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Genetics

Genetic analysis of mutations at the fused locus in the mouse

(developmental mutants/restriction fragment length polymorphisms/gene dosage)

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ABSTRACT **Mutations at the fused (*Fu*) locus on chromosome 17 of the mouse disrupt embryonic development by altering the organization of the neurectoderm. We have examined the interactions among several independent fused mutations, including a deletion of the locus, to define the nature of the mutant defects. Closely linked restriction fragment length polymorphisms made possible the unambiguous identification of genotype in all progeny. Tests with the deletion, as well as interactions among alleles, indicate that all three spontaneous mutations are "gain of function" defects. Comparisons of relative viabilities of the various mutant combinations rank them into a series of increasing severity and place constraints on possible modes of gene action.**

The embryonic origins of the mammalian nervous system appear to mirror those of amphibians and birds, in which an inductive signal from mesodermal tissue defines a region of overlying ectoderm as the neural plate and establishes the primary regional plan for the nervous system. This picture of early neurectodermal organization has emerged from embryonic grafting experiments in amphibians (1, 2) and birds (3). Although developmental manipulations in these lower vertebrates are informative about the phenomenology of tissue interactions, they cannot identify the actual genes involved. The mouse, on the other hand, does offer the potential for genetic analysis of these early embryonic events, by dint of genetic variants that alter ectodermal organization.

Mutations of the fused (*Fu*) locus on chromosome 17, when homozygous, produce excessive amounts of neurectoderm, usually resulting in embryonic lethality (4–6). The phenotype often manifests itself as multiple neural tubes or neural axes. In heterozygotes, all alleles are viable and have a mild skeletal abnormality of the tail, reflected in two of their names: kinky (Fu^{Ki}) and knobbly (Fu^{Kb}). The presence of dominant phenotypes allowed these spontaneous mutations to be recognized originally (7–9).

Mutations inform us about normal gene function only when we can discern how they differ from wild type. Muller introduced a powerful method for the characterization of mutant alleles, at a time when the nature of the gene had yet to be defined, based on varying the dosage of mutant and wild-type gene copies (10). Comparison between mutant alleles and a deletion of a locus reveals whether the mutation causes a "gain" or "loss" of gene function, an essential distinction for any inference concerning normal function. If a mutation causes a loss of function it will be equivalent to a deletion of the locus and show no gene activity. If, on the other hand, it produces a gain of function, such as excessive or ectopic expression, it will differ from a deletion. The strategy remains forceful, particularly when a gene's product is not known, as is the case with the fused locus. Extensive use has been made of this approach in *Drosophila*, where it has been particularly informative about complex homeotic genes (11, 12). It has been little exploited in the mouse.

The fused locus is well suited for such analysis. In addition to multiple alleles, a putative deletion of the fused locus arose as the apparent product of an unequal crossover involving rearranged variants of chromosome 17 (*t* haplotypes, refs. 9 and 13). It was subsequently found to uncover the locus for a cloned globin pseudogene, *Hba-ps4* (14). The combination of a deletion and a tightly linked molecular marker, for which several restriction fragment length polymorphisms (RFLPs) exist (15), provides the requisite tools for analyzing the character of the fused mutations. The marker makes possible the scoring of progeny genotypes without further test crosses and with no risk of ambiguity due to the incomplete penetrance of heterozygous dominant phenotypes (cf. refs. 6 and 7). The deletion makes possible the testing of mutant and wild-type alleles in one or two doses. In this paper, we exploit these tools to demonstrate that all three spontaneous fused mutations are "gain of function" defects.

MATERIALS AND METHODS

Genetic Strains. A strain carrying the kinky allele (Fu^{Ki}) was obtained from Salome Gluecksohn-Waelsch (Albert Einstein College of Medicine, Bronx, NY) and crossed onto an AKR background in our colony. The knobbly allele (Fu^{Kb}) and the partial *t* haplotype t^{h20} were obtained from Mary Lyon (Medical Research Council, Chilton, England). A strain carrying the fused (*Fu*) locus on the strain 129 background was obtained from The Jackson Laboratory as were all inbred strains used in the study as sources of the three different alleles of *Hba-ps4*: SM (*c* allele), C57BL/6 (*b* allele), and AKR (*a* allele).

Genotype Scoring and Analysis. Chromosome 17 genotypes were scored by means of RFLPs for the globin pseudogene *Hba-ps4* (15) and for a sequence from the proximal region of chromosome 17, Tu66, derived by microdissection (16, 17). Recombinant clones containing these sequences were provided by Lee Silver (Princeton Univ.). High molecular weight DNA was extracted from tail skin (18), digested with *Taq* I (Boehringer Mannheim) for scoring of *Hba-ps4* (15) or *T66E* genotype (17), and analyzed by routine Southern hybridization techniques. Analyses were repeated on any individuals producing ambiguous results or exhibiting rare genotypes.

DNA was prepared from progeny of the various crosses at ≈3 weeks of age. Few cases of perinatal lethality were observed in any of the crosses, and these were not correlated with any particular genotype, so viability as measured at weaning age was considered reliable. Statistical analysis of progeny classes consisted of χ^2 tests for goodness of fit to expectations for standard Mendelian segregation (1:2:1). Percent viability was calculated as the (number of survivors of a particular genotype/number of expected progeny of that genotype) × 100.

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Abbreviation: RFLP, restriction fragment length polymorphism.

RESULTS

Interactions Among Alleles. To test the viability of various mutant combinations, it was first necessary to determine the *Hba-ps4* RFLP genotype of each chromosome in the various crosses. We mated each mutant strain to the SM strain, bearing the rare *c* allele of *Hba-ps4*, recovered F_1 mice clearly carrying and capable of transmitting the dominant mutations, and analyzed DNA obtained from their tail skin. The results, shown in Fig. 1, indicate that Fu^{Ki} and Fu^{Kb} are linked to $Hba\text{-}ps4^a$, whereas *Fu* is linked to $Hba\text{-}ps4^b$. The t^{h20} chromosome is deleted for *Hba-ps4*, as reported (14).

Table 1 presents the progeny recovered from crosses of the various mutant heterozygote combinations. All mice used in the crosses, the F_1 mice described above, carried the $Hba\text{-}ps4^c$ allele on the normal homolog of chromosome 17. This meant that, excepting rare crossovers, the genotypes of progeny were unambiguously scorable. Heterozygosity of all the parents in Table 1 for $Hba\text{-}ps4^c$ also served to normalize for differences in genetic background—that is, all of the fused alleles were initially on different backgrounds (see *Materials and Methods*), but as a consequence of placing each mutant chromosome in *trans* with $Hba\text{-}ps4^c$, all were now heterozygous for the rest of the SM genome as well.

Homozygotes for Fu^{Ki} failed to survive, as expected (8), whereas *Fu* homozygotes had essentially normal viability. Homozygosity for Fu^{Kb} was usually lethal, yielding only 3 of an expected 16 in the homozygous class (equivalent to 19% of normal viability). The combination of Fu^{Ki}/Fu^{Kb} was fully lethal, and the combination of Fu^{Ki}/Fu was mostly lethal, with only 2 of an expected 22 surviving (9% viability). The combination of Fu^{Kb}/Fu showed normal viability.

Since the globin pseudogene maps approximately 1 centimorgan distal to the fused locus (16), rare recombinational events will separate them and confound the results. To confirm that rare survivors were bona fide survivors and not merely crossover products, we employed a second genotypic marker, *T66E* proximal to the fused locus (17).

Taq I digestion of DNA from the parental genotypes in the crosses revealed RFLPs for the Tu66 marker (Fig. 2). The patterns for the Fu^{Ki} and Fu^{Kb} chromosomes were identical and distinguishable from those of the SM and *Fu* chromosomes. By means of this analysis, we eliminated one bogus individual from the Fu^{Kb} homozygous class, leaving three confirmed homozygotes (see Table 1). Likewise for the Fu^{Ki}/Fu class, three recombinants were eliminated, leaving the two confirmed genotypes shown in Table 1. Restriction patterns for these mice are shown in Fig. 2.

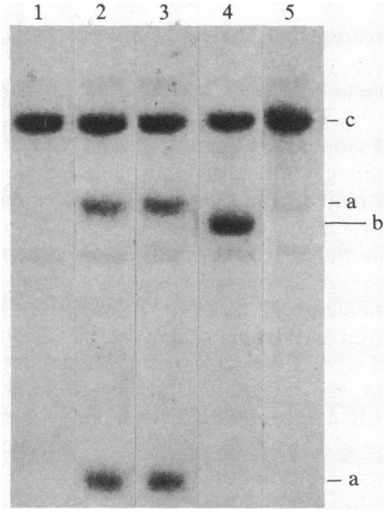

FIG. 1. DNA polymorphisms for *Hba-ps4*. Genomic DNA was digested with *Taq* I, electrophoresed, blotted, and hybridized with the *Hba-ps4* clone. The letters a, b, and c indicate the RFLPs for each allele. Lane 1, *c/c* (SM strain); lane 2, *a/c* ($Fu^{Ki}/+$); lane 3, *a/c* ($Fu^{Kb}/+$); lane 4, *b/c* (*Fu/+*); lane 5, *Df/c* ($t^{h20}/+$).

Table 1. Viabilities of allelic combinations

Parents	Genotype of offspring* m/m	m/+	+/+	χ^2	P
Homoallelic					
$Fu^{Ki}/+ \times Fu^{Ki}/+$	0	39	26	21.64	<0.001
$Fu^{Kb}/+ \times Fu^{Kb}/+$	3	38	21	12.23	<0.01
$Fu/+ \times Fu/+$	9	27	14	0.89	>0.05
Heteroallelic					
$Fu^{Ki}/+ \times Fu^{Kb}/+$	0	54	26	25.08	<0.001
$Fu^{Ki}/+ \times Fu/+$	2	56	28	22.09	<0.001
$Fu^{Kb}/+ \times Fu/+$	19	59	19	3.92	>0.05

χ^2 values are calculated for two degrees of freedom.
*Genotype of offspring as reflected by *Hba-ps4* and *T66E* genotype in the cross, where "*m*" denotes the mutation-bearing chromosome and "+" the normal, SM-derived homolog.

We determined that there were no unusual effects of segregation or dominant lethality associated with any of the relevant chromosomes or genetic background mixtures present in the crosses (see Table 2). These controls used wild-type siblings of the mutant heterozygotes, bearing the normal homologs from the original mutant strains heterozygous with SM chromosomes.

Deletion Tests of Fused Alleles. To test the t^{h20} chromosome for lethality with fused alleles, we made use of the same DNA polymorphic markers described above. The t^{h20} chromosome was usually balanced with the *c* allele of *Hba-ps4* (but was sometimes balanced with the *a* or *b* alleles when the genotype of the other parent was compatible). Genotypic scoring in these crosses relied upon the absence of any globin pseudogene allele on the t^{h20} chromosome. Thus, inheritance of the deletion chromosome (symbolized by *Df*) could be unambiguously inferred from the absence of the *Hba-ps4* allele carried by the wild-type homolog of the t^{h20} parent. In this fashion,

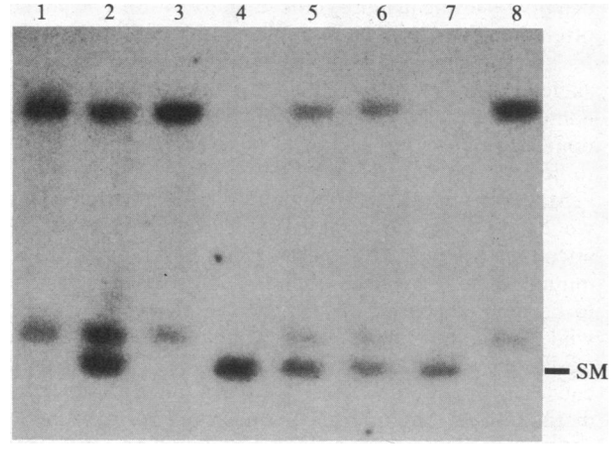

FIG. 2. DNA polymorphisms for *T66*. Genomic DNA was digested with *Taq* I, electrophoresed, blotted, and hybridized with the Tu66 clone. The RFLP specific to the SM strain is indicated. Lower molecular weight fragments are not shown (cf. ref. 17), as they were identical in all genotypes. Lane 1, true Fu^{Kb} homozygote; lane 2, bogus Fu^{Kb} homozygote (due to presence of "SM" band); lane 3, Fu^{Kb}/Df; lane 4, SM strain; lane 5, $Fu^{Ki}/+$; lane 6, $Fu^{Kb}/+$; lane 7, *Fu/+*, in which only the SM (+) chromosome is present, since the *Fu* chromosome lacks all of these fragments; lane 8, Fu^{Kb}/Fu, showing only the knobbly chromosome pattern, since the *Fu* chromosome lacks all of these fragments.

Table 2. Controls for segregation of mutant chromosomes

Parents†	Genotype of offspring* $+^m/m$	$+^m/+^{SM}$ or $m/+^{SM}$	$+^{SM}/+^{SM}$	χ^2	P
$+^{Ki}/+^{SM} \times Fu^{Kb}/+^{SM}$	15	24	12	0.28	>0.05
$+^{Ki}/+^{SM} \times Fu/+^{SM}$	18	28	14	0.51	>0.05
$+^{Kb}/+^{SM} \times Fu^{Ki}/+^{SM}$	7	31	15	3.22	>0.05
$+^{Kb}/+^{SM} \times Fu/+^{SM}$	16	27	12	0.34	>0.05
$+^{Fu}/+^{SM} \times Fu^{Ki}/+^{SM}$	16	34	19	0.12	>0.05
$+^{Fu}/+^{SM} \times Fu^{Kb}/+^{SM}$	12	34	12	1.25	>0.05

χ^2 values are calculated for two degrees of freedom.
*Genotypes as reflected by *Hba-ps4* and *T66E* genotype in the cross, where "m" denotes mutation-bearing chromosome, "$+^m$" denotes wild-type homolog from original mutant strain, and "$+^{SM}$" denotes wild-type chromosome 17 derived from the SM strain.
†"$+^{Ki}$" denotes wild-type homolog from original kinky strain, "$+^{Kb}$" denotes wild-type homolog from original knobbly strain, "$+^{Fu}$" denotes wild-type homolog from original fused strain, and "$+^{SM}$" denotes wild-type chromosome 17 derived from SM strain.

any progeny surviving with the deletion chromosome and a mutant chromosome were detectable by the presence of the *Hba-ps4* allele on the mutant chromosome only.

Table 3 presents the results of these crosses, demonstrating that the deletion shows full lethality with Fu^{Ki} and nearly complete lethality with Fu^{Kb}. When heterozygous with *Fu*, the animals have normal viability. Confirmation that the lone Fu^{Kb}/Df survivor was not the result of a crossover in one parent between Fu^{Kb} and *Hba-ps4* came from analyzing its *T66E* genotype (Fig. 2). The absence of the *Hba-ps4* allele derived from the other parent was an absolute marker for the presence of the t^{h20} chromosome.

DISCUSSION

The primary goal of this study was the characterization of the three extant alleles of the fused locus as either "loss of function" or gain of function mutations, depending upon their interaction with and similarity to a deletion of the locus. Our results indicate that they are all gain of function, based primarily on their nonequivalence to the deletion. In Muller's original formulation (10), a complete loss of function ("amorphic") allele should exhibit genetic characteristics similar to a deletion.

Crucial to this discussion is the demonstration that the t^{h20} chromosome deletes the fused locus. In Lyon's original study of t^{h20}, she reported that it failed to complement knobbly (9), based upon distinguishing the double dominant phenotype of brachyury (*T*) and knobbly from either single phenotype. A difficult distinction, it relies on penetrance, which is not always complete (cf. ref. 6). There is no question that t^{h20} is a deletion, since it deletes *Hba-ps4* (14) and also fails to complement the neighboring recessive coat mutation tufted (*tf*; ref. 9). Moreover, our results indicate that t^{h20} fails to complement the lethality of kinky and knobbly, as demonstrated by means of a RFLP marking system not subject to problems of penetrance (cf. refs. 6 and 19). The failure of the t^{h20} chromosome to provide wild-type activity for the fused locus, in conjunction with its previously described deletion characteristics, support the conclusion that it deletes the fused locus.

Table 3. Tests with deletion t^{h20}

Parents	Genotype of offspring* m/Df	$m/+$	Df or $+/+$	χ^2	P
$Fu^{Ki}/+ \times t^{h20}/+$	0	32	47	21.72	<0.001
$Fu^{Kb}/+ \times t^{h20}/+$	1	27	53	23.17	<0.001
$Fu/+ \times t^{h20}/+$	26	13	58	6.5	<0.05†

χ^2 values are calculated for two degrees of freedom.
*Genotype as reflected by *Hba-ps4* and *T66E* genotype in the cross, where "m" denotes mutation-bearing chromosome, "Df" denotes deletion t^{h20}, and "+" denotes normal chromosome.
†The deviation from expected normal Mendelian ratios in this cross is due to the "$m/+$" class, not the "m/Df" class, which exhibits normal viability.

The expression of alleles of the fused locus clearly differs from that of the deletion. $t^{h20}/+$ heterozygotes have completely normal tails (Fig. 3), whereas heterozygotes for all fused alleles have dominant tail defects (Fig. 3, and refs. 7–9). Thus, they cannot be loss of function defects.

The interactions among the dominant alleles further argue against loss of function, whether partial or complete. If, for example, one postulated that fused was due to partial loss of function and kinky due to complete loss, then the combination of Fu/Fu^{Ki} should be similar to Fu/Df, but it is not. In fact, the combination of fused and kinky is far more lethal. Finally, if any of the alleles were the result of partial loss, then the deletion heterozygote, $Df/+$, should have the most severe dominant phenotype, as opposed to the actual situation where it has a normal phenotype.

One final argument against a loss of function character for these mutations is that no radiation-induced alleles of this locus (i.e., dominant visible alleles) have ever been recovered among the many thousands of progeny screened in such

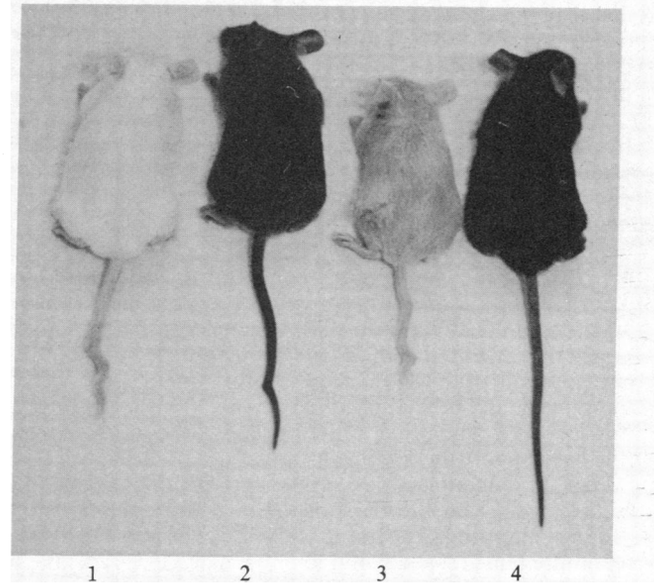

FIG. 3. Tail phenotypes of mice (anesthetized) heterozygous for variants of the fused locus. Mouse 1, $Fu^{Ki}/+$; mouse 2, $Fu^{Kb}/+$; mouse 3, $Fu/+$; mouse 4, $t^{h20}/+$.

experiments (ref. 20; M. Lyon, personal communication). In contrast, new alleles of other loci for which heterozygous deletions produce dominant phenotypes, such as white (*W*; ref. 21) and brachyury (*T*; ref. 22) appear regularly in such experiments (20, 21). This is consistent with the expectation that, in general, loss of function alleles are the most commonly induced, particularly with radiation (cf. ref. 12). The simplest interpretation for the absence of radiation-induced alleles of the fused locus is not that they are harder to induce but rather that they have no dominant phenotype.

Allelism has been determined previously for the combinations of kinky with fused and kinky with knobbly. Dunn and Gluecksohn-Waelsch (19) test-crossed progeny of Fu/Fu^{Ki} mice and found no evidence for recombination between the two alleles. Jacobs-Cohen *et al.* (6) examined embryos from crosses between heterozygous $Fu^{Ki}/+$ and $Fu^{Kb}/+$ and found a class of nearly 25% abnormal embryos, phenotypically similar to homozygotes for either mutation. Previous studies on this locus also reported reduced viability of Fu/Fu^{Ki} (19, 23) and nearly normal viability of Fu/Fu (5, 7, 19), based on progeny tests to determine genotype. Our results are in agreement with these findings.

In the absence of a tight marking system, it has heretofore been laborious to obtain accurate quantitative information on the relative viabilities of different genotypes and often impossible to recognize very rare survivors. *Drosophila* geneticists have obviated this problem by means of "balancer chromosomes"—that is, chromosomes carrying reliable, dominant markers and multiple inversions for the suppression of crossing-over (24). Lacking balancer chromosomes in the mouse, tightly linked DNA polymorphisms provide a suitable alternative. In the present study, we have exploited such markers to realize a quantitative ranking of the various fused genotypes and also to reveal rare classes of survivors not detected previously. The relative viabilities of the various genotypes can be ordered into a series of decreasing severity: $Fu^{Ki}/Df = Fu^{Ki}/Fu^{Ki} = Fu^{Ki}/Fu^{Kb} > Fu^{Kb}/Df = Fu^{Kb}/Fu^{Kb} = Fu/Fu^{Ki} > Fu/Fu^{Kb} = Fu/Df = Fu/Fu$. The greater severity of kinky as opposed to knobbly is shown repeatedly, by comparison of lethality as homozygotes, comparison of lethality with the deletion, and comparison of their interactions with *Fu*. The rare survivors of knobbly either homozygous or with t^{h20} provide additional clues as to the nature of the knobbly defect. The lack of difference between one or two doses of the mutant gene suggests that it is not a simple overproducer ("hypermorph"; ref. 10), since an overproducer ought to be less severe in a single dose (Fu^{Kb}/Df) than in two doses (Fu^{Kb}/Fu^{Kb}).

An alternative view of the allelic interactions is suggested by focusing on the failure of kinky to complement either knobbly or fused, whereas knobbly and fused complement each other completely. This pattern of complementation is characteristic of complex loci such as Bithorax (11) and rudimentary (25) in *Drosophila* and suggests separable functions within the gene. In this context, the alleles fused and knobbly appear to represent defects in separate functions, whereas kinky affects both. The validity of this view depends upon the extent to which Fu^{Kb}/Fu is truly normal. It is already known that homozygosity for *Fu* and for Fu^{Kb} can produce neural tube duplications (5, 6). If this is not the case for Fu^{Kb}/Fu, such that all individuals are normal, it would further support the argument.

For the detection of rare survivors (see Tables 1 and 3), our scoring relies on the flanking DNA markers *Hba-ps4* and *T66E*, mapping approximately 1 centimorgan and 7 centimorgans, respectively, to either side (16, 17). Thus, the probability of being misled by a double crossover is 0.0007, assuming no interference. Intervals of this size in *Drosophila* never support double crossovers (26). Lyon observed no survivors of the genotype Fu^{Kb}/t^{h20} out of 64 progeny (9). Our finding of 1 out of 81 (Table 3) is not statistically different. In the case of knobbly homozygotes, a low survival rate in the expected homozygous class would not have been statistically discernible in the embryo counts performed by Jacobs-Cohen *et al.* (6), in the absence of a definitive marker.

Gain of function mutations in developmental genes often give rise to ectopic or supernumerary structures, as with the transformation of wing into haltere by Contrabithorax in *Drosophila* (11, 27) or the multiple sensory "hooks" of *lin-12* males in *Caenorhabditis* (28). In these homeotic genes, loss of function alleles produce phenotypes that are the opposites of gain of function alleles. It is not yet known if this is true for the fused locus, but isolation of a loss of function allele will help resolve the question.

One explanation for supernumerary structures resulting from a gain of function might be inappropriate spatial or cell-type specific expression of an essential gene. Such an effect has been demonstrated in the case of the Contrabithorax mutation in *Drosophila*, which causes ectopic expression of a product from the Ultrabithorax region of the Bithorax complex (27). If the mechanism of neural determination in mammals involves mesodermal induction of ectoderm, as seems likely by analogy to other vertebrates (1–3), then the production of excessive neurectoderm or supernumerary neural tubes by gain of function mutations would be a predicted phenotype caused by the relevant genes. It is conceivable that a putative defect in either signal or response, spread over too large an area, could produce such a phenotype.

The genetic characteristics of the fused mutations contrast with those of a class of mutants in *Drosophila* that give rise to excess neural tissue (29). Although superficially similar to the fused locus defects, the expansion of neurectoderm in mutants such as Notch and Delta represents loss of gene function, resulting in a surfeit of ectodermal precursors becoming neuroblasts and a concomitant deficit in dermatoblasts (29, 30). The differences are not surprising in view of the lack of morphogenetic similarity between insect neurogenesis and that of vertebrates (1, 31) and the apparent lack of a crucial role for mesodermal induction in determining the insect nervous system (32). Phyletic differences in developmental mechanisms will reflect differences in developmental genes and their actions. Further analysis of the fused locus may prove useful in defining such differences as a by-product of elaborating its role in mammalian embryogenesis.

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