced mutations at the *b* locus, and many of the mutations induced at Oak Ridge have been preserved in breeding stocks. Among this set of radiation- and chemical-induced *b* mutations are those that are lethal in homozygotes. These lethal mutations are chromosomal deletions of varying length that encompass at least a portion, if not all, of the *b* (*Tyrp1*) locus itself (RINCHIK *et al.* 1994).

Combining genetic and molecular analyses of regions of the mouse genome that are associated with radiation- or chemical-induced deletion mutations at specific loci represents a powerful strategy for developing integrated physical and functional maps for megabase stretches of the mammalian genome (reviewed in RINCHIK and RUSSELL 1990). Such studies on the *dilute* (*d*) region of chromosome *9* (*e.g.*, RUSSELL 1971; RINCHIK *et al.* 1986; JENKINS *et al.* 1989; KINGSLEY *et al.* 1990, 1992; RINCHIK and RUSSELL 1990), the *albino* (*c*) region in chromosome *7* (*e.g.*, GLUECKSOHN-WAELSCH 1979; RUSSELL *et al.* 1982; NISWANDER *et al.* 1989; RINCHIK *et al.* 1990b; RINCHIK and RUSSELL 1990; SHARAN *et al.* 1991; KELSEY *et al.* 1992; KLEBIG *et al.* 1992a,b; RINCHIK *et al.* 1993a,b) and the *pink-eyed dilution* (*p*) region, also in chromosome *7* (*e.g.*, RINCHIK and RUSSELL 1990; LYON *et al.* 1992; GARDNER *et al.* 1992; RINCHIK *et al.* 1993c; NICHOLLS *et al.* 1993; CULIAT *et al.* 1993), have contributed to the dissection of the physical and functional makeup of these genomic regions of substantial size. Similar combined genetic and molecular analyses of lethal *b*-region deletions should likewise result in the generation of a regional map that will provide a framework for the further in-depth genomic characterization of this segment of the mouse chromosome *4*.

This report summarizes the results of genetic analyses of a large collection of lethal *brown* deletions. The analyses included a series of pseudodominance tests designed to determine whether lethal *brown* deletions (b^l) cover phentoypically defined loci closely linked to the *b* locus. This study also included a large number of pairwise crosses between individual b^l deletions to identify *b*-region loci whose deficiency results in lethality. Combining the results of these genetic analyses

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with molecular mapping studies presented in a companion study (RINCHIK *et al.* 1994) has resulted in the definition of four new phenotype-defined loci within a refined molecular/complementation map of the region surrounding the *b* locus.

MATERIALS AND METHODS

Mice: The origin and initial molecular characterization of radiation- and chemical-induced lethal *brown* (*b*) deletion mutations maintained at the Oak Ridge National Laboratory is described in a companion study (RINCHIK *et al.* 1994). The majority of lethal *b* deletions [*Df(b)*^{l}, hereafter designated b^l] were typically maintained by crossing $+/b^l$ mice to +/+ mice, with a testcross in each generation of the resultant $+/b^l$ and +/+ progeny to *b/b* mice (typically from the closed colony stock 24A) to assure the continued presence of b^l in the stock. A few b^l mutations were maintained by intercrossing B^w/b^l heterozygotes. Mice homozygous for the B^w mutation [*White-based brown* (HUNSICKER 1969; JACKSON *et al.* 1990)] are lighter in color than are B^w/b^l segregants, which allows easy maintenance of b^l mutations. The *whirler* (*wi*) mutation (LANE 1963) was maintained by crosses of *wi b/+ b* females to *wi b/wi b* males in a stock called WI. Breeding pairs of the C3H/HeJ inbred strain and a B6C3 stock segregating the *depilated* (*dep*) mutation (MAYER *et al.* 1976) were purchased from The Jackson Laboratory, Bar Harbor, Maine.

Complementation tests: Pseudodominance tests designed to determine whether a given b^l deletion includes the closely linked *wi* and *dep* loci were carried out by crossing *wi b/wi b* males or *+dep/+dep* males to b/b^l females (where the "+" represents the wild-type allele at the *b* locus). At least 15 F_1 offspring of these crosses were inspected for the expression of the whirler or depilated phenotype; such expression would signify inclusion of the respective locus within a particular b^l deletion.

Trans complementation testing between individual b^l deletions was carried out by crossing mice heterozygous for independent b^l deletions ($+/b^{l-1} \times +/b^{l-2}$). The progeny from each of these crosses were examined for the presence of brown progeny (*i.e.*, b^{l-1}/b^{l-2}). The large numbers of $+/b^l$ segregants required for these crosses were obtained by crossing b/b^l mice to C3H/HeJ (+/+) mice. Appropriate $+/b^l$ segregants were identified in the progeny by Southern blot analysis as those animals not carrying a *b*-specific 5.2-kb *Taq*I fragment recognized by MT4.Pv.25, which is a 250-bp *Pvu*II probe isolated from the pMT4 *Tyrp1* cDNA clone (SHIBAHARA *et al.* 1986; JACKSON 1988; RINCHIK *et al.* 1994) (see Figure 1 and RESULTS). DNA preparation, blotting, and hybridization were carried out as described previously (RINCHIK *et al.* 1990a, 1994).

Assay for late fetal/neonatal death in selected b^l/b^l homozygotes: A gross estimate of the time of death experienced by embryos/fetuses homozygous for a number of b^l deletions was determined by intercrossing b/b^l males and females within stocks and sacrificing females at day 13.5–15.5 of gestation (E13.5–E15.5, where E0.5 is the morning of finding a vaginal plug). Uterine contents (live implants, dead but somewhat developed implants, resorption moles) were examined, and DNA was prepared from a portion of any recognizable fetus (*i.e.*, all uterine contents except for resorption moles). These DNAs were then digested with *Taq*I, electrophoresed, blotted to nylon, and hybridized with MT4.Pv.25 and, as a loading control, 34-1-111, a cDNA clone from the chromosome 7 locus *D15S9h-1* (now designated *Znf127*) (NICHOLLS *et al.* 1993).

TABLE 1

Deletion mapping of the *whirler* (*wi*) and *depilated* (*dep*) loci with lethal *brown* (b^l) deletions

	Cross			
	$b^l/b \times wi\ b/wi\ b$		$b^l/b \times +dep/+dep$	
b^l	Brown, whirler	Brown	Depilated	Normal
b^{11R30M}	19[a]	16	0[a]	27
b^{11PU}	0	19	26	21
b^{12PU}	0	21	13	26
b^{8PUb}	0	19	26	21
b^{3YPSh}	0	39	12	17
b^{46UThc}	0	28	9	19
b^{13R75M}	0	33	5	6
b^{4ACRg}	0	28	10	11
b^{5CHLe}	0	40	15	13
$b^{37FrThc}$	0[a]	19	19[a]	20
$b^{47DThWb}$	0	21	0	41
$b^{51DThWb}$	0	17	0	34
b^{26R60L}	0	20	0	33
b^{55CoS}	0	15	0	28
b^{331K}	0	41	0	34
$b^{1THO-IV}$	0	27	0	47
b^{33G}	0	15	0	25
b^{13DT}	0[a]	19	0[a]	27
b^{9PU}	0	46	0	31
b^{9R75VH}	0	21	0	33
b^{173G}	0	37	0	34
b^{37PUb}	0	46	0	36
b^{1OZ}	0	26	0	26
b^{3YPSc}	0	35	0	30
b^{37DTD}	0	24	0	26
b^{1DFiOD}	0	23	0	36
b^{49HATh}	0	30	0	32

If a given deletion includes the test locus (*i.e.*, *wi* or *dep*), approximately one-half of the progeny of these crosses should manifest the phenotype (assuming equal transmission of *b* and b^l). Under this assumption, a binomial distribution predicts that $P = 0.0002$ that 12 progeny will fail to show the *wi* or *dep* phenotype if the locus is deleted by the mutation. In all cases where pseudodominance was not observed, substantially more than 12 progeny were classified for each cross.

[a] Data for these mutations were previously reported in RINCHIK and co-workers (1991).

RESULTS

Genetic localization of the *whirler* (*wi*) and *depilated* (*dep*) loci within the *brown* (*b*) deletion complex: Complementation testing of radiation- and chemical-induced lethal *b* deletions (b^l) was initiated by testing whether particular b^l deletions include loci closely linked to *b* that are associated with previously identified visible phenotypes. Thus, $+\ b/b^l$ females from 27 different deletion stocks were crossed to *wi b/wi b* males, which were homozygous both for *b* and for the mutation *whirler* (*wi*), which maps 1.5–5 cM proximal to *b* (LANE 1963; DAVISSON *et al.* 1989). Table 1 shows that only the neutron-induced b^{11R30M} (RINCHIK *et al.* 1991) produced whirler progeny in these crosses, indicating that this deletion includes the *wi* locus and, therefore, extends at least 1.5–5 cM proximal to *b*.

A similar pseudodominance test was performed with the *depilated* (*dep*) gene, thought to map ~2 cM distal to *b* (MAYER *et al.* 1976). The mutation defining this

locus affects the skin epidermis and is associated with hair loss in juvenile animals (MAYER *et al.* 1976). $b+/b^{l}$ mice from the 27 different mutant lines were crossed to $+dep/+dep$ homozygotes. Nine of the 27 *b* deletions (b^{3YPSh}, b^{8PUb}, b^{11PU}, b^{12PU}, $b^{37FrThc}$, b^{13R75M}, b^{46UThc}, b^{4ACRg} and b^{5CHLe}) were found to include the *dep* locus since depilated mice were found among the progeny of these crosses (Table 1). Thus, nine *b* deletions extend at least 2 cM distal to *b*. It is significant that b^{11R30M} deletes *wi* but not *dep*, which confirms that the *b* gene does indeed map between the *wi* and *dep* loci.

Complementation testing for prenatal lethality: Each of the 27 deletions listed in Table 1 is lethal in the homozygous state (RINCHIK *et al.* 1994). Therefore, to initiate the process of placing recessive-lethal loci defined by these deletions onto the developing molecular and genetic maps, pairwise *trans* complementation crosses were performed for numerous combinations of deletions. The breeding strategy used for these crosses is shown in Figure 1. $+/b^{l}$ segregants carrying independently generated *b* deletions (*e.g.*, b^{l-1} and b^{l-2}) were identified in the progeny of the cross $b/b^{l} \times +/+$ (mating 1) by Southern blot analysis of tail biopsy DNA with the MT4.Pv.25 *b* locus cDNA clone. This probe detects 4.0- and 1.2-kb wild-type *Taq*I fragments and detects a 5.2-kb *b*-specific fragment (JACKSON 1988; RINCHIK *et al.* 1994). Thus, $+/b^{l}$ segregants (as opposed to $+/b$) could be identified as those offspring from the $b/b^{l} \times +/+$ cross that did not carry the 5.2-kb *b*-specific fragment. The Southern blot in Figure 2A shows, for example, the analysis of segregants from each of three $b/b^{l} \times +/+$ crosses. For b^{26R60L} and $b^{37FrThc}$, four $+/b^{l}$ and two $+/b$ segregants are present, whereas for $b^{47DThWb}$, there are five $+/b^{l}$ segregants *vs.* one $+/b$. In this way, hundreds of $+/b^{l}$ segregants carrying one of 25 lethal mutations listed in Table 1 were generated (b^{33G} and b^{9PU} were not included in the complementation analyses).

Appropriate $+/b^{l-1}$ and $+/b^{l-2}$ segregants were then mated, and at least 23 progeny of this cross were examined for the presence of brown (b^{l-1}/b^{l-2}) offspring (Figure 1, mating 2). [If one assumes equal transmission of the *b* and b^{l} alleles, a binomial distribution predicts that the probability (*P*) of these crosses producing no brown progeny by chance in 23 classified progeny is 0.0013. Nonetheless, more than 23 progeny were raised in the majority of combinations.] If no brown progeny were observed in these crosses, it was assumed that the corresponding deletions overlapped at (at least) one gene required for normal prenatal development (*i.e.*, they failed to complement for prenatal lethality). If brown progeny were produced in these crosses, the two deletions must not overlap at the same gene or genes required for normal prenatal development (*i.e.*, they complemented for prenatal lethality). In complementing combinations, DNA from brown progeny was analyzed by Southern blot analysis to test for total deletion

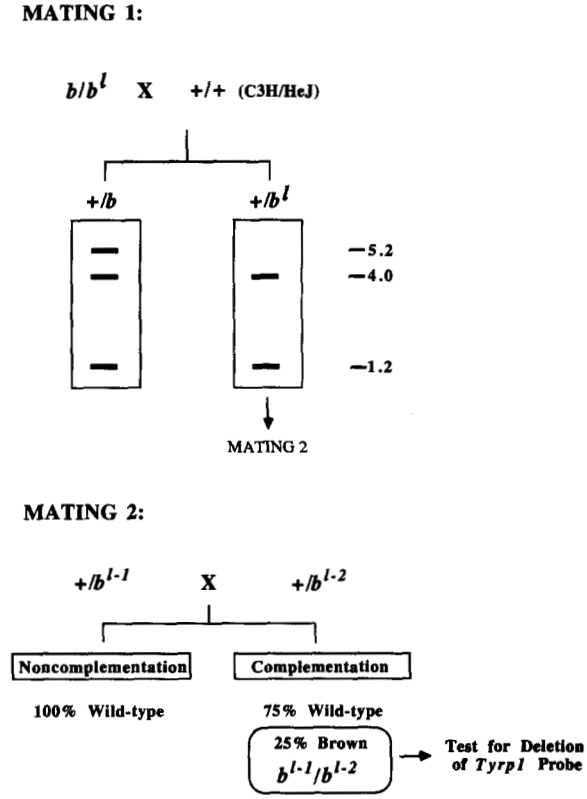

FIGURE 1.—Breeding strategy used in complementation analyses of lethal brown deletions. *Mating 1*, Southern blots of *Taq*I-digested DNAs prepared from progeny of the indicated cross are hybridized with a *Tyrp1* (*b*) cDNA clone. The fragment patterns shown in the two boxes represent the two possible outcomes of this cross: presence of the 5.2-kb *b*-associated *Taq*I fragment, which identifies $+/b$ segregants (left); and absence of the 5.2-kb fragment, which identifies $+/b^{l}$ segregants (right) (JACKSON 1988; RINCHIK *et al.* 1994). The 4.0- and 1.2-kb *Taq*I fragments are present in both classes. *Mating 2*, $+/b^{l}$ segregants identified in the progeny of mating 1 are crossed, and their progeny are examined for the presence of brown (b^{l-1}/b^{l-2}) offspring. "Noncomplementation" and "complementation" refer to whether deletions complement for prenatal lethality. DNA prepared from any brown progeny recovered in these crosses was subjected to Southern blot analysis with a *Tyrp1* probe to confirm homozygous deletion of the sequence recognized by the probe. The 3:1 ratio of wild-type *vs.* brown progeny in complementing combinations represents an idealized situation; often there was a deficit of brown progeny due to the variable expression of the baf phenotype (see text).

of *Tyrp1* sequences recognized by the MT4.Pv.25 probe. This analysis was included as a control for any accidental mistyping of $+/b$ parents as $+/b^{l}$. Figure 2B shows a representative analysis of a number of animals from combinations that did produce b^{l-1}/b^{l-2} brown offspring. For the five compound-heterozygous combinations shown in Figure 2B, no *Tyrp1* sequences could be detected. However, *Tyrp1* sequences were detected in the $+/b$ heterozygous and $+/b^{37DTD}$ hemizygous controls, and hybridization of the same blot with a probe from the chromosome 7 locus *Znf127* (*D15S9h-1*) confirmed that there was hybridizable DNA in each lane.

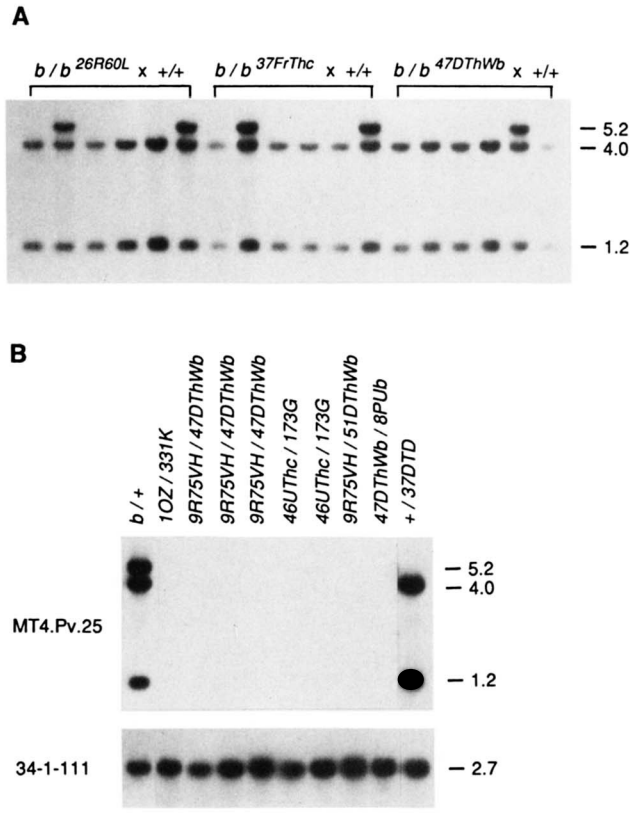

FIGURE 2.—Molecular analysis of segregants from the b^{l} complementation crosses. Tail DNAs from progeny either from the indicated crosses or of the indicated genotypes were digested with *Taq*I, electrophoresed, blotted to nylon, and hybridized with the *Tyrp1* cDNA subclone MT4.Pv.25. The 2.7-kb *Taq*I fragment recognized by the 34-1-111 chromosome 7 control probe after rehybridization of the blot shown in (B) demonstrates that there is hybridizable DNA in each lane.

Of a total of 300 possible combinations of lethal *b* deletions, 142 were tested in this series of complementation crosses (Figure 3). The majority of crosses (107) in the complementation grid did not produce brown offspring in 23 or more progeny (denoted by the black boxes in Figure 3), which indicated that these deletions overlap at at least one gene essential for completion of prenatal development. Thirty-five combinations (white boxes) did produce brown progeny, but these brown progeny were not completely wild type. In every case of complementation for prenatal lethality, the b^{l-1}/b^{l-2} brown offspring were, to variable degrees, runted and less fit than their non-brown littermates. This variable-fitness phenotype in juveniles ranged from a state of being extremely small and moribund to nearly wild type, and the variability was observed even among brown progeny from the same cross; therefore, the fitness problem was not restricted to specific alleles or to specific combinations of alleles. This phenotype of decreased fitness and viability will be discussed in more detail below.

Construction of a complementation map: The complementation data presented in Figure 3 provide evidence for two lethal factors, one proximal to *b*, designated *l(4)1Rn*, and one distal to *b*, designated *l(4)2Rn*. The inference of these two lethals, as well as the construction of an initial map, can be most easily accomplished by analyzing a subset of the complementation data (Table 2).

Two deletions, b^{1OZ} and b^{9R75VH}, fail to complement each other as well as any of the nine *Df(b dep)* mutations tested in the grid (b^{11PU}, b^{12PU}, b^{8PUb}, b^{3YPSh}, b^{46UThc}, b^{13R75M}, b^{4ACRg}, b^{5CHLe} and $b^{37FrThc}$). However, the b^{13DT} deletion, which is the only mutation that was tested with every other deletion in the grid, complements both b^{1OZ} and b^{9R75VH}, and also complements three of the *Df(b dep)* deletions (b^{8PUb}, b^{46UThc} and b^{4ACRg}). Therefore, b^{13DT} defines a proximal prenatal-lethal factor, *l(4)1Rn*, located between *b* and *wi*, whereas b^{1OZ} and b^{9R75VH} define a distal prenatal lethal, *l(4)2Rn*, located between *b* and *dep*. Of the 16 deletions tested in the grid that did not include *dep*, b^{37PUb} was the only one that included both the *l(4)1Rn* and *l(4)2Rn* loci.

The variable decrease in fitness observed in the brown progeny obtained from all crosses that complemented for prenatal lethality (*e.g.*, b^{13DT}/b^{1OZ} or b^{13DT}/b^{9R75VH}) suggests the presence of another locus, *brown-associated fitness* (*baf*), that is closely linked to *b* (*Tyrp1*). Because all brown progeny recovered from the complementation crosses exhibited the baf phenotype, it is not possible to order *baf* with respect to *b* from these data. In fact, because each of the 35 combinations that complemented for prenatal lethality also showed the baf phenotype in b^{l-1}/b^{l-2} offspring, it is probable that this variable decrease in fitness results from the deletion of a locus mapping very close to *b* (see DISCUSSION).

Table 2 and Figure 3 present additional data confirming these core complementation data. For example, three deletions (b^{26R60L}, b^{173G} and b^{3YPSc}) fail to complement b^{13DT}, none of them includes *wi* or *dep*, and they all behave like b^{13DT} in complementation crosses. Consequently, although these three deletions were not tested in pairwise crosses with all of the other deletions (as was the case for b^{13DT}), they can be placed into the same group as b^{13DT} (*i.e.*, *Df[l(4)1Rn b]*), and their overall complementation characteristics provide supporting data for the construction of an initial complementation map, which is shown in Figure 4. It is noteworthy that the outcome of each of the 142 crosses summarized in Figure 3 is consistent with the initial complementation map presented in Figure 4.

Evidence for at least two distal lethals contained within *Df(b dep)* deletions: : Using only the very gross phenotype of prenatal lethality [*i.e.*, whether any brown (b^{l-1}/b^{l-2}) progeny are obtained in crosses of $+/b^{l}$ heterozygotes], the complementation data presented in Figure 3 can provide evidence for only two genes associated with prenatal lethality [*i.e.*, *l(4)1Rn* and *l(4)2Rn*]. However, because *b* deletions can be large [*e.g.*, the 1–5-cM $Df(wi\ b)^{11R30M}$ deletion], it is possible,

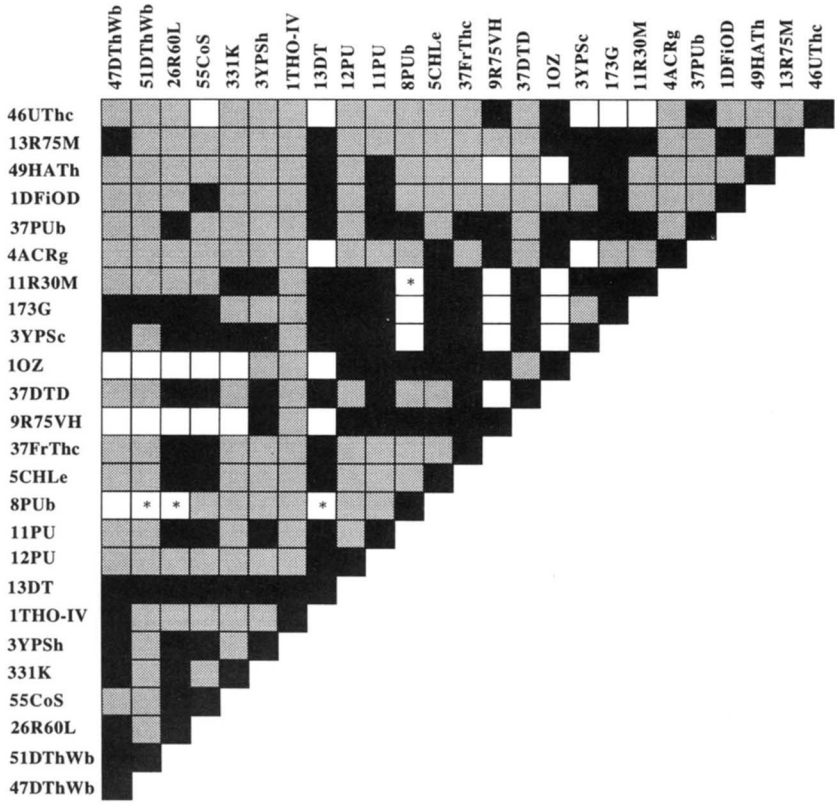

FIGURE 3.—Summary of the crosses performed for the b^{l} complementation test. Open boxes denote combinations that complement for prenatal lethality, in which brown (b^{l-1}/b^{l-2}) progeny could be recognized after birth. In each such case, a variable number of brown offspring were runted to different degrees. Black boxes denote combinations in which no brown offspring were observed in at least 23 progeny. The black boxes along the diagonal did not result from crosses performed in this study, but are a reflection of the prenatal lethality determined earlier to be associated with homozygosity for each of these mutations (RINCHIK *et al.* 1994). Stippled boxes denote crosses that were not made. The asterisks indicate complementing combinations in which extremely runted brown progeny were noted before weaning, but whose DNA was not tested for total deletion of the *Tyrp1* probe sequence. Data from reciprocal crosses were pooled for this summary; in the 15 combinations in which reciprocal crosses could be scored (*i.e.*, where there were sufficient progeny from each cross), there were no differences in outcome.

and even probable, that a number of genes required for normal prenatal development are deleted in any one mutation. Knowledge of the general complementation characteristics of the deletions (Figures 3 and 4) provides a basis for comparing the lethal phenotype in fetuses homozygous for deletions of different complementation groups. For example, comparing the gross time and/or cause of death in different deletion homozygotes within the currently defined complementation groups can aid in refining the estimate of the number of genes required for prenatal development that are absent in any one deletion. This type of analysis also allows the next step to be taken in investigating the nature of the lethal phenotype exhibited by embryos/fetuses homozygous for a particular deletion.

Consequently, a gross estimate of the gestational time at which embryos/fetuses homozygous for each of 13 deletions die was determined by assaying for the presence of deletion homozygotes at mid-gestation. At least 23 fetuses from the cross $b/b^{l} \times b/b^{l}$ were collected at E13.5–E15.5. DNAs prepared from these fetuses were digested with *Taq*I, blotted to nylon, and hybridized with the *Tyrp1* cDNA clone MT4.Pv.25 as well as with the 34-1-111 control probe (from chromosome 7). Figure 5 shows representative blots analyzing 10 fetal DNAs obtained from crosses involving the b^{1OZ}, b^{9R75VH} and b^{46UTHc} deletions. The 2.7-kb chromosome 7 control fragment is present in each DNA sample, but it is evident that segregants 1, 4, 6 and 7 from the b^{1OZ} intercross, segregants 1, 5 and 8 from the b^{9R75VH} crosses, and segregants 3, 5, 6 and 10 from the b^{46UThc} crosses are completely deleted for *Tyrp1* sequences (the 5.2-kb *b*-associated fragment in each case). Thus, b^{1OZ}/b^{1OZ}, b^{9R75VH}/b^{9R75VH} and b^{46UThc}/b^{46UThc} fetuses are present and are externally normal at mid-gestation (*i.e.*, at E14.5), and these fetuses must die sometime between E14.5 and shortly after birth.

Table 3 summarizes the results obtained from all 13 crosses. Homozygous deletion of *l(4)1Rn* results in early gestation death, since fetuses homozygous for b^{13DT}, b^{3YPSc} and b^{26R60L} cannot be found at E13.5–E15.5. However, as discussed above, it is evident that the lethal period for three mutations, b^{1OZ}, b^{9R75VH} and b^{46UThc} is later in gestation than is the lethal period for the other 10 mutations. Of particular interest are the results from the b^{46UThc} crosses. Because b^{46UThc} also deletes the *dep* locus, there cannot be any gene between *b* and *dep* that is required for early gestational development. This result therefore defines *l(4)2Rn* as a gene whose deletion results in late gestation/neonatal death. Moreover, the earlier gestational death of embryos homozygous for the b^{4ACRg} and b^{8PUb} deletions provides evidence for a third lethal, *l(4)3Rn*, whose deletion kills the fetus before midgestation. *l(4)3Rn* must map distal to *dep* because the b^{4ACRg} and b^{8PUb} deletions both complement the prenatal lethality of b^{13DT} and other b^{13DT}-like deletions. Thus, the earlier lethality exhibited by embryos homozygous for b^{4ACRg} and b^{8PUb} cannot be due to the deletion of the *l(4)1Rn* locus. On the other hand, since deletion of *l(4)1Rn* is also associated with early gestation lethal-

TABLE 2

A subset of complementation crosses among lethal *brown* (b^l) deletions

b^{l-1}	b^{l-2}					
	b^{13DT}	b^{3YPSc}	b^{173G}	b^{26R60L}	b^{1OZ}	b^{9R75VH}
b^{11R30M}	0/33	0/37	0/37	0/19[a]	26/78	6/41
b^{11PU}	0/33	0/87	0/47	0/28	0/61	0/54
b^{12PU}	0/37	0/60	0/24	ND	0/31	0/34
b^{8PUb}	1/33	2/13	3/10	3/35	0/52	0/25
b^{3YPSh}	0/35	0/43	ND	0/47	0/20	0/27
b^{46UThc}	7/43	2/23	6/14	ND	0/30	0/42
b^{13R75M}	0/61	0/40	0/52	ND	0/26	0/18[a]
b^{4ACRg}	6/32	1/9	ND	ND	0/24	0/41
b^{5CHLe}	0/40	0/48	0/53	0/30	0/31	0/44
$b^{37FrThc}$	0/57	0/66	0/35	0/50	0/30	0/38
$b^{47DThWb}$	0/45	0/41	0/118	0/50	2/11	7/38
$b^{51DThWb}$	0/39	ND	0/30	ND	6/15	2/14
b^{26R60L}	0/50	0/37	0/56	ND	7/47	11/46
b^{55CoS}	0/37	0/31	0/47	0/42	4/25	1/17
b^{331K}	0/40	0/33	ND	0/40	7/42	8/33
$b^{1THO-IV}$	0/30	ND	ND	ND	ND	ND
b^{13DT}	0/61	0/30	0/24	0/50	12/58	18/58
b^{9R75VH}	18/58	2/30	2/18	11/46	0/33	0/24
b^{173G}	0/24	ND	ND	0/56	7/26	2/18
b^{37PUb}	0/25	0/33	0/43	0/75	0/37	0/60
b^{1OZ}	12/58	5/33	7/26	7/47	ND	0/33
b^{3YPSc}	0/30	ND	ND	0/37	5/33	2/8
b^{37DTD}	0/22[a]	0/57	0/35	0/47	9/33	9/29
b^{1DFiOD}	0/34	ND	0/37	ND	ND	ND
b^{49HATh}	0/51	0/37	0/34	ND	6/19	6/30

Shown are the total number of brown progeny (b^{l-1}/b^{l-2})/total number of progeny classified from the cross $+/b^{l-1} \times +/b^{l-2}$. In every case of complementation for prenatal lethality (shown by the underline), the brown progeny were less fit than their littermates and expressed the baf (*b*-associated fitness) phenotype to variable degrees (see text). DNA from at least one (and usually more) of the brown progeny from complementing combinations was analyzed by southern blotting with a *Tyrp1* (*b*) cDNA probe (MT4.Pv.25) to confirm that the *b* locus was totally deleted. A binomial distribution (assuming Mendelian transmission of both the wild-type and mutant alleles) predicts that crosses producing no browns in 23 progeny are suggestive of prenatal lethality (and thus non-complementation) at the $P = 0.0013$ level; nonetheless, more than 23 progeny were produced in the majority of noncomplementing combinations. Two deletions listed in Table 1 (b^{33G} and b^{9PU}) were not analyzed in the complementation crosses. ND, not done.

[a] Noncomplementing ($P \leq 0.005$).

ity, it was not possible to determine whether the two other *Df(b dep)* deletions (b^{5CHLe} or b^{13R75M}), also analyzed in the experiments summarized in Table 3, include the early gestation lethal *l(4)3Rn* because these deletions fail to complement the *l(4)1Rn* phenotype (Figures 3 and 4).

Since the object of these particular crosses was to determine whether there was any late gestation lethality (which could be shown statistically with only 23 fetuses), complete information is not yet available about whether the lethal period for embryos homozygous for the nine deletions that include *l(4)1Rn* and/or *l(4)3Rn* is during preimplantation, peri-implantation, or postimplantation stages. A more exact determination of the time of death for embryos homozygous for these nine mutations would require the analysis of a larger number of fetuses, resorption moles, and corpora lutea from each cross.

Integration of the complementation and molecular maps: Figure 6 presents a composite map of the *wi–Ifa* region of mouse chromosome *4* that combines molecular mapping data (RINCHIK *et al* 1994) with the complementation data reported here. The *D4Rck52* locus, defined by a chromosome-microdissection clone, is currently genetically inseparable from the *l(4)1Rn* early-gestation lethal–each of the 19 mutations that fail to complement the *l(4)1Rn* lethality also deletes the *D4Rck52* locus. The *D4Rck140* locus, identified by another microclone, has been placed proximal to the *l(4)2Rn* locus because of the existence of mutations (*e.g.*, b^{3YPSc}, b^{55CoS}, b^{26R60L}, b^{49HATh} and b^{1DFiOD}) that delete *D4Rck140* but are able to complement the *l(4)2Rn* late gestational lethality. Because no b^l deletion tested to date includes the *Ifa* locus (RINCHIK *et al.* 1994), the *dep* locus must map between *D4Rck140* and *Ifa* on the molecular map, and the *l(4)3Rn* early gestation lethal must map between *dep* and *Ifa* at the distal end of the *b* deletion complex.

The b^{4ACRg} mutation, found in this study to affect the *b*, *baf*, *l(4)2Rn*, *dep* and *l(4)3Rn* loci, had not been included in the molecular analyses presented in a companion study (RINCHIK *et al.* 1994). Nonetheless, we were able to determine that b^{4ACRg} is deleted for the *D4Rck140* locus, but not the *D4Rck4* and *D4Rck52* loci, by hybridizing Southern blots of *Taq*I digests of DNA prepared from b^{3YPSc}/b^{4ACRg} mice obtained from the complementation crosses (Figure 3) to the relevant probes. b^{3YPSc} is deleted for each of these probes (RINCHIK *et al.* 1994), but DNA from the b^{3YPSc}/b^{4ACRg} compound

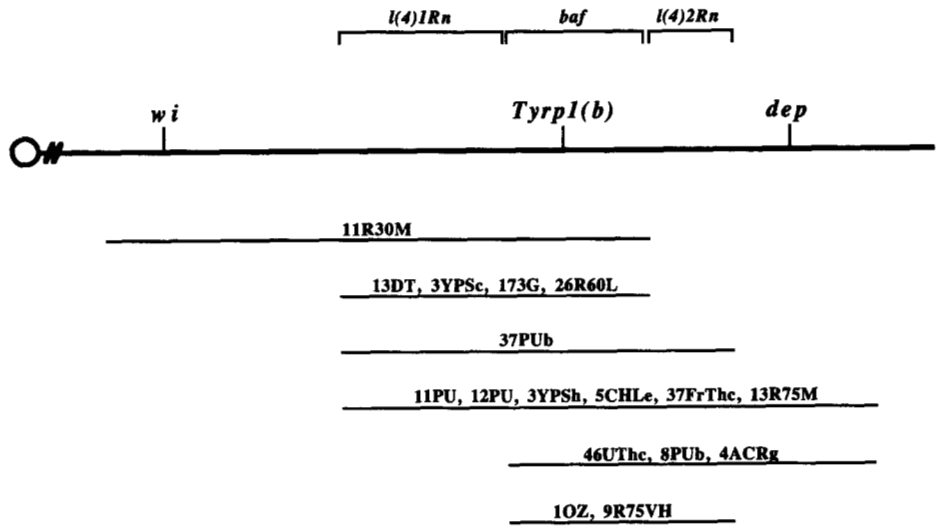

FIGURE 4.—A simple complementation map of the *b* region. Loci defined by complementation analyses are indicated above the brackets and are defined in the text. Other loci include: *whirler* (*wi*); *tyrosinase-related protein-1* (*brown*) [*Tyrp1* (*b*)]; and *depilated* (*dep*). The solid lines below the map represent the genetic extent of individual deletions, and no correlation with physical distance is implied. Deletions indicated on the same line cannot be discriminated solely from the data presented in Table 2 and Figure 3. The $b^{47DThWb}$, $b^{51DThWb}$, b^{1DFiOD}, b^{37DTD}, b^{49HATh}, b^{55CoS} and b^{331K} deletions (Figure 3) are in the same complementation group as are the b^{13DT}, b^{3YPSc}, b^{173G} and b^{26R60L} deletions but have been omitted from this map for clarity. The centromere is indicated by the circle on the left, and estimates of genetic distances are: *wi*–*b*, a range of 1.8 ± 0.6 cM to 5.5 ± 0.6 cM (LANE 1963; DAVISSON *et al.* 1989); and *b*–*dep*, 1.9 ± 0.5 cM (MAYER *et al.* 1976). The order of the *Tyrp1* (*b*) and *baf* loci cannot be determined from the present complementation data.

heterozygote was found to hybridize to probes for *D4Rck4* and *D4Rck52*, but not to a *D4Rck140* probe (data not shown). It was not possible, however, to determine if the b^{4ACRg} mutation deletes the *Adfp* or *Ifa* loci (see Figure 6).

DISCUSSION

Genetic and molecular analyses of radiation- and chemical-induced deletion mutations recovered in specific-locus mutagenesis experiments are components of one strategy for generating physical and functional maps of megabase stretches of the mouse genome (reviewed in RINCHIK and RUSSELL 1990). This type of analytical strategy has now also been applied to the *wi–Ifa* region of mouse chromosome 4. Heritable, lethal, radiation- or chemical-induced deletions of the brown (*b*; *Tyrp1*) locus (RINCHIK *et al.* 1994) are available for use as experimental reagents to map molecularly and phenotypically defined loci in the region immediately surrounding the *b* locus. The combined molecular and complementation map of the *b* region presented in Figure 6 can provide a framework on which to build future physical and mutation maps of this chromosomal segment. Perhaps the most significant result of the present study is the definition and initial placement of six loci associated with developmental abnormalities into intervals of the deletion map.

The existence of the *l(4)1Rn* locus is inferred from the prenatal lethality exhibited by mice homozygous for proximally extending *b* deletions that can complement several lethal *b* deletions that also include the distal *dep* locus. Lethality caused by deficiency of the *l(4)1Rn* product must occur early in gestation (either preimplantation or early postimplantation) because deletion homozygotes lacking *l(4)1Rn* cannot be found at 14.5 days of gestation. The complete correlation between deletion of *D4Rck52* and the *l(4)1Rn* locus (Figure 6) suggests that *D4Rck52* may serve well as a physical map nucleation point with which to begin the search for the *l(4)1Rn* gene. At least a portion of *l(4)1Rn* should be found on the physical map between the more distally located proximal breakpoint of the b^{3YPSh} or b^{11PU} deletions and the most proximally located of the five deletion breakpoints that map between the [*D4Rck52*, *l(4)1Rn*] cluster and the [*Tyrp1*/*baf*] cluster (see Figure 6).

The recombination frequency for the *b* (*Tyrp1*)–*dep* interval has been estimated to be 2.2 ± 0.7% in females and 1.4 ± 0.7% in males (MAYER *et al.* 1976). Applying a standard (but not necessarily accurate) average conversion factor for the mouse of 2 megabases (Mb) for every one percent recombination frequency, this region may be on the order of 2–6 Mb in physical length. Within these genetic and (grossly estimated) physical intervals lies the late gestation/neonatal lethal *l(4)2Rn* and the *dep* locus, as well as a physical map nucleation point *D4Rck140*. The *l(4)2Rn* locus is defined by the late gestation/neonatal lethality of the b^{1OZ} and b^{9R75VH} deletions, whose distal breakpoints become important physical landmarks for the analysis of this region. The more proximal of these two distal breakpoints will define the distance one must travel distally on the physical map from *D4Rck140* to ensure that at least a segment of the *l(4)2Rn* gene has been covered. The more distal of the two breakpoints will define the proximal border

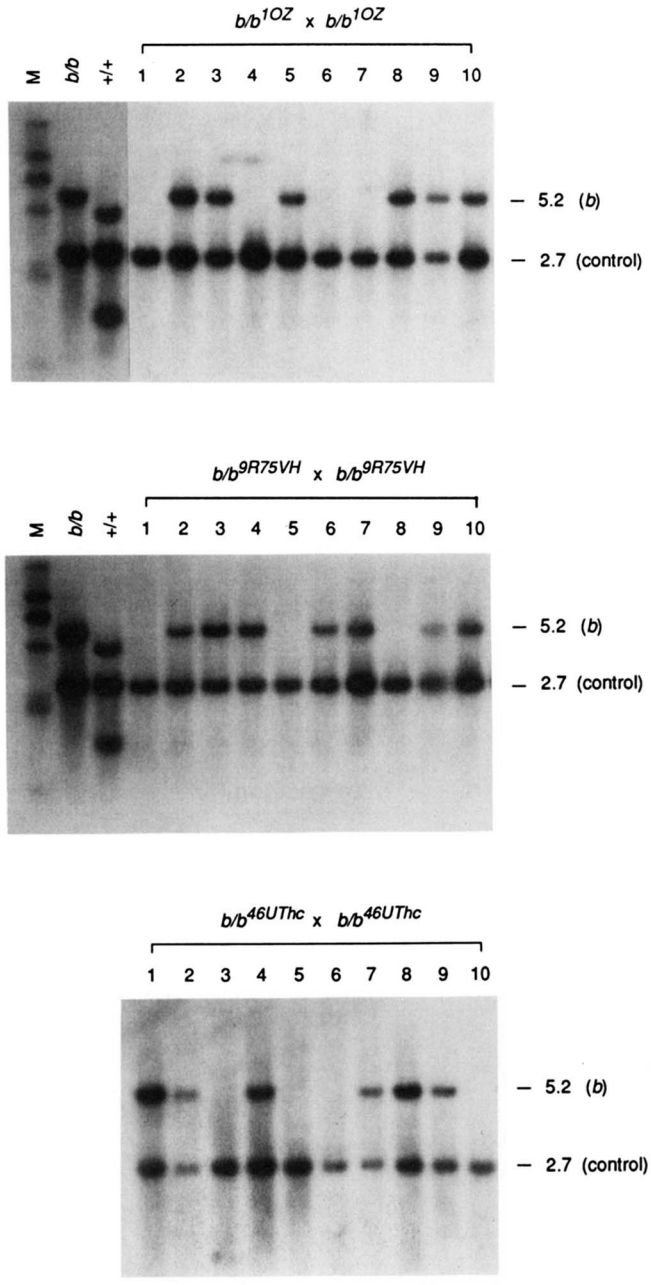

FIGURE 5.—Identification of mid-gestation lethality in certain distally extending b^l deletions. DNAs from either +/+ or *b/b* mice as well as from individual E14.5 fetuses from the indicated crosses were digested with *Taq*I, electrophoresed in agarose, blotted to nylon and hybridized with the *Tyrp1* cDNA subclone MT4.Pv.25. M denotes *Hin*dIII fragments of bacteriophage λ, and fragment sizes are given in kilobases. The 2.7-kb fragment recognized by the chromosome 7 control probe from the *Znf127* (*D15S9h-1*) locus (Probe 34-1-111) is also indicated.

of the interval that should contain the *dep* locus. Similarly, the distal breakpoint of the b^{46UThc} deletion, which is the only *Df(b dep)* deletion currently known *not* to include the *l(4)3Rn* locus (in contrast to b^{8PUb} and b^{4ACRg}, which do include it), will define the minimum physical segment extending distally that should contain at least a portion of the *dep* gene. However, it is possible that the distal breakpoint of one of the six *Df(b dep)* deletions (b^{3YPSh}, b^{11PU}, b^{12PU}, b^{13R75M}, $b^{37FrThc}$ and b^{5CHLe}) not yet characterized with respect to their inclusion of the *l(4)3Rn* locus could lie more proximal than that of b^{46UThc} and thus be more useful in this regard.

Thus, the genetic reagents available for positional cloning of genes within this region provide a significant rationale for the further study of the *l(4)2Rn* and *dep* phenotypes. Other than determining the gross time of death, the lethal phenotype of late gestation fetuses/neonates homozygously deleted for *l(4)2Rn* has not yet been characterized in any detail. Genetically detemined late gestation/neonatal lethality can have diverse, often unexpected, and usually quite interesting etiologies. For example, mice homozygous for the c^{14CoS} deletion at the *albino* locus die within a few hours of birth, presumably from abnormalities in liver and kidney enzyme function (reviewed in GLUECKSOHN-WAELSCH 1979). These abnormalities are due to deficiency of fumarylacetoacetate hydrolase, an important enzyme in tyrosine metabolism (KLEBIG *et al.* 1992b; RUPPERT *et al.* 1992; KELSEY *et al.* 1993). Mice homozygous for the p^{cp} deletion (PHILLIPS 1973) or the $p^{4THO\text{-}II}$ deletion (CULIAT *et al.* 1993) at the *pink-eyed dilution* locus have a cleft palate and die at birth, most likely from simple aspiration of milk. Mice with severe macrocytic anemias, such as those resulting from mutations at the *Dominant white-spotting* (*W*; c-*kit*) or *Steel* (*Sl*) loci, often die during late gestational or neonatal life (reviewed in RUSSELL 1979). Thus, it will clearly be of interest to examine b^{1OZ}/b^{1OZ} and/or b^{9R75VH}/b^{9R75VH} fetuses by both histological and biochemical techniques to determine the cause of their demise. In addition, it will be of interest to determine whether these mice are indeed born, and, if so, how long they can survive into early neonatal life.

The *dep* locus is defined by a single, spontaneous mutation that affects hair growth in the juvenile mouse (MAYER *et al.* 1976). Depilated mice have normal hair and hair follicles at day 5 after birth; but by day 8, disorientation and degeneration of hair follicles becomes evident. The degeneration of hair follicles can result in juvenile mice losing their fur completely, although the phenotype is highly variable and can range from complete fur loss to a more trivial shortening of the hair. Skin transplantation studies have shown that the epidermis, rather than the dermis, is the defective tissue (MAYER *et al.* 1976). Bracketing of the *dep* gene within *b* deletion breakpoints, as reported here, provides a means for identifying transcripts whose candidacy (for *dep*) could later be evaluated by testing for expression in epidermis.

The most distal breakpoint of the group of *Df(b dep)* deletions that define the distal border of the *dep* interval likewise defines the proximal border of the *l(4)3Rn* locus. Homozygous deletion of *l(4)3Rn* results in death of the embryo before midgestation stages, but, as in the case of *l(4)1Rn*, it is not yet known whether this death

TABLE 3

Assay for midgestation survival in fetuses homozygous for selected lethal *brown* (b^l) deletions

Mutation (b^l)	Genotype[a]	No. midgestation fetuses		Revised genotype[b]
		b^l/b^l	Total	
b^{11R30M}	*Df[wi l(4)1Rn b baf]*	0	28	*Df[wi l(4)1Rn b baf]*
b^{1OZ}	*Df[b baf l(4)2Rn]*	10	34	*Df[b baf l(4)2Rn]*
b^{9R75VH}	*Df[b baf l(4)2Rn]*	7	35	*Df[b baf l(4)2Rn]*
b^{46UThc}	*Df[b baf l(4)2Rn dep]*	8	21	*Df[b baf l(4)2Rn dep]*
b^{8PUb}	*Df[b baf l(4)2Rn dep]*	0	45	*Df[b baf l(4)2Rn dep l(4)3Rn]*
b^{4ACRg}	*Df[b baf l(4)2Rn dep]*	0	32	*Df[b baf l(4)2Rn dep l(4)3Rn]*
b^{5CHLe}	*Df[l(4)1Rn b baf l(4)2Rn dep]*	0	34	—[c]
b^{13R75M}	*Df[l(4)1Rn b baf l(4)2Rn dep]*	0	28	—[c]
b^{13DT}	*Df[l(4)1Rn b baf]*	0	36	*Df[l(4)1Rn b baf]*
b^{26R60L}	*Df[l(4)1Rn b baf]*	0	32	*Df[l(4)1Rn b baf]*
b^{3YPSc}	*Df[l(4)1Rn b baf]*	0	30	*Df[l(4)1Rn b baf]*
b^{37DTD}	*Df[l(4)1Rn b baf]*	0	23	*Df[l(4)1Rn b baf]*
b^{37PUb}	*Df[l(4)1Rn b baf l(4)2Rn]*	0	36	*Df[l(4)1Rn b baf l(4)2Rn]*

DNAs prepared from individual E13.5–E15.5 fetuses generated from the cross $b/b^l \times b/b^l$ (or $B^w/b^l \times B^w/b^l$ in the case of b^{37DTD} and b^{4ACRg}) were analyzed by Southern blot analysis with a *Tyrp1* cDNA probe to ascertain whether any b^l/b^l fetuses were present at these mid-gestation stages.

[a] This genotype is based solely on the complementation analyses presented in Table 1 and Figure 3.

[b] This genotype indicates whether the corresponding deletion includes the *l(4)3Rn* lethal, deduced from the data in this table (see also Fig. 6).

[c] It cannot be determined from this analysis whether these mutations affect the *l(4)3Rn* lethal.

occurs in the pre-, peri- or postimplantation stages of embryogenesis. Analysis of the uterine contents of additional females from crosses that can produce *Df(b dep)*^{8PUb} or *Df(b dep)*^{4ACRg} homozygotes will be necessary to make such a determination. Once a more specific time of death is determined, more detailed histological analyses of mutant embryos can be performed to elucidate further the nature of the *l(4)3Rn* lethal phenotype.

The genetic definition of the *l(4)1Rn* and *l(4)3Rn* loci by the complementation analyses reported here provide significant additions to the *b*-region functional and mutation map. However, their definition also highlights one limitation of building functional maps based solely on proximally and distally extending deletions of a single, specific locus. For example, the early gestation lethality observed in embryos homozygously deleted for *l(4)1Rn* precludes the discovery of phenotypes that could appear later in development as a result of deletion of loci proximal to *l(4)1Rn*. The same arguments apply for additional phenotypes specified by genes mapping distally to *l(4)3Rn* but still within any of the *Df(b dep)* deletions that include *l(4)3Rn*.

Several different strategies can be used to overcome at least some of the functional mapping roadblocks created in these situations (see RINCHIK and RUSSELL 1990). One strategy, which is merely a modification and refinement of one already used in this study, involves comparing the lethal phenotype associated with homozygosity for deletions currently within the same complementation group. For example, any specific differences in lethal phenotypes among early gestation embryos homozygous for b^{13DT}, b^{3YPSc} and b^{37DTD}, for example, or compound heterozygous embryos deleted for less DNA than the corresponding homozygotes (*e.g.*, b^{13DT}/b^{11PU}), may allow the mapping of multiple loci required for early development and could associate each locus with distinct early gestation processes. This type of strategy has been successful in subdividing a series of prenatally lethal *albino* (*c*)-locus deletions into those that cause death during preimplantation development when homozygous *vs.* those that cause death shortly after implantation (RUSSELL and RAYMER 1979; RUSSELL *et al.* 1982; RINCHIK *et al.* 1993a). This type of strategy has also been successful in subdividing the series of postimplantation lethal *c* deletions and has provided evidence for at least two loci (necessary for extraembryonic and embryonic ectoderm development, respectively), by identifying deletions that included either the latter locus or both loci (NISWANDER *et al.* 1989). Indeed, the early *vs.* mid/late gestation lethality among *b* deletion homozygotes has already been useful in defining and mapping the *l(4)2Rn* locus within the *b* deletion complex, suggesting that more detailed examination of individual lethal phenotypes in deletion homozygotes or in specific compound heterozygotes may prove to be a useful strategy for analyzing and refining the functional content of other *b* deletions.

The hypothesized *b-associated fitness* (*baf*) locus (Figures 4 and 6) is defined by the variable decrease in fitness observed in brown (b^{l-1}/b^{l-2}) mice carrying overlapping deletions that complement the *l(4)1Rn*- or *l(4)2Rn*-associated prenatal lethalities. This fitness problem is evident to some degree in every combination of deletions that complement for prenatal lethality, where its severity ranged from extreme runtiness, resulting in death before weaning, to slight runting, which had very little effect on the well being or fertility of the animal. In fact, testing of both males and females of a number of different, arbitrarily chosen b^{l-1}/b^{l-2} com-

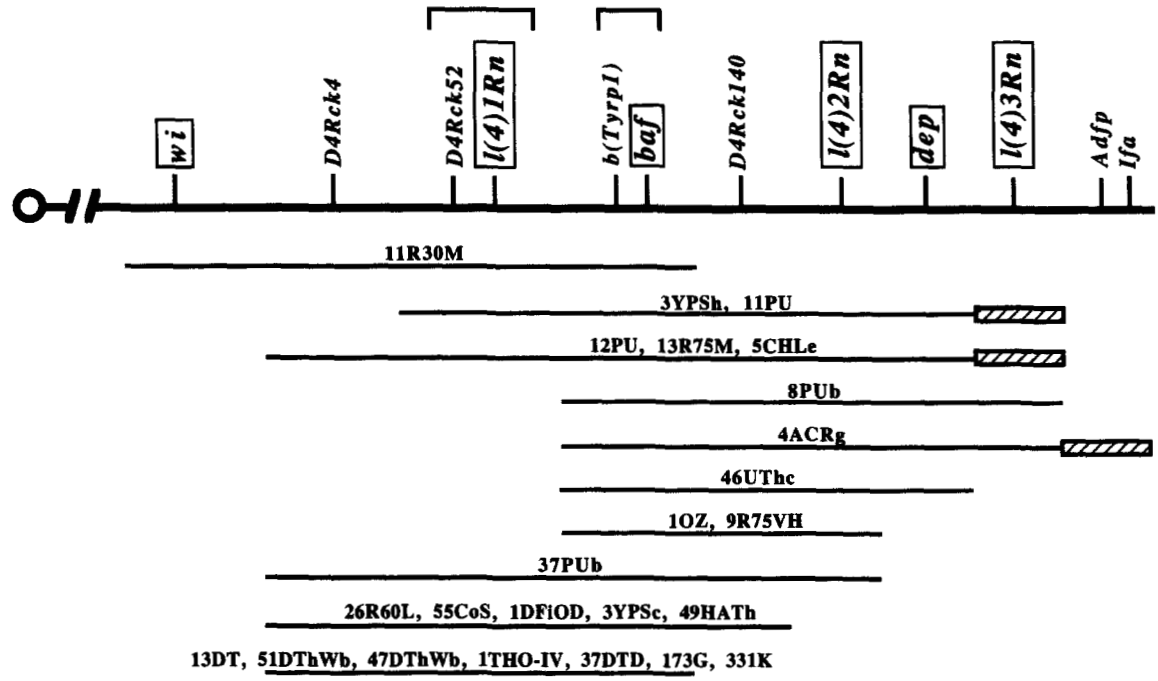

FIGURE 6.—A combined molecular and complementation map of the *wi–Ifa* region in mouse chromosome *4*. This map represents an interdigitation of the molecular map presented in a companion study (RINCHIK *et al.* 1994) with the complementation map generated from data presented in this report. See RINCHIK and co-workers (1994) for descriptions of molecularly defined loci. Loci mapped into deletion intervals by complementation analyses are shown in boxes above the chromosome. Loci grouped within a bracket cannot be ordered by deletion analysis. The diagonally striped boxes shown at the ends of six deletions denote current uncertainties about the distal extents of these deletions. Deletions indicated on the same line cannot at present be distinguished by either molecular or complementation data. No correlation with any physical distance is implied by the spacing of loci or by the length of lines showing the genetic extent of deletions.

pound heterozygotes (males b^{26R60L}/b^{1OZ}, b^{13DT}/b^{1OZ}, b^{37DTD}/b^{1OZ}, b^{13DT}/b^{4ACRg}, b^{11R30M}/b^{9R75VH}, b^{173G}/b^{1OZ}, b^{331K}/b^{9R75VH}, b^{11R30M}/b^{1OZ} and females b^{26R60L}/b^{9R75VH}, b^{37DTD}/b^{1OZ}, b^{331K}/b^{9R75VH}, b^{173G}/b^{1OZ}, b^{13DT}/b^{1OZ}, b^{11R30M}/b^{1OZ}, b^{13DT}/b^{9R75VH}) showed that at least some mice from each tested combination of complementing deletions are fertile. Not surprisingly, the fertility of the animal was, in general, negatively correlated with the severity of the runting phenotype: both male and female b^{l-1}/b^{l-2} animals that were mildly affected tended to be fertile.

The fact that each of the 35 different b^{l-1}/b^{l-2} compound heterozygotes (Figure 3) exhibited the baf runting phenotype to some degree suggests that *baf* is very closely linked to *Tyrp1* and cannot be separated from it by any one of the deletion breakpoints that break either proximal or distal to it. It is unlikely that total deletion of *Tyrp1* itself is responsible for the baf phenotype. Molecular analysis (JACKSON *et al.* 1990) of the *White-based brown* (B^w) mutation (HUNSICKER 1969) provides evidence that *baf* is a separate, but closely linked, locus. B^w/B^w skin expresses no detectable *Tyrp1* transcript, and the only phenotype evident in B^w/B^w homozygotes is that they have brown hairs with a white base, which results from melanocyte cell death during hair growth. Otherwise, B^w/B^w homozygotes are completely viable and are not at all runted, unlike mice exhibiting the baf phenotype. Thus, the absence of detectable *Tyrp1* expression in these non-baf animals suggests that the *baf* locus is a separate gene(s) that happens to be very closely linked to *Tyrp1*. The deletion breakpoints that flank the *baf* locus both proximally and distally (Figure 6) should therefore provide useful tools both for delimiting the *baf* gene(s) on the physical map and for dissecting the nature of the baf phenotype(s).

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