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A Tripartite Interaction Among Alleles of *Notch*, *Delta*, and *Enhancer of split* During Imaginal Development of *Drosophila melanogaster*

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ABSTRACT

A dramatic example of a phenotypic interaction that involves neurogenic loci during *Drosophila* imaginal development is the synergistic impact of *split* (*spl*), a recessive allele of the *Notch* locus, and $E(spl)^D$, a dominant gain-of-function allele of the *Enhancer of split* locus, on morphogenesis of the compound eye. Screens for mutations that relieve the enhancing effect of $E(spl)^D$ on *spl* have yielded three classes of mutations: intragenic revertants of the $E(spl)^D$ allele, extragenic suppressors that are allelic to the neurogenic gene *Delta* (*Dl*) and unlinked extragenic modifiers. Analysis of the suppression of the *spl*-$E(spl)^D$ interaction by various *Dl* alleles indicates that this modification is sensitive to the dosage of the *Dl* locus. This tripartite interaction illustrates the combinatorial action of *N*, *Dl* and *E*(*spl*) during imaginal development.

PHENOTYPIC interactions that involve members of the zygotic neurogenic gene set suggest that products of three genes may interact directly during embryonic and imaginal development of *Drosophila melanogaster*. These interactions involve alleles of *Notch* (*N*, 1-3.0, 3C7), *Delta* (*Dl*, 3-66.2, 92A1-2) and *Enhancer of split* [*E*(*spl*), 3-89.1, 96F11-14] (WELSHONS 1956; VASSIN, VIELMETTER and CAMPOS-ORTEGA 1985; DE LA CONCHA *et al.* 1988; ALTON *et al.* 1989).

Comparison of the development of wild-type embryos and neurogenic mutant embryos indicates that neurogenic loci participate in the regulation of the segregation of the ectoderm into neural and epidermal lineages (POULSON 1937, LEHMANN *et al.* 1983). Cell transplantation (TECHNAU and CAMPOS-ORTEGA 1987) and ablation (DOE and GOODMAN 1985) experiments imply that at least some neurogenic loci provide functions that mediate cell-cell interactions central to establishment of the epidermal lineage within the ectoderm. Molecular analyses of *N* (WHARTON *et al.* 1985; KIDD, KELLEY and YOUNG 1986) and *Dl* (VASSIN *et al.* 1987, KOPCZYNSKI *et al.* 1988) imply that products encoded by these genes are structurally analogous to proteins known or believed to be involved in cell-cell communication in other organisms. Analysis of the *E*(*spl*) region (HARTLEY, PREISS and ARTAVANIS-TSAKONAS 1988) has led to the identification of another gene product that is essential for embryonic neurogenesis and that could also be involved in mediating cell-cell interactions.

The first imaginal genetic interaction to be described that involves an allele of a zygotic neurogenic gene is between mutations in *N* and *E*(*spl*) (WELSHONS 1956). As the name implies, *E*(*spl*) was originally identified on the basis of a dominant gain-of-function allele, $E(spl)^D$, that leads to a severe reduction in the size of the compound eye in males hemizygous for the recessive *N* allele *split* (*spl*). More recently, genetic analysis has revealed a set of pairwise interactions among alleles of *N*, *E*(*spl*) and *Dl* that affect development of the wing (VASSIN, VIELMETTER and CAMPOS-ORTEGA 1985). The observation that adults heterozygous for loss-of-function mutations that affect any one of these three loci exhibit dominant wing phenotypes reveals the sensitivity of wing development to the dosage of each of these genes. Enhancement or suppression of the haploabnormal phenotype associated with any member of this triumvirate can apparently result from the appropriate alteration of the gene dosage of any other member of this set. The existence of these phenomena suggests that these three loci exert combinatorial effects on imaginal ectodermal development.

Embryonic interactions that involve neurogenic genes are observed in animals heterozygous for loss-of-function mutations that affect *E*(*spl*) and either *N* or *Dl*. Such animals exhibit embryonic lethality and partially penetrant neural hypertrophy even though they still possess one wild type copy of each mutated locus (VASSIN, VIELMETTER and CAMPOS-ORTEGA 1985). This result is unanticipated because adults heterozygous for a loss-of-function mutation in any one of these loci exhibit neither reduced viability nor the embryonic neurogenic phenotype (POULSON 1937; LEHMANN *et al.* 1983). The same study (VASSIN, VIELMETTER and CAMPOS-ORTEGA 1985), as well as one by DE LA CONCHA *et al.* (1988), demonstrated that the

phenotype of embryos hemizygous for loss-of-function mutations in *N* can also be modified by increasing either the zygotic dosage of *Dl* or the maternal dosage of *E(spl)*. The interpretation of these studies must be qualified by noting that the duplications and deficiencies employed alter the dosage of a number of loci in addition to the specific genes in question. Evidence that we present suggests a new interpretation for previous data regarding inferred embryonic interactions between either *N* or *Dl* and rearrangements that affect *E(spl)*.

We have isolated loss-of-function mutations that affect *E(spl)* in order to further characterize the trans-heterozygous embryonic lethal interaction between alleles of *Dl* and *E(spl)*. A common method of obtaining loss-of-function mutations for a locus within which one initially possesses only dominant gain-of-function mutations involves reversion of the dominant phenotype associated with the mutation in question (ANDERSON, JURGENS and NUSSLEIN-VOLHARD 1985). Reversion of such mutations most frequently involves inactivation of the aberrant expression of the locus and concomitant generation of a loss-of-function mutation that affects the gene of interest. To our surprise, we found that screens for mutations that lead to reversion of the imaginal *spl-E(spl)^D* double mutant phenotype yielded not only intragenic lesions that affect *E(spl)*, but also a set of linked extragenic suppressors of this interaction. Combined genetic, cytogenetic and physical analyses indicate that these suppressor mutations are *Dl* alleles. These results reveal that the morphogenesis of the ectodermally derived adult eye is sensitive to the combined action of the products of *N*, *Dl* and *E(spl)*. We also deduce that the role of the *E(spl)* function, *per se*, in heterozygous embryonic lethal interactions between mutations that affect *E(spl)* and either *N* or *Dl* is questionable. However, these lethal interactions may involve one or more loci that map in proximity to and are distinct from *E(spl)*.

MATERIALS AND METHODS

Drosophila stocks: The markers employed have been described in LINDSLEY and GRELL (1968) unless otherwise noted. A stock isogenic for a single third chromosome marked with *E(spl)^D* (map position 89.1, LEHMANN *et al.* 1983) and *tx* (map position 91.0, LINDSLEY and GRELL 1968) was generated in our laboratory by meiotic recombination and appropriate crosses, beginning with an isogenic third chromosome carrying the *E(spl)^D* allele derived from a stock provided by WILLIAM WELSHONS (Iowa State University). A compound first chromosome marked with mutations in *yellow*, *white* and *forked* [*C(1)RM, y w f*; abbreviated *ywf:=*] was employed in some procedures. The *Dl* alleles *3* and *Df(3R)Cha^{M9}* and the isogenic stock *ss e^4 ro* have been described in ALTON *et al.* (1988). The balancer chromosomes *TM6B, Hu e Tb ca* (*TM6B*) and *In(3R)C, Sb e l(3)e* (*IS*) and other *Dl* alleles are described in ALTON *et al.* (1989) unless otherwise noted. The *N* allele *81k1* has been described in GRIMWADE *et al.* (1985).

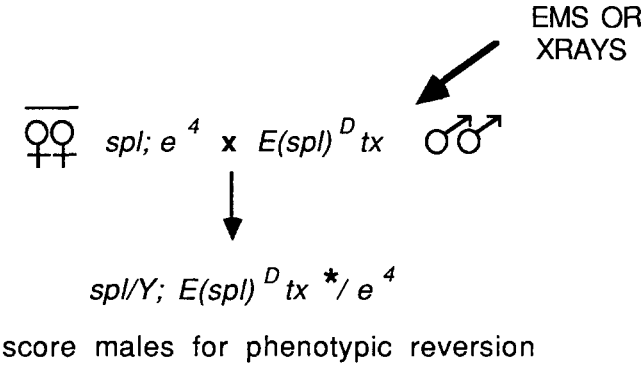

FIGURE 1.—Mutagenesis scheme used to recover phenotypic revertants of the *spl/Y;E(spl)^D tx/e^4* double mutant phenotype. Details are given in MATERIALS AND METHODS. Asterisk indicates mutagenized chromosome.

Terminology: Normal development of the compound eye leads to the establishment of a geometric array of ommatidia that serve as the primary mediators of photoreception in the adult fly (READY, HANSON and BENZER 1976). We will use the term "facet" to refer to the external manifestation of partial or complete ommatidial development within the compound eye. We employ this less specific term in our descriptions because ultrastructural studies of the eye in adult males of the genotype *spl/Y;e^4/e^4* and *spl/Y;E(spl)^D tx/e^4* indicate that ommatidial development is deranged, although the facets observed by scanning electron microscopy are substantially normal in these animals (D. W. WILLIAMS, T. R. TOKARSKI and M. A. T. MUSKAVITCH, unpublished data).

Mutagenesis: Males from an isogenic *E(spl)^D tx* stock were collected and mutagenized, then mated (Figure 1) to virgin females from a stock marked with mutations in *Notch* and *ebony* (*spl;e^4*) and brooded as described in ALTON *et al.* (1988). The resulting first generation (F_1) male progeny (46,100 third chromosomes from EMS mutageneses and 24,800 third chromosomes from X-ray mutageneses) that eclosed before 19 days postoviposition were scored for phenotypic reversion of the reduction in eye size associated with the *spl/Y;E(spl)^D tx/e^4* genotype (Figure 2). Males partially or fully reverted for the phenotype associated with this genotype were crossed to *ywf:=* virgin females to assess transmissibility of the revertant phenotype. Stocks isogenic for single third chromosomes of the genotype *Dl$^-$ E(spl)^D tx* or *E(spl)$^-$ tx* were constructed with *TM6B* or *IS* balancers; final stocks generated were free of first chromosomes carrying the *spl* mutation. Allele designations (Table 1): *BE*, *B*loomington *E*MS-induced; *BX*, *B*loomington *X*-ray-induced; *CE*, *C*ambridge *E*MS-induced; *CS*, *C*ambridge *S*pontaneous.

Scanning electron microscopy: Adult flies of the appropriate genotypes were generated in crosses involving the stocks *spl;e^4* and *E(spl)^D tx* and various reversion-bearing stocks. Flies were stored in 70% (v/v) ethanol in water and dehydrated serially in 80% (v/v, one wash), 95% (v/v, one wash) and 100% (v/v, three washes) ethanol in water. Specimens were then critical point dried in a Pelco model H critical point dryer. Fly heads were mounted on stubs, sputter coated with gold for 4 min in a Polaron SEM Coating Unit, then examined and photographed using a Cambridge Stereoscan 250 MK2 scanning electron microscope.

Complementation tests: Tests for viability of specific allele combinations generally involved the mating of virgin females and males from stocks in which lethal-bearing chromosomes were balanced over *TM6B*. Parents were mated in

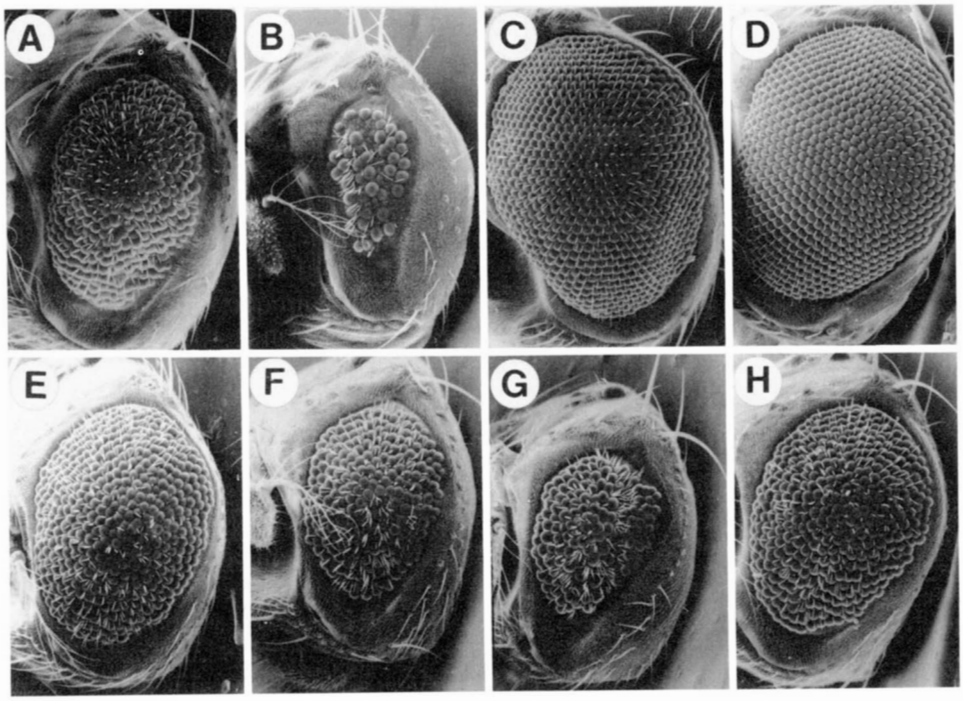

FIGURE 2.—Imaginal eye phenotypes resulting from various allele combinations involving mutations in *Notch*, *Delta* and *Enhancer of split*. Eyes were prepared for scanning electron microscopy as described in MATERIALS AND METHODS. Anterior is to the left and dorsal is up in each panel. A, *spl/Y;e*^{4}. B, *spl/Y;E(spl)*^{D} *tx/e*^{4}. C, *E(spl)*^{D} *tx* male. D, *Oregon-R* (Stanford isolate) male. E, *spl/Y;Dl*^{CS20}*/e*^{4}. F, *spl/Y;E(spl)*^{D} *tx/Dl*^{CS20}. G, *spl/Y;E(spl)*^{D} *tx/Df(3R)E(spl)*^{BX36}. H, *spl/+;E(spl)*^{D} *tx/e*^{4}.

vials and pupal (before day 14 postoviposition) and adult (before day 19 postoviposition) progeny were scored for exhibition of the Tubby^{+} phenotype. The appearance of such animals indicated that a given allele pair was viable to the pupal or adult stage of development, respectively. In some crosses, one of the parental stocks carried the balancer *IS* instead. Only adult progeny were scored in such crosses, and the presence of Stubble^{+} Tubby^{+} adult progeny was taken as an indicator of viability of the allele pair. For complementation tests involving *N*, crosses designed to yield maternal contribution of the *N* allele were performed with N^{81k1}*/FM7* virgin females and *E(spl)*$^{-}$*/TM6B* males. Crosses designed to yield paternal contribution of *N* involved *E(spl)*$^{-}$*/TM6B* virgin females and N^{81k1}*/Y;Dp(1;2)w*$^{+51b7}$*/+* males (GRIMWADE *et al.* 1985). The presence of Bar^{+} Tubby^{+} adult progeny in the former crosses and Bar^{+} Curly^{+} Tubby^{+} adult progeny in the latter crosses was taken as an indication of the viability of the allele pair. A minimum of 100 adult progeny were scored for each cross. Criteria for viability of allele combinations were: lethality, less than 5% of expectation; semilethality, between 5% and 15% of expectation; viability, greater than 15% of expectation.

Meiotic separability: Males from stocks heterozygous for reversion-bearing chromosomes and the balancer *TM6B* (*IS* for *BX23*) were mated to virgin females from the *ywf:=* stock. Virgin female progeny with the Tubby^{+} phenotype (Stubble^{+} for *BX23*) were mated to males of the genotype *spl/Y* in bottles. Male progeny of this cross were scored for occurrence of the reduced eye phenotype associated with the *spl/Y;E(spl)*^{D} genotype (Figure 2B). If the phenotypic reversion were due to a modifying mutation extragenic to the *E(spl)* locus, then we predicted that meiotic recombination would lead to separation of the modifying mutation from the *E(spl)*^{D} allele and subsequent expression of the severe double mutant phenotype in the progeny. Since we could reliably detect only one product resulting from such recombination events, we calculated the apparent separability of the modifying mutation from *E(spl)*^{D} as a fraction equal to $2n^{sE}/n^{t}$ in which n^{sE} equals the number of males exhibiting the *spl/Y;E(spl)*^{D} phenotype and n^{t} equals the total number of males scored. A minimum of 600 male progeny were scored for each reversion-bearing chromosome. Since the separability value approximates the meiotic map distance between *E(spl)* (map position 89.1) and the modifying locus, we anticipated a value on the order of 0.23 for modifying mutations within *Dl* (map position 66.2). Conversely, intragenic reversion of the *E(spl)*^{D} allele (or a modifying mutation mapping very close to the locus) would yield a separability approximating zero. Those alleles listed in Table 1 for which separability values of <0.01 are given yielded no apparent recombinants among over 600 male progeny scored in each case.

Phenotypic criteria: Phenotypic reversion of the *spl/Y; E(spl)*^{D} *tx* eye phenotype was classified as: weak, 50–150 facets/eye; moderate, 150–250 facets/eye; strong, 250–350 facets/eye or full, 350–450 facets/eye. Terminal cuticular phenotypes were assessed in mixed populations of embryos resulting from brooding of balanced stocks of the genotype *Dl*$^{-}$ *E(spl)*^{D} *tx/TM6B*, for extragenic suppressors, or *E(spl)*$^{-}$ *tx/TM6B*, for apparent intragenic revertants. Phenotypes for embryos homozygous for mutated *Dl* alleles were classified as weak hypomorph, intermediate hypomorph or amorph as described in ALTON *et al.* (1988). Phenotypes of embryos homozygous for mutated *E(spl)* alleles were classified as: mild, equivalent to the weak hypomorph class of Delta^{-} phenotypes; moderate, equivalent to the intermediate hypomorph class of Delta^{-} phenotypes; and severe, corresponding to loss of ventral, lateral and the majority of dorsal cuticle (only two small patches of unfused posterodorsal cuticle are usually observed).

Other procedures: Drosophila culture conditions, methods of cytological analysis, meiotic mapping of Delta^{-} wing phenotypes, cuticular preparations and direct immunofluorescence analysis of embryo whole mounts have been described in ALTON *et al.* (1988).

RESULTS AND DISCUSSION

Identification of two classes of phenotypic revertants: Hemizygosity for the *spl* mutation (*spl/Y*, Figure

TABLE 1

Extragenic suppressors and intragenic revertants of *E(spl)^D*

Allele	Degree of suppression or reversion[a]	Embryonic phenotype[b]	Viability in *trans* to *Dl*[c]	Separability[d]	Allelism
CS20[e]	M	I	L	0.23	*Dl*
CE21	S	A	L	0.30	*Dl*
CE23[e]	S	A	L	0.21	*Dl*
CE33[e]	S	I	L	0.21	*Dl*
CE34	M	I	L	0.19	*Dl*
CE37	M	I	L	0.21	*Dl*
CE43	M	I	L	0.34	*Dl*
BE21	M	I	L	0.27	*Dl*
BE23	W	W	L	0.28	*Dl*
BE24	M	I	L	0.24	*Dl*
BE26	W	W	L	0.25	*Dl*
BX32[e]	M	I	L	0.25	*Dl*
BX35[e,f]	M	I	L	0.02	*Dl*
BX38	S	A	L	0.23	*Dl*
BX39	S	A	L	0.23	*Dl*
BX40	M	I	L	0.24	*Dl*
BX41[e,f]	M-S	I	L	0.08	*Dl*
BX43[e,f]	M	I	L	0.04	*Dl*
BX44	S	A	L	0.21	*Dl*
BX45[e]	M	I	L	0.22	*Dl*
BX46[e]	W-M	W-I	L	0.22	*Dl*
BE22	F	MI	V	<0.01	*E(spl)*
BE25	F	MI	V	<0.01	*E(spl)*
BX37	S	wt	V	<0.01	*E(spl)*
BX21	M-S	MOD	V	<0.01	*E(spl)*
BX22	S-F	MOD	V	<0.01	*E(spl)*
BX23	W	MI	V	<0.01	*E(spl)*
BX36	F	SEV	V	<0.01	*E(spl)*
R1	F	SEV	V	<0.01	*E(spl)*
R2	F	SEV	V	<0.01	*E(spl)*
8D06	F	SEV	V	<0.01	*E(spl)*

The alleles listed were isolated in our laboratory with the exception of *R1* and *R2* (LEHMANN *et al.* 1983) and *8D06* (JURGENS *et al.* 1984). Cytological analysis of the *E(spl)* alleles listed above yielded the following cytogenetic definitions: *BE22*, *BE25*, *BX22* and *BX37* [normal]; *BX21* [T(2;3)41;96F10-12 (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988)]; *BX23* [not determined]; *BX36* [Df(3R)96F1;97B1]; *R1* [Df(3R)96F2;97A associated with In(3R)97A;99C (*ibid.*)]; *R2* [Df(3R)96F5;97A9 (*ibid.*)]; *8D06* [Df(3R)96F;97A3-4 (*ibid.*)].

[a] W = weak, M = moderate, S = strong, F = full; see MATERIALS AND METHODS for criteria for each class.

[b] Neurogenic phenotype of embryos homozygous for the allele listed [each *Dl* allele was linked to *E(spl)^D* and *tx*]: wt = wild type, W = weak hypomorph, I = intermediate hypomorph, A = amorph, MI = mild, MOD = moderate, SEV = severe; see MATERIALS AND METHODS for criteria for each class.

[c] Viability of the listed allele when *trans*-heterozygous with *Dl*^{3} in a cross in which the *Dl*^{3} allele is contributed maternally; L = lethal (viability <5% of expectation), V = viable (viability >15% of expectation); see MATERIALS AND METHODS for a description of the crosses performed.

[d] Approximate frequency of separation of the *E(spl)^D* allele from any modifying mutation on the third chromosome; see MATERIALS AND METHODS for procedures and interpretation.

[e] Correlated molecular lesion defined within the *Dl* locus (KOPCZYNSKI *et al.* 1988).

[f] Associated with a chromosomal translocation.

TABLE 2

Relationship between genotype and eye phenotype

Genotype[a]	Facets/eye[b] average ± SE	*n*[c]
Wild-type male	599 ± 25	14
spl/Y;e^4/e^4	373 ± 17	20
E(spl)^D tx/+ male	655 ± 16	6
spl/Y;E(spl)^D tx/e^4	45 ± 6	9
spl/Y;E(spl)^D tx/Dl^{CS20}	178 ± 18	5
spl/Y;E(spl)^D tx/Df(3R)Dl^{M2}	182 ± 28	5
spl/Y;E(spl)^D tx/Df(3R)E(spl)^{BX36}	139 ± 12	5
spl/Y;e^4/Dl^{CS20}	407 ± 32	5
spl/Y;e^4/Df(3R)Dl^{M2}	439 ± 43	5
spl/Y;e^4/Df(3R)E(spl)^{BX36}	469 ± 26	5
Wild-type female	634 ± 33	14
spl/spl;e^4/e^4	461 ± 46	12
E(spl)^D tx/+ female	666 ± 39	3
spl/+;E(spl)^D tx/e^4	391 ± 31	7
spl/+;*e^4*/+	646 ± 26	5

[a] The designations wild type and +, for third chromosomes, refer to the strain Oregon-R and chromosomes therefrom, respectively. The chromosome designated + in the genotype *spl/+;E(spl)^D tx/e^4* is derived from the isogenic *E(spl)^D tx* stock described in MATERIALS AND METHODS.

[b] The values presented represent the simple average of the values obtained by counting facets in scanning electron micrographs of eyes bearing the listed genotype, prepared as described in MATERIALS AND METHODS. The standard error presented represents the 99% confidence limit ($P < 0.01$) calculated using the formula SE = $2.576(\sigma_{n-1})/\sqrt{n}$ in which SE equals the standard error, n equals the sample size analyzed for a given genotype and σ_{n-1} equals the standard deviation of the values in the sample.

[c] Number of eyes for which facets were counted for the genotype listed.

2A) leads to a reduction in the size of the eye to approximately 370 facets (Table 2) and to distinct disorganization of the facet array and duplication of some interommitidial bristles. In comparison, the eye of a wild-type adult male (Figure 2D) contains approximately 600 facets (Table 2) in a regular geometric array. The dominant gain-of-function allele *E(spl)^D* fails to affect the size of the eye (Table 2) or the regularity of the ommatidial array in males (Figure 2C) or females carrying only wild type *N* alleles. However, males of the genotype *spl/Y;E(spl)^D tx/e^4* exhibit eyes (Figure 2B) that consist of only 45 facets (Table 2). This interaction is sufficiently strong that the split$^-$ phenotype is expressed in a pseudodominant fashion in females of the genotype *spl/+;E(spl)^D tx/e^4* (Figure 2H). The eye in such females is reduced in size to that of a *spl/Y* male (Table 2) and is similarly disorganized. These quantitative and qualitative data illustrate the synergistic effect of the *spl* and *E(spl)^D* mutations on the development of the compound eye.

We utilized this synergistic phenotype as the basis for a screen in which we isolated a set of 34 revertants of the *spl/Y;E(spl)^D tx/e^4* phenotype (Figure 1, Table 1). The extent of phenotypic reversion varies widely between marginal improvements in the phenotype and complete reversions of the *spl/Y;E(spl)^D tx/e^4* phenotype to one approximating the *spl/Y* phenotype. Parallel effects are exerted on the pseudodominant

expression of the split$^-$ phenotype observed in females heterozygous for *spl* and *E(spl)^D* (data not shown). Analyses described below indicate that 28 of these apparent reversions were correlated with third chromosomal mutations. We also obtained a set of six dominant modifiers of the *spl-E(spl)^D* interaction that map to the second chromosome; these will not be considered below.

Genetic lesions correlated with reversion of the *spl/Y;E(spl)^D tx/e^4* phenotype fall into two groups based on phenotypes observed in animals heterozygous for the mutated chromosome and a wild-type third chromosome. Class I mutations (21 alleles) yield wing venation phenotypes similar to those associated with heterozygosity for *Dl* mutations, and class II mutations (seven alleles) yield wild type wing venation. Complementation tests reveal that chromosomes that carry class I mutations are invariably lethal when heterozygous with *Dl^3* (Table 1) and, with one exception (*BX43*), lethal or semi-lethal when heterozygous with *Df(3R)Cha^{M9}* (data not shown). When contributed paternally, chromosomes that carry class II mutations are each viable over both of these *Dl* alleles (Table 1). These phenotypic properties and complementation patterns raise the possibility that a significant fraction of the phenotypic reversions are the result of mutations that affect *Dl*, not *E(spl)*.

***Dl* is a modifier of the *spl-E(spl)^D* interaction:** The first apparent reversion of the *E(spl)^D* mutation we identified resulted from the class I mutation *CS20* (Figure 2F), which arose spontaneously during the construction of the isogenic *E(spl)^D tx* stock. Meiotic mapping of the mutation responsible for the wing phenotype associated with the *CS20*-bearing chromosome in relation to the third chromosomal markers *spineless* (*ss*, map position 58.5), *ebony* (*e*, map position 70.7) and *rough* (*ro*, map position 91.1) revealed that the dominant wing venation phenotype associated with *CS20* maps to a position 62% distal from *ss* within the *ss-e* interval, corresponding to approximate map position 66 (58 recombinants within the *ss-e* interval were assessed). Parallel mapping of *Dl^3* revealed that the associated wing venation defect maps at a position 64% distal from *ss* within the *ss-e* interval, corresponding to approximate map position 66 (50 recombinants within the *ss-e* interval were assessed). Males carrying recombinant chromosomes obtained during the *CS20* meiotic mapping experiment were mated to *spl; E(spl)^D* virgins to determine the cosegregation frequency of the dominant wing phenotype and the apparent reversion of the *spl/Y;E(spl)^D tx/e^4* phenotype. Within a set of 11 recombinant chromosomes tested (four yielding Delta$^-$ phenotypes and seven yielding Delta$^+$ phenotypes), phenotypic reversion of the *spl/Y;E(spl)^D tx/e^4* phenotype correlated exactly with the dominant wing phenotype that maps on the *CS20* chromosome. These data strongly suggest that the phenotypic reversion observed for the *CS20* chromosome reflects an extragenic suppression of the *spl-E(spl)^D* interaction by a mutation within *Dl*.

We then determined the frequency with which each of the other putative suppressing class I mutations could be separated from the *E(spl)^D* mutation by meiotic recombination (Table 1). We found that 18 of the 21 chromosomes carry suppressing mutations that separate from the *E(spl)^D* mutation with an average frequency of 0.24 ± 0.02 (±SE, $n = 18$). Cytogenetic analysis of the three chromosomes that exhibit very low frequencies of separation (*BX38*, *BX41* and *BX43*) reveals that each has suffered a translocation involving one breakpoint within the 92A interval. Since the meiotic map distance between *Dl* and *E(spl)* is approximately 23 map units and the cytological location of *Dl* is 92A1-2, these data are consistent with the hypothesis that class I mutations are *Dl* alleles.

Molecular analysis of class I mutations reveals that at least nine suppressing mutations are correlated with physical alterations within *Dl* (Table 1; KOPCZYNSKI *et al.* 1988). Each of these 21 chromosomes yields a terminal embryonic phenotype in homozygotes that involves the neural hypertrophy and reduction of the epidermis characteristic of loss-of-function mutations in zygotic neurogenic loci (LEHMANN *et al.* 1983). *Dl* is the only known neurogenic locus that is located at a distance from *E(spl)* that would be consistent with the meiotic separability exhibited by the majority of suppressing class I mutations.

Finally, we find that the suppression of the *spl-E(spl)^D* interaction by class I mutations can be mimicked by the deficiency *Dl^{M2}* (Table 2) and other *Dl* alleles tested (data not shown). While a number of independent amorphic *Dl* alleles (*3*, *BE32*, *Df(3R)bxd^{110}* and *Df(3R)Cha^{M9}*; ALTON *et al.* 1988) tested act as suppressors of this interaction, a set of independent hypomorphic *Dl* alleles assayed (*BE34*, *ibid.*; *BX8* and *HD82*, ALTON *et al.* 1989) do not. These results imply that suppression of the *spl-E(spl)^D* interaction is dependent on the degree of reduction in the function of the *Dl* locus and is not necessarily mediated by a specific class of *Dl* alleles.

In contrast to a previous report (KNUST *et al.* 1987), we find no alleles of the neurogenic locus *neuralized* (*neu*, 3-50.5, 86CD) among 21 independent class I mutations we have isolated. Each of the class I mutations we have characterized is viable in heterozygous combination with the amorphic allele *neu^{1F65}* (LEHMANN *et al.* 1983) (data not shown).

Genetic analyses in *Drosophila* and other organisms have demonstrated that phenotypic interactions between mutations in independent loci often identify genes that encode functionally related products. Some mutations that fail to complement β-tubulin mutations

map to α-tubulin genes (STEARNS and BOTSTEIN 1988) or loci that appear to encode products involved in microtubule function (REGAN and FULLER 1988), and numerous interactions can be defined among loci known or believed to encode products involved in the structure and function of muscle (PARK and HORVITZ 1986, HOMYK and EMERSON 1988). Such intergenic interactions may be allele-specific or dosage-dependent for different pairs of loci, respectively. The interactions we observe among *N*, *Dl* and *E(spl)* mutations may therefore reflect the functional relatedness of products derived from these genes during imaginal development.

Intragenic reversion of *E(spl)^D*: All class II mutations are viable in transheterozygous combination with Dl^3 or $Df(3R)Cha^{M9}$ when either of these *Dl* alleles is contributed maternally (Table 1). This suggests that class II mutations are genetically distinct from the class I mutations. All attempts to recover the $E(spl)^D$ allele from each of the class II revertant chromosomes by meiotic recombination failed (Table 2), which implies that these mutations are intragenic revertants of $E(spl)^D$.

The *BX21* mutation is associated with a translocation that affects the chromosomal interval 96F10-12, while the *BX36* mutation is correlated with a large deficiency (96F1;97B1) that removes this same interval (Table 1). The cytogenetic analysis of the previously isolated *E(spl)* mutations *R1*, *R2* (LEHMANN *et al.* 1983) and *8D06* (JURGENS *et al.* 1984) indicates that each of these alleles is correlated with an extensive deficiency that affects the same region of the chromosome (Table 1). Therefore, two of the class II mutations, as well as these three previously identified alleles, are correlated with rearrangements that affect the chromosomal interval within which *E(spl)* maps (ZIEMER *et al.* 1988; PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Complementation tests indicate that *BX21*, *BX22* and *BX36* each fail to complement *groucho* (*gro*, map position 90) a recessive mutation that appears to be an allele of *E(spl)* (*ibid.*, KNUST *et al.* 1987). These combined data indicate that these three class II mutations are alleles of *E(spl)*.

Inter se complementation analysis among the seven newly isolated class II mutations and three preexisting *E(spl)* alleles reveals the existence of a number of qualitatively distinct alleles that affect *E(spl)* (Figure 3). The chromosome bearing *BX37* is homozygous viable and viable over all other mutations tested. *BE22* and *BE25* are similarly viable over all other mutations tested, but the chromosomes bearing each of these alleles are lethal in homozygotes. These homozygotes exhibit a mild neurogenic phenotype (Table 1). We have not yet determined whether this phenotype results from some partial impairment of the wild-type *E(spl)* function or from accessory lethal mutations on each chromosome. Certain pairwise combinations of alleles *BX21*, *BX22* and *BX23*, which yield neurogenic phenotypes in homozygotes ranging from mild to moderate (Table 1), exhibit *inter se* complementation behavior that varies depending on which allele is contributed maternally. The *BX23/BX21* allele pair exhibits impaired viability only when *BX23* is contributed maternally, and the *BX22/BX23* allele pair exhibits lethality only when *BX22* is contributed maternally. The latter case can be explained by invoking the maternal effect that has been reported to be associated with *E(spl)* (KNUST *et al.* 1987) and noting that *BX22* is a more severely affected allele than *BX23* (Table 1). The explanation for the former case may be analogous if the variegation associated with the translocation *BX21* is such that *BX21* can be less severely affected than *BX23* in a significant fraction of females in the crosses performed. Allele *BX36* behaves exactly as the preexisting deficiencies *R1*, *R2* and *8D06*. It is viable in combination with *BX37*, *BE22* and *BE25* and lethal when heterozygous with all other alleles tested. The alleles we have isolated therefore range from a putative exact revertant (*BX37*) to complete loss-of-function mutations.

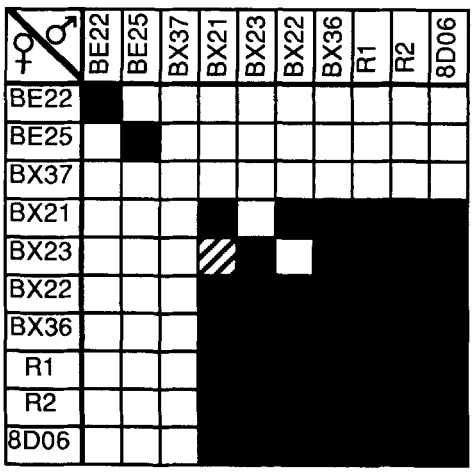

FIGURE 3.—Complementation table for heterozygous combinations of apparent intragenic reversions of $E(spl)^D$. Details are given in MATERIALS AND METHODS. Males for a given cross are listed along the horizontal axis, and virgin females for a given cross are listed along vertical axis. Open box, viable allele combination; hatched box, semilethal allele combination; filled box, lethal allele combination. See MATERIALS AND METHODS for criteria for each class.

We note that the severity of the neurogenic phenotype differs for independent mutations each of which appears to affect the *E(spl)* locus (Table 1). *BX22* has been correlated with a 14-kilobase (kb) deletion and an immediately adjacent 14-kb inversion in distal 96F (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Embryos homozygous for this mutation exhibit a moderate phenotype. *BX36*, and the other large, multiband deficiencies that remove the locus, yield a severe phenotype in homozygotes involving an

even greater reduction in the epidermis than that associated with amorphic *Dl* mutations.

Other authors have argued that the *E(spl)* locus is genetically complex and is comprised of multiple functions that map within the distal portion of cytological interval 96F (ZIEMER *et al.* 1988) based, in part, on the observation that large deficiencies that affect 96F yield more severe neurogenic phenotypes than smaller deficiencies that affect 96F. We observe the same trend. However, *BX22* eliminates or severely truncates the DNA that encodes each of six transcripts; among these six, only two, which appear to have the same coding capacity and arise from a single transcription unit (HARTLEY, PREISS and ARTAVANIS-TSAKONAS 1988), have been directly demonstrated to play a role in embryonic neurogenesis to date (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). The more severe neurogenic phenotype associated with larger deficiencies must therefore reflect the elimination of an independent locus or loci, in addition to those affected by *BX22*, that also affect(s) ectodermal differentiation and map(s) in close proximity to the *E(spl)* locus.

Vital interactions among *N*, *Dl* and mutations that affect *E(spl)* may not involve *E(spl)*, *per se*: Given the reported maternal-dependence of the heterozygous lethal interactions that occur among *N*, *Dl* and *E(spl)* (VASSIN, VIELMETTER and CAMPOS-ORTEGA 1985), we conducted complementation tests among alleles of these loci by performing each cross in two directions: maternal or paternal contribution of each *E(spl)* allele (Table 3). We found that *BX36* and other large deficiency alleles of *E(spl)* are semilethal or lethal in heterozygous combination with either Dl^3 or $Df(3R)Cha^{M9}$ when the *E(spl)* allele is maternally contributed to zygotes. The extent of the reduction in viability is correlated with the extent of the reduction in *Dl* function in these crosses (Table 4). Fully penetrant lethality is observed only when the *Dl* allele present is one of a number of amorphic alleles tested. This result implies that this lethal interaction is dependent on relative gene dosage and is not mediated by a specific class of *Dl* alleles. We do not, however, observe a significant reduction in the viability of heterozygotes that carry any of the other *E(spl)* mutations tested, independent of the direction of the cross (Table 3).

We have also analyzed the allele- and maternal-dependence of the lethal phenotypic interaction observed in zygotes heterozygous for mutations that affect *N* or *E(spl)*. We find that heterozygotes that carry the amorphic mutation N^{81k1} exhibit reduced viability only when they also carry a third chromosomal mutation that involves extensive deletion of material encompassing *E(spl)* (*e.g.*, *BX36*, *R1*, *R2* or *8D06*), just as we observe for the *Dl-E(spl)* interaction.

TABLE 3

Viability[a] of animals heterozygous for *E(spl)* and *Dl* or *N* mutations

E(spl) allele	Dl^3 male	Dl^3 female	$Df(3R)Cha^{M9}$ male	$Df(3R)Cha^{M9}$ female	N^{81k1c} *female*
BX37 (wt)[b]	+	+	+	+	+
BE22 (MI)	+	+	+	+	+
BE25 (MI)	+	+	+	+	+
BX21 (MOD)	+	+	+	+	+
BX22 (MOD)	+	+	+	+	+
BX36 (SEV)	–s	+	–	+	–
R1 (SEV)	–	+	–	+	–
R2 (SEV)	–s	+	–	+	–
8D06 (SEV)	–s	+	–s	+	–

Heterozygotes were generated by crossing sets of flies, from balanced stocks, heterozygous for different single neurogenic gene mutations; see MATERIALS AND METHODS for a description of the crosses performed. Crosses involving the alleles Dl^3 and $Df(3R)Cha^{M9}$ were performed in both polarities, *i.e.*, the *Dl* mutation was contributed either paternally ("Dl^3 male") or maternally ("Dl^3 female").

[a] Viability: – = lethal, –s = semilethal, + = viable; see MATERIALS AND METHODS for numerical criteria for different complementation behaviors.

[b] Neurogenic phenotype of animals homozygous for the *E(spl)* mutation listed: wt = wild type; MI = mild; MOD = moderate, SEV = severe. Criteria for the various phenotypic classes listed are described in MATERIALS AND METHODS.

[c] Identical results were obtained in crosses in which the N^{81k1} allele was contributed paternally, as described in MATERIALS AND METHODS.

The *BX22* mutation is not lethal in heterozygous combination with N^{81k1}, nor are any of the remaining *E(spl)* alleles that have been tested. Crosses that involve *N* mutations and mutations that affect *E(spl)* also reveal that the reduction in viability observed is the same for a given allele pair independent of the direction of the cross in which zygotes are generated. We do observe a marginal number of heterozygous escapers in some crosses involving paternal contribution of large deficiency alleles of *E(spl)* and no escapers in crosses involving maternal contribution (data not shown). However, crosses in both directions yield levels of survival below our lethal criterion (5% of expectation). It appears that the combinatorial zygotic requirement for *N* and functions that map in proximity to *E(spl)* is such that neither the maternal contributions from *N* nor those from loci in the vicinity of *E(spl)* can compensate for the deficit in zygotic expression of these loci.

Neither the *N* nor the *Dl* mutations we have tested are lethal in heterozygous combination with *BX22*, yet some of these same mutations are lethal when heterozygous with large deficiencies that affect *E(spl)* (Table 3). Lethality is therefore observed only when functions in addition to those affected by *BX22* are eliminated from the chromosome. This genetic behavior may reflect the synergistic impact of reduction in the level of *N* or *Dl* function *and* the function of *E(spl)* and flanking loci, since the *BX22* lesion presum-

TABLE 4

Viability[a] of animals *trans*-heterozygous for *Df(3R)E(spl)^{BX36}* and different *Dl* alleles

Allele	Embryonic phenotype[b]	*Trans*-heterozygotes as fraction of total progeny (*n*)[c]	Viability
HD9[d]	W	0.37 (242)	+
BE33[e]	W	0.34 (326)	+
BE31[e]	W	0.30 (307)	+
9K23[f]	W	0.21 (136)	+
6B37[f]	I	0.22 (140)	+
HD40[d]	I	0.19 (167)	+
BE38[e]	I	0.10 (370)	+
HD82[d]	I	0.06 (217)	+
HD62[d]	I	0.04 (189)	–s
BE32[e]	A	0.04 (317)	–s
Df(3R)Dl^{12e}	A	0.04 (179)	–s
3[e]	A	0.04 (143)	–s
Df(3R)Cha^{M9e}	A	0.02 (194)	–s
Df(3R)Dl^{M2e}	A	0.02 (106)	–s
Df(3R)Dl^{HD28e}	A	0.01 (227)	–
9D27[f]	A	0.01 (202)	–
9M46[f]	A	0.01 (179)	–
Df(3R)bxd^{110e}	A	<0.01 (184)	–
9P39[f]	A	<0.01 (155)	–

Trans-heterozygotes were generated by crossing virgins of the genotype *Df(3R)E(spl)^{BX36}/TM6B* to males of the genotype *Dl^{-}/TM6B*, as described in MATERIALS AND METHODS.

[a] Viability: – = lethal, –s = semilethal, + = viable; see MATERIALS AND METHODS for numerical criteria for different complementation behaviors.

[b] Neurogenic phenotype of embryos homozygous or hemizygous for the *Dl* allele listed: W = weak hypomorph, I = intermediate hypomorph, A = amorph. See MATERIALS AND METHODS for criteria for each class.

[c] The maximum fraction of the first generation progeny expected to be *trans*-heterozygotes was 0.33 for each cross as performed for this analysis. *n* = total number of progeny scored from a given cross.

[d] YEDVOBNICK *et al.* (1985).

[e] ALTON *et al.* (1988).

[f] LEHMANN *et al.* (1983).

ably affects a function or functions essential for *E(spl)* activity (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). However, we currently favor the more conservative interpretation that the observed heterozygous lethality reflects the combinatorial requirement for products of *N* or *Dl* and loci *other than E(spl)* that map in proximity to *E(spl)* (see below).

The nature of the *E(spl)* locus: The *E(spl)* locus was originally defined on the basis of the gain-of-function allele *E(spl)^{D}* (WELSHONS 1956). LEHMANN *et al.* (1983) subsequently proposed that *E(spl)* is a member of the zygotic neurogenic gene set based on their observation that certain revertants of the *E(spl)^{D}* mutation yielded severe neurogenic mutant phenotypes in homozygotes. The observations of VASSIN, VIELMETTER and CAMPOS-ORTEGA (1985) that amorphic alleles of *Dl* and *N* were lethal in heterozygous combination with the revertant allele *E(spl)^{R1}* and that a fraction of these inviable embryos exhibited neural hypertrophy were presented as further support for this contention. However, subsequent cytogenetic analyses (Table 1; PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988) revealed that the *R1* allele and other alleles that yield severe phenotypes in homozygotes are large deficiencies that remove multiple polytene chromosome bands and, presumably, multiple genetic loci. In fact, no well-defined point mutations that specifically affect *E(spl)* and yield a severe neurogenic phenotype in homozygous or hemizygous configuration have been described in the literature (LEHMANN *et al.* 1983; ZIEMER *et al.* 1988; PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988; this work). As a result, no one has directly demonstrated that the function(s) that is altered by the *E(spl)^{D}* mutation and that mediates enhancement of the imaginal split^{-} phenotype is required during embryonic neurogenesis.

We suggest that the most parsimonious definition of the *E(spl)* locus is the transcription unit (or units) that encodes the product which, when altered by the *E(spl)^{D}* mutation, leads to enhancement of the split^{-} phenotype. Only when this product has been identified and the developmental consequences of mutations that eliminate only this gene product have been defined will we know whether *E(spl)* is indeed a neurogenic gene. Once this uncertainty is resolved, we will be able to conclusively assert whether the imaginal interaction we have described among *Dl*, *N* and *E(spl)^{D}* alleles constitutes an interaction among three neurogenic genes or among two neurogenic genes and the *E(spl)* locus.

Two groups have published molecular analyses of the *E(spl)* region (KNUST, TIETZE and CAMPOS-ORTEGA 1987; PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Data from these analyses that are relevant to the interpretation of some of our results are summarized in Figure 4. It is clear that the mutation *E(spl)^{BX22}* deletes portions or the entirety of genomic sequences that contribute to at least six distinct transcripts. Within the interval defined by the *BX22*-associated deletion, only the product that is encoded by the m9/m10 transcription unit and is homologous to β-transducin (HARTLEY, PREISS and ARTAVANIS-TSAKONAS 1988) has been directly shown to be essential for embryonic neurogenesis to date (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). PREISS, HARTLEY and ARTAVANIS-TSAKONAS (1988) have shown that a transposon that contains the intact m9/m10 transcription unit, but no other intact transcription units, can rescue the lethality associated with heterozygosity for the *E(spl)^{D}* allele and deficiencies that affect the *E(spl)* region. However, it is not yet known whether the product of this transcription unit, as affected within the context of the *E(spl)^{D}* chromosome, is sufficient to mediate enhancement of the imaginal split^{-} phenotype.

We observe that heterozygotes that carry certain

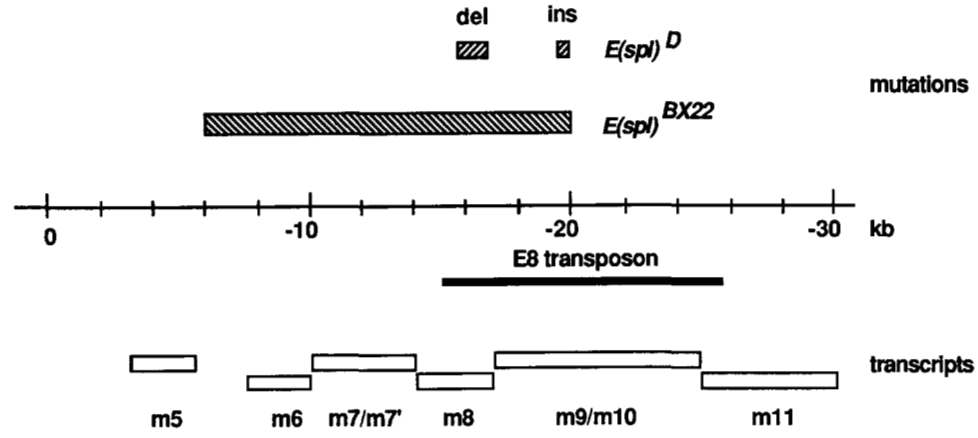

FIGURE 4.—The molecular map of a chromosomal interval within the *E(spl)* region. This figure summarizes portions of the molecular data presented by KNUST *et al.* (1987) (reference A) and PREISS, HARTLEY and ARTAVANIS-TSAKONAS (1988) (reference B). The coordinate system is that utilized in reference B. kb, kilobases. The chromosome that carries the $E(spl)^{D}$ mutations possesses a deletion (del) of approximately 0.4 kb of chromosomal DNA and an insertion (ins) of approximately 5 kb of DNA in comparison to wild type chromosomes within the intervals indicated by the rightward cross-hatched boxes. The $E(spl)^{BX22}$ allele is associated with a deletion of approximately 14 kb of chromosomal DNA indicated by the leftward cross-hatched box. The chromosomal segment indicated by the solid bar labelled "E8 transposon" has been employed to fully or partially rescue certain mutant phenotypes associated with the $E(spl)^{D}$ mutation (reference B). The maximum extents of chromosomal intervals that have been shown to cross-hybridize with particular embryonic transcripts are indicated by open boxes below the coordinate scale. The correspondences between transcript data presented in the two source references (A and B) are: m5, 1.0 kb (A), 1.4 kb (B); m6, 1.4 kb (A), 1.5 kb (B); m7, 1.5 kb (A), 1.5 kb (B); m7′ (not reported in A), 1.2 kb (B); m8, 1.0 kb (A), 1.2 kb (B); m9, 3.3 kb (A), 3.0 kb (B); m10, 4.4 kb (A), 4.0 kb (B); m11, 2.1 kb (A), 2.2 kb (B).

mutations in *N* or *Dl* and mutations that affect *E(spl)* are inviable when the mutation that affects the *E(spl)* region deletes a large chromosomal segment, but are viable if the *E(spl)* mutation present is *BX22* (Table 3). This result implies that the lethal interactions observed between *N* or *Dl* mutations and large *E(spl)* deficiencies cannot strictly reflect the combinatorial requirement for *N* or *Dl* and any of the functions eliminated by *BX22*. Therefore, if future analyses indicate that one or more of the functions affected by *BX22* are sufficient to mediate enhancement of the split⁻ phenotype, as we might expect based on the extent of reversion associated with the *BX22* mutation (Table 1), then the lethal interactions we and others observe will not reflect the specific combinatorial requirement for *N* or *Dl* and the *E(spl)* gene. Based on this reasoning, we currently favor the hypothesis that these lethal interactions reflect the combinatorial requirements for *N* or *Dl* and functions other than *E(spl)* that map in proximity to the *E(spl)* locus.

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