Two Genetically and Molecularly Distinct Functions Involved in Early Neurogenesis Reside Within the *Enhancer of split* Locus of *Drosophila melanogaster*

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ABSTRACT

Molecular correlation of the genetic aspects of the function of the neurogenic gene *Enhancer of split* [*E(spl)*] has previously been hampered by the densely transcribed nature of the chromosomal region within which it resides. We present data indicating that two distinct molecular species contribute to *E(spl)* function. Analysis of new *E(spl)* alleles has allowed us to define two complementing functions within the locus. Subsequent phenotypic analysis of different *E(spl)* deficiencies combined with *P* element-transformed constructs has demonstrated that these two functions correspond to: (1) a family of helix-loop-helix (HLH) protein-encoding genes and (2) the single copy gene *E(spl) m9/10*, whose product shares homology with G-protein β subunits. The zygotically active *E(spl)* HLH genes can, at least partially, substitute for one another's functions and their total copy number determines the activity of the locus. *E(spl) m9/10* acts synergistically with the *E(spl)* HLH genes and other neurogenic genes in the process of neurogenesis. The maternal component of *E(spl) m9/10* has the most pronounced effect in neurogenesis, while its zygotic component is predominantly required during postembryonic development. The lethality of *trans*-heterozygotes of null *E(spl)* deficiency alleles with a strong *Delta* point mutation is a result of the concomitant reduction in activity of both *E(spl)* HLH and *m9/10* functions. Immunocytochemical localization of the E(spl) m9/10 protein has revealed that it is a ubiquitously distributed nuclear component in embryonic, larval and imaginal tissues.

EMBRYONIC neurogenesis in Drosophila is an elaborate developmental process, in which progenitor neural cells (neuroblasts), in addition to acquiring their individual fates, must be sorted out from the ectodermal epithelium, where they are intermingled with prospective epidermal progenitor cells. The ectodermal domains from which neuroblasts arise are termed neurogenic regions and they consist of the ventral region of the trunk and two dorsolateral regions of the head (HARTENSTEIN and CAMPOS-ORTEGA 1984; CAMPOS-ORTEGA and HARTENSTEIN 1985). Within these regions, the neural fate is not preassigned to specific cells, rather intercellular communication is involved in deciding between neural and epidermal differentiation for each cell. From laser ablation studies in the analogous developing nervous system of the grasshopper, it appears that this communication consists in differentiating neuroblasts inhibiting their immediate neighbors from also acquiring a neural fate, thus committing them to epidermal differentiation (DOE and GOODMAN 1985). Although a similar study has not been performed in Drosophila, the genetics and molecular biology of a class of mutations, termed neurogenic mutations, are consistent with the existence of a signaling pathway to mediate exactly such a lateral inhibition mechanism. Loss of function of any of the neurogenic genes results in embryonic lethality due to hypertrophy of the nervous system and concomitant loss of ventral and cephalic epidermis (*i.e.*, the neurogenic phenotype), presumably due to the disruption of lateral inhibition and consequent misrouting of presumptive dermoblasts into neural differentiation. This gene class comprises the six zygotic loci *Notch*, *Delta*, *Enhancer of split*, *mastermind*, *neuralized* and *big brain*, as well as a number of less well characterized, maternally active genes (LEHMAN *et al.* 1983; PERRIMON and MAHOWALD 1986; PERRIMON, ENGSTROM and MAHOWALD 1989).

In accordance with the putative function of their products as interacting components of a signalling pathway, most neurogenic genes have been observed to exhibit numerous allele-specific and dosage-dependent interactions (VÄSSIN, VIELMETTER and CAMPOS-ORTEGA 1985; DE LA CONCHA *et al.* 1988; XU *et al.* 1990). In addition to deregulating embryonic neurogenesis, neurogenic mutations have pleiotropic effects disrupting other structures of the fly, such as the adult wing, eye and notum, suggesting that components of the proposed signaling mechanism are also active in other developmental decisions. The availa-

bility of such extensive genetic data has facilitated the molecular cloning and characterization of most of these genes. The best characterized to date are *Notch* (*N*) and *Delta* (*Dl*), both of which have been shown to encode transmembrane proteins containing EGF-like repeats in their extracellular domains (WHARTON *et al.* 1985; VÄSSIN *et al.* 1987; JOHANSEN, FEHON and ARTAVANIS-TSAKONAS 1989). In support of a direct molecular interaction (FEHON *et al.* 1990) showed that the products of *N* and *Dl* expressed on the surface of cultured Drosophila cells induce specific adhesion between *N* and *Dl* expressing cells.

Enhancer of split [*E(spl)*] was discovered as a dominant mutation that enhances the eye and bristle phenotypes of *spl*, a recessive viable allele of *Notch* (WELSHONS 1956). Although $E(spl)^D$ has no neurogenic phenotype on its own, its subsequent reversion revealed that deletions of *E(spl)*, presumably resulting in complete loss of function, exhibit a neurogenic phenotype (LEHMAN *et al.* 1983). These null alleles exhibit further interactions with neurogenic genes, specifically embryonic lethality as *trans*-heterozygotes with null alleles of either *N* or *Dl* (VÄSSIN, VIELMETTER and CAMPOS-ORTEGA 1985). A recessive viable allele, *groucho* (*gro*), that affects the adult head bristles, has also been characterized, while other lethal alleles have shown dominant interactions with *N* alleles *notchoid* (*nd*) and *Ax* (KNUST *et al.* 1987; XU *et al.* 1990). Although at the time it was not unambiguously shown that all of these phenotypes were due to defects in the same gene, we could nonetheless conclude that the *E(spl)* locus is intimately associated with the signaling pathway of embryonic neurogenesis as well as with other developmental decisions that involve neurogenic genes.

In order to develop a molecular definition of the *E(spl)* gene and further illuminate the lateral inhibition signal transduction mechanism in general, we undertook the cloning of the region encoding *E(spl)* using chromosomal breakpoints associated with *E(spl)* mutations as guides (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Unfortunately, breakpoint mapping did not unambiguously identify the transcription unit responsible for *E(spl)* functions, primarily due to the densely transcribed nature of the 96F10-14 region under study and the inability to recover point mutations in most of these transcription units. Indeed, all recessive lethal *E(spl)* alleles exhibiting extreme, fully penetrant neural hypertrophy, proved to be deletions of the entire cloned region. From extensive mutageneses for lethals uncovered by *E(spl)* deficiencies only one point-mutable complementation group was unambiguously identified; this corresponds to transcription unit *m9/10* (as named by KNUST, TIETZE and CAMPOS-ORTEGA 1987). Although these mutations exhibited neural hypertrophy, the expressivity and penetrance were much less than that observed with *E(spl)* deficiency alleles (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Additionally, ZIEMER *et al.* (1988) reported two classes of putative *E(spl)* hypomorphs: first, mutations that produced neural hypertrophy only over deficiencies but yielded a mild or wild-type embryonic lethal phenotype as homozygotes (weak hypomorphs); and, second, mutations with no effect on the neural/epidermal partitioning, that were included as *E(spl)* alleles because of their complete or partial lethality over other *E(spl)* mutations. We shall not consider the latter group any further, as it probably consists of lesions in neighboring vital genes that are affected by the *E(spl)* rearrangements with which they fail to complement. Even the weak hypomorphs were in most cases shown to be either translocations or cytologically invisible lesions with detectable restriction fragment alterations distributed across the cloned region (KNUST, TIETZE and CAMPOS-ORTEGA 1987), and thus could not adequately delimit the *E(spl)* gene.

Therefore, the only gene in this region unambiguously identified as having a role in embryonic neurogenesis was *m9/10*, a transcription unit producing two alternatively polyadenylated transcripts, encoding the same protein product (HARTLEY, PREISS and ARTAVANIS-TSAKONAS 1988). The carboxy-terminal half of this protein contains a repeated motif present in the β-subunit of G-proteins, a structure compatible with a putative signal-transducing molecule, but also found in non-G-proteins, such as the *Saccharomyces cerevisiae* proteins CDC4 involved in cell-cycle control (YOCHEM and BYERS 1987) and PRP4, a component of the spliceosome (PETERSEN-BJÖRN *et al.* 1989; DALRYMPLE *et al.* 1989). Given these molecular and genetic data, it was unlikely that *m9/10* alone could account for the entirety of *E(spl)* function. Nonetheless, a precise subset of the genetic functions of *E(spl)* could be attributed to *m9/10* using a *P* element construct containing only the wild-type *m9/10* gene (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). First, we were able to rescue all alleles of the single lethal, (weakly) neurogenic complementation group to adulthood even as heterozygotes over null *E(spl)* alleles (large deficiencies), thus defining the correspondence of the point lethal complementation group and *m9/10*. Second, two more phenotypes associated with *E(spl)* were also fully rescued: the recessive visible defects produced by *groucho*, and the lethality of the dominant $E(spl)^D$ allele over deficiencies of the locus. Although the embryonic neurogenic phenotype of less severe deficiency alleles was significantly improved, that of the most severe alleles was completely unaffected. This led to the conclusion that *m9/10* is responsible for only a subset of the genetic functions of *E(spl)* and that neighboring genes are also involved in

producing the extreme deficiency phenotype.

The participation of additional genes was suggested by the identification of the transcription unit responsible for the dominant enhancement of *spl*. KLÄMBT *et al.* (1989) showed that a transformed copy of *m8* (the gene immediately proximal to *m9/10*; see Figure 1) derived from the $E(spl)^D$ chromosome enhances *spl*. Therefore, the enhancement of *spl* is not due to the mutation in *m9/10*, which is, however, responsible for the lethality of $E(spl)^D$ over deletions. Indeed, molecular alterations in both *m8* and *m9/10* have been identified on the $E(spl)^D$ chromosome (KNUST, TIETZE and CAMPOS-ORTEGA 1987; PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Furthermore, KLÄMBT *et al.* (1989) reported the sequence of *m8* and three other neighboring genes whose expression is highest within the neurogenic ectoderm: *m4*, *m5* and *m7*. Interestingly, *m5*, *m7* and *m8* show significant sequence homology among themselves and are members of the helix-loop-helix (HLH) protein superfamily (MURRE, SCHONLEBER MCCAW and BALTIMORE 1989). Although no functional evidence was furnished regarding the involvement of these genes in neurogenesis, the interaction of an allele of *m8* with an allele of *Notch* made such participation a possibility, especially since this interaction is further modified by mutations in *Delta* (SHEPARD, BROVERMAN and MUSKAVITCH 1989). The similarity among these proteins raises the possibility that these genes could have a vital role, that would not be revealed as a result of a point mutation, since one member may be able to substitute for another. The fact that the amorphic state of *E(spl)* is only achieved by deficiencies could be explained by invoking such a cluster of redundant genes as responsible for a subset of *E(spl)* functions. Other HLH proteins are master regulatory factors for tissue differentiation, *e.g.*, members of the MyoD family induce muscle differentiation in mammals (WEINTRAUB *et al.* 1991). It is thus conceivable that *m5*, *m7* and *m8* play a central role in inducing the epidermal fate.

To summarize, the *E(spl)* chromosomal region encompasses several classes of genes, the best characterized of which are the HLH cluster and the single-copy G-protein-like *m9/10*. Insofar as only *m9/10* exhibited a lethal neurogenic phenotype as a result of point mutation, but at the same time *m8* was shown to interact with other neurogenic genes, we were confronted with the possibility that both of these classes might play a role in neurogenesis and further that members of the HLH class be functionally redundant. We decided to test this hypothesis using transformation constructs in combination with a variety of deletions. We undertook the analysis of a relatively broad region of 25 kb, which contains both *m9/10* and all three members of the HLH family. We present evidence from this analysis indicating that the HLH protein encoding genes are functional components of *E(spl)*. Although expression of these genes is strictly zygotic, the nearby *m9/10* gene is also expressed maternally (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Our data suggest that the function of *m9/10* in embryonic neurogenesis is primarily provided by its maternal component. Accordingly, we show that the zygotic component of *m9/10* is primarily required for postembryonic development. In accordance with the pleiotropy exhibited by *m9/10*, we have been able to detect the m9/10 protein in the nuclei of all cells by immunocytochemical staining. Our genetic and transformation data further predict that the *E(spl)* locus must contain an additional function, most probably one or more additional copies of HLH protein coding genes, proximal to the 25-kb region we have analyzed. We have relied on the embryonic neurogenic phenotype as our criterion for *E(spl)* activity, since other phenotypic traits associated with *E(spl)* have already been attributed to *m8* or *m9/10* exclusively (as mentioned above). We have further obtained evidence that the combined dosage effects of maternal *m9/10* and (zygotic) HLH *E(spl)* genes contribute to the lethality observed in animals *trans*-heterozygous for *E(spl)* deficiencies and a *Dl* point mutation.

MATERIALS AND METHODS

Fly strains: All genetic markers and chromosomes referred to in the text are described in LINDSLEY and GRELL (1968), except $E(spl)^{BX22}$ (SHEPARD, BROVERMAN and MUSKAVITCH 1989), $E(spl)^{E48}$ and $E(spl)^{E73}$ (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988), *Df(3R)8D06* (JÜRGENS *et al.* 1984), and $Df(3R)boss^{14}$ and $Df(3R)boss^{15}$ (HART *et al.* 1990). *Df(3R)P709* was a gift from A. HART and S. L. ZIPURSKY (University of California, Los Angeles).

Physical mapping of deficiency chromosomes: Cytological analysis of salivary gland polytene chromosomes was performed after crossing *TM6B*-balanced deficiency strains to *cn* ; ry^{506} and selecting Tb^+ (*ry/Df*) third instar larvae. To detect restriction map alterations, whole genome DNA blot hybridizations of heterozygous (*ry/Df*) mutant flies were performed. Fly DNA was prepared as described in PREISS, HARTLEY and ARTAVANIS-TSAKONAS (1988) and analyzed with at least four different restriction enzymes (*Eco*RI, *Hin*dIII, *Sal*I, *Xho*I). Probes spanning the entire cloned region were used to determine restriction site polymorphisms not present in the parental chromosomes. Alterations clustered in the same area were finely mapped using additional restriction enzymes and smaller probes.

Generation of transformation constructs: P[ry^+, *C2.8*] was generated by cloning a 2.8-kb *Cla*I genomic fragment containing *E(spl) m8* into the *Hpa*I site of Carnegie 20 (RUBIN and SPRADLING 1983), after filling in the 5′ overhangs of the insert using the Klenow fragment of DNA polymerase. P[ry^+, *X5.6*] was similarly created by cloning a 5.6-kb *Xho*I genomic fragment into the compatible *Sal*I site of Carnegie 20. P[ry^+, *Ep*] and P[ry^+, *E25R*] were generated in several steps. The inserts were built stepwise in Bluescript vectors (Stratagene) from a *Bam*HI fragment (−1 to −13 of the genomic walk coordinates, see Figure 1), a partial *Bam*HI-*Xho*I fragment (−13 to −16) and an *Xho*I fragment (−16 to −26). The *Not*I sites from the Bluescript polylinkers

were then used to subclone the respective fragments into the pDM30 vector (D. MISMER and G. RUBIN, personal communication).

Fly transformation: Injection mixes contained the *P* element constructs at a concentration of 300–500 ng/μl and helper plasmid pπ25.7wc (KARESS and RUBIN 1984) at 100 ng/μl. Precellularization *cn ; ry*^{506} or *ry*^{506} embryos were injected as described by RUBIN and SPRADLING (1982). Individual *ry*$^{+}$ progeny of the injected flies were backcrossed to *cn ; ry*^{506} to generate transformant stocks and the presence of a single copy of the construct was confirmed by Southern analysis (not shown). The chromosome carrying each insertion was determined by crossing to balancer carrying strains and observing the segregation of the *ry*$^{+}$ marker from the relevant dominant marker, or by *in situ* hybridization to polytene chromosomes. Homozygous transformant stocks were obtained from the balancer crosses used to identify their chromosomal position, or by pair mating *ry*$^{+}$ flies from the transformant stock and ensuring that no *ry*$^{-}$ progeny were obtained after two generations.

Phenotypic analysis: For allelic combinations producing no adult offspring, judging from standard segregation analysis, we determined the lethal period. For this purpose, we first outcrossed the balanced *E(spl)* strains to be tested to a *cn ; ry*^{506} strain to obtain *ry/E(spl)* individuals, which were then crossed in the appropriate combinations to generate the desired genotype. In this way we avoided balancer chromosomes in the test cross, which might have introduced additional lethal classes complicating the analysis. From the test cross, *ry/E(spl)*^{a} × *ry/E(spl)*^{b}, where *a* and *b* represent *E(spl)* alleles to be tested, we collected approximately 200 embryos laid on apple-juice agar Petri dishes and determined the percentage of hatching after correcting for unfertilized eggs. When hatching was close to 100%, showing that the mutant class is not embryonic lethal, we collected the first instar larvae into standard fly medium (cornmeal-agar-yeast-molasses) vials and scored the number of eclosing adult flies to confirm that 25% of the progeny is absent by the adult stage. Inspection of these vials for uneclosed pupae determined whether the mutant class was larval or pupal lethal.

In the cases of embryonic lethality, we assessed the cuticular phenotype and in some cases the anti-HRP staining pattern, which enables us to visualize the nervous system (JAN and JAN 1982). In all cases tested, the extent of neural hypertrophy judged by anti-HRP staining inversely correlated with the amount of cuticle remaining. We decided to base our phenotypic analysis on the cuticular phenotype rather than that of the nervous system, as the former allowed the distinction among subtler phenotypic gradations among individuals that appeared identical in their neural phenotypes. Mutant embryos were collected from a cross like the one described for lethal period determination [*ry/E(spl)*^{a} × *ry/E(spl)*^{b}], except that longer periods of egg laying were performed, the embryo collections were incubated for a further 36–48 hr at 25° and the hatching larvae were periodically cleared. The unhatched embryos remaining were dechorionated in 50% Clorox and mounted on microscope slides in Hoyer's medium (WIESCHAUS and NÜSSLEIN-VOLHARD 1986). After clearing at 60° for 24 hr, the cuticles of the dead embryos were observed under phase contrast optics using a Leitz Orthoplan microscope.

In the case of transformant-deficiency combinations, we outcrossed stocks homozygous for the transformant and heterozygous for the deficiency to the original transformant stock (*e.g., Ep/Ep ; TM3/E(spl)* × *Ep/Ep ; ry*^{506}) to obtain transformant-homozygous flies carrying the deficiency, but lacking the balancer, which were then used for determination of the lethal period and embryonic cuticular phenotype as described above. As most transformant stocks were generated in a *cn ; ry*^{506} background, we used this strain instead of a wild type for outcrossing flies containing no transformed constructs.

Immunocytochemistry: The fixation, staining and microscopy of embryos were performed as described in FEHON *et al.* (1991), except that 0.1% Triton X-100 was used in the place of saponin in the preincubation and staining buffers. Polyclonal rabbit and mice antisera were used at a dilution of 1:100 to 1:1000, while culture supernatants from anti-m9/10 monoclonal hybridoma lines were used either undiluted or at a dilution of 1:10.

RESULTS

In the following molecular genetic analysis of the *E(spl)* locus, the reader is referred to Figure 1, which summarizes the physical structure of the locus, including the positions of transcription units and breakpoints of deficiencies used in the present study.

Mapping and complementation of new deficiencies: To facilitate the transformation analysis, we had to extend our gamut of available deletion breakpoints, since originally we had only two classes of deficiencies judging by phenotype and breakpoint mapping (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988): (1) deficiencies removing all the cloned region, which result in extreme neural hypertrophy with only a small patch of posterior-dorsal cuticle present (*e.g.*, Figure 2, A, B, C); and (2) deficiencies breaking in the vicinity of *m5* and extending distally (*e.g., E(spl)*^{BX22}, see Figure 1), which exhibit a milder neural hypertrophy (intermediate phenotype) and carry an extensive shield of dorsal cuticle (from the posterior tip to about 75% embryo length) with occasional strips connecting it to a small patch of cephalic cuticle (*e.g.*, Figure 2, G, H, I, J).

We obtained three new deficiencies generated in an attempt to create alleles of the nearby *bride of sevenless* (*boss*) gene: *Df(3R)boss*^{14}, *Df(3R)boss*^{15} (HART *et al.* 1990) and *Df(3R)P709* which is *Df(3R)boss*$^{+}$ (A. HART and S. L. ZIPURSKY, personal communication). Cytological and genomic DNA blot analyses have identified a breakpoint common to all three deficiencies located in the *E(spl)* region at 96F10/11, close to the transcription start site of *m7*. Our data suggest that this is associated with an insertion element upstream of *m7* in the parent chromosome and that all three deficiencies have one of their breakpoints within that element: *Df(3R)boss*^{14} and *Df(3R)boss*^{15} delete DNA proximal to the insertion element, while *Df(3R)P709* deletes distally (Figure 1), in agreement with the location of the *boss* gene proximal to *E(spl)* (HART *et al.* 1990).

We have tested the complementation behavior of these three deficiencies inter-se as well as against *E(spl)m9/10* point mutations, the small deficiency *E(spl)*^{BX22} and large deficiencies such as *Df(3R)8D06*. A wide variation in the severity of neurogenic phe-

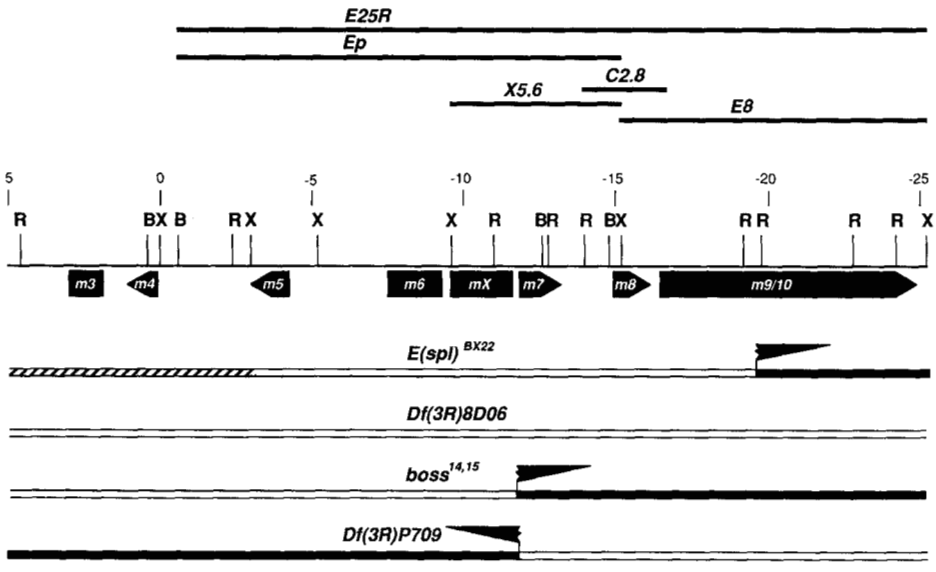

FIGURE 1.—A restriction map of approximately 30 kb of the *E(spl)* genomic region is shown, proximal is to the left, the numbering is given in kilobases and corresponds to the coordinates of our genomic walk (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). The different transcription units are indicated with their orientations when known; their nomenclature is according to KNUST, TIETZE and CAMPOS-ORTEGA (1987). The extents of different deficiencies are shown below the restriction map: empty boxes denote deleted DNA, filled boxes denote intact DNA, the striped box corresponds to an inverted DNA segment present next to the deletion in $E(spl)^{BX22}$. The broken triangles at the breakpoints of $E(spl)^{BX22}$, $Df(3R)boss^{14,15}$ and *Df(3R)P709* represent insertion elements present in the parent chromosomes and partially deleted in the deficiencies. The breakpoints of *Df(3R)P709*, $Df(3R)boss^{14}$ and $Df(3R)boss^{15}$ are within the same insertion element, but the former deletion extends distally while the latter two extend proximally. Cytologically, both *Df(3R)boss* chromosomes delete 96F3/4-96F10/11 and *Df(3R)P709* deletes 96F10/11-97D1/2. *Df(3R)8D06* [Df(3R)96E10/12-97A3/4; JÜRGENS *et al.* 1984) deletes the whole of the region indicated and indeed the whole *E(spl)* gene, since it behaves as a null. Above the restriction map we have indicated the extent of the different fragments used in transformation constructs for the present study. B, *Bam*HI; R, *Eco*RI; X, *Xho*I.

notypes was obtained from the various embryonic lethal combinations tested. To facilitate their description, we have arbitrarily divided the continuous range of neurogenic defects into five categories, as opposed to the three of LEHMAN *et al.* (1983); representative examples are given in Figure 2. **Extreme** neurogenic embryos (Figure 2, A, B, C) have only a miniscule patch of dorsoposterior cuticle or two patches of posterior lateral cuticle (due to failure in dorsal closure) along with a trace of pharyngeal cuticle. Occasionally, a telson (anal plates and anal tuft) can be seen extending posterior to the dorsal patch or bridging the two lateral patches. Such phenotypes are exemplified by embryos homozygous for large deficiencies of the locus, such as *Df(3R)8D06*, and presumably reflect complete absence of *E(spl)* function. **Moderate-extreme** embryos (Figure 2, D, E, F), such as *Df(3R)boss* homozygotes, have a dorsal cuticular shield extending from the posterior end (telson always present), and covering from ~50–75% of the total embryo length. Most of these dorsal shields are very narrow, covering no more than half of the width of the embryo. Occasionally, however, wider, shorter patches are produced, which in many cases are incompletely formed (with holes, *e.g.*, Figure 2F). Posterior spiracles are always present connected to the tracheae, usually at the very lateral edge of the cuticle shield, and accordingly Filzkörper are present albeit not well formed in most cases. Our extreme, moderate-extreme and some of the intermediate phenotypes correspond to the extreme class of LEHMAN *et al.* (1983). The **intermediate** class (Figure 2, G, H, I, J, K) is characterized by wider dorsal shields, spanning the width of the embryo, with well formed telson and Filzkörper. Most of these dorsal shields contain anterior projections at the ends of the anterior edge; in some cases one (Figure 2H) or both (Figure 2, I, J, K) of these projections actually connects with the remnants of pharyngeal cuticle at the anterior tip of the embryo. These are probably derived from the maxillary segment, as proposed by LEHMAN *et al.* (1983), who named this latter category intermediate. Embryos exhibiting a **weak-intermediate** phenotype (Figure 2, L, M) have laterally extended dorsal cuticular shields with two pharyngeal connections. Some ventral cuticle is present as reflected by denticles at the edges of the dorsolateral shield (small arrows in Figure 2L) or thin ventral cuticle bridges across the edges of the dorsolateral shield. Occasionally, embryos have a more well formed cephalic region with detectable head skeleton material. The remaining neurogenic defects are grouped under the **weak** category (Figure 2, N, O, P, Q, R). They include holes of variable size in the ventral trunk and/or the dorsal cephalic region, the latter presumably resulting from hypertrophy of the brain. Head and ventral defects not accompanied by

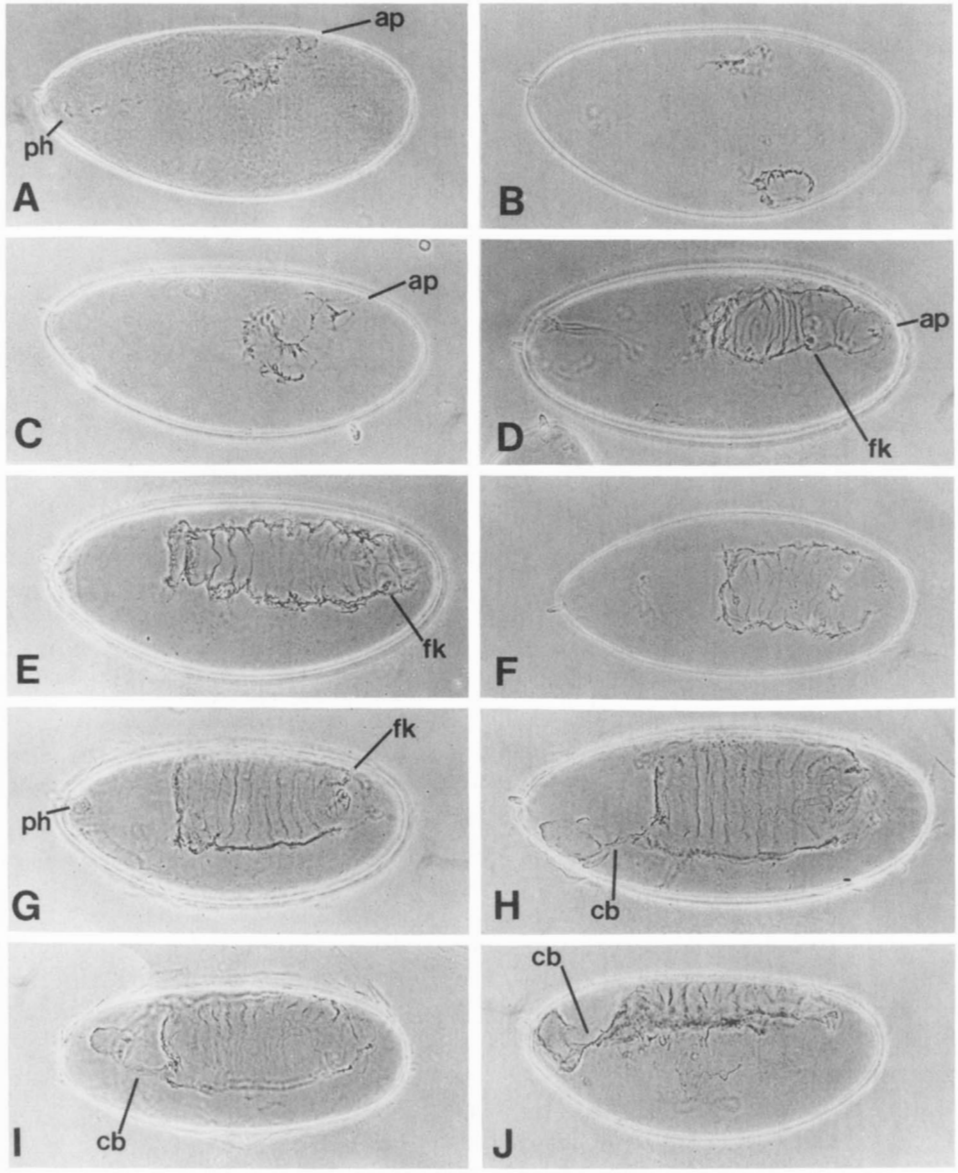

FIGURE 2.—Part I. A series of mutant embryos representing the full range of phenotypes obtainable by *E(spl)* alleles is shown in order of decreasing phenotypic severity. All embryos are oriented with the anterior to the left and for side views (**A**, **J**, **K**, **R**, **S**, **T**) with the dorsal side up. **A–C**: Extreme phenotypes (ex). Only small patches of dorsal and pharyngeal (ph) cuticle remain. The only discernible structures are occasional anal plates (ap). The embryo in **B** was unable to undergo dorsal closure and as a result has two lateral cuticle patches. **D–F**: moderate-extreme phenotypes (mex). The cuticle patches are more extensive than those of extreme embryos and anal plates (ap) as well as filzkörper (fk) are always discernible. Note that the filzkörper are always far apart and disorganized (compare with the normal filzkörper of **G** or **H**). Narrow patches (short or long; **D** and **E**, respectively) or wider ones with frequent holes (**F**) are seen. **G–K**: intermediate phenotypes (int). Embryos produce large dorsal cuticle shields with normal filzkörper and telsons. 0 (**G**), 1 (**H**) or 2 (**I**, **J**) cephalic cuticle bridges (cb) connect this dorsal shield to the cephalic cuticle. **J** is a side view of an embryo similar to that in **I**.

cuticle holes, but similar to those associated with small cuticle holes (*e.g.*, Figure 2, S *cf.* with P and R), are placed in a separate "no hole" category. These are probably due to slight defects in the neurogenic pathway, given the continuity of their phenotypes with the weak neurogenic phenotype (see also LEHMAN *et al.* 1983).

Df(3R)P709 homozygotes display weak neurogenic defects or no-hole phenotypes (Figure 2, R-S), as well as thin denticles and minor problems in dorsal closure with variable penetrance. The latter two defects need not be attributed to genes near the *m7* breakpoint since this deficiency extends as far as cytological division 97D (see legend of Figure 1 for cytology). The two *Df(3R)boss* deficiencies exhibit a much more severe phenotype (moderate-extreme), consisting mostly of long narrow dorsal shields (Figure 2E). We shall collectively refer to $Df(3R)boss^{14}$ and $Df(3R)boss^{15}$ as *Df(3R)boss*, since they behave identically in all combinations tested. Regarding their complementation behavior (Table 1), the three alleles *Df(3R)8D06*, $E(spl)^{BX22}$ and *Df(3R)P709* show neurogenic phenotypes in any combination irrespective of cross direction. In fact, they behave as an allelic series, with *Df(3R)8D06* representing the amorphic state, $E(spl)^{BX22}$ an intermediate hypomorph and *Df(3R)P709* a weak hypomorph. In contrast to these mutations, the behaviors of *Df(3R)boss* as well as the point mutations in *m9/10* depend on cross direction. When *Df(3R)boss* is paternally supplied, it behaves as a strong hypomorph in combination with *Df(3R)8D06*, $E(spl)^{BX22}$ and *Df(3R)P709*, as it produces a somewhat less severe phenotype than the respective allele over *Df(3R)8D06*. When, however, *Df(3R)boss* is maternally supplied, the phenotypes of the same zygotic genotypes are significantly improved, although the order of severity is maintained, with *Df(3R)boss/Df(3R)8D06* exhibiting the strongest (moderate-extreme) and *Df(3R)boss/*

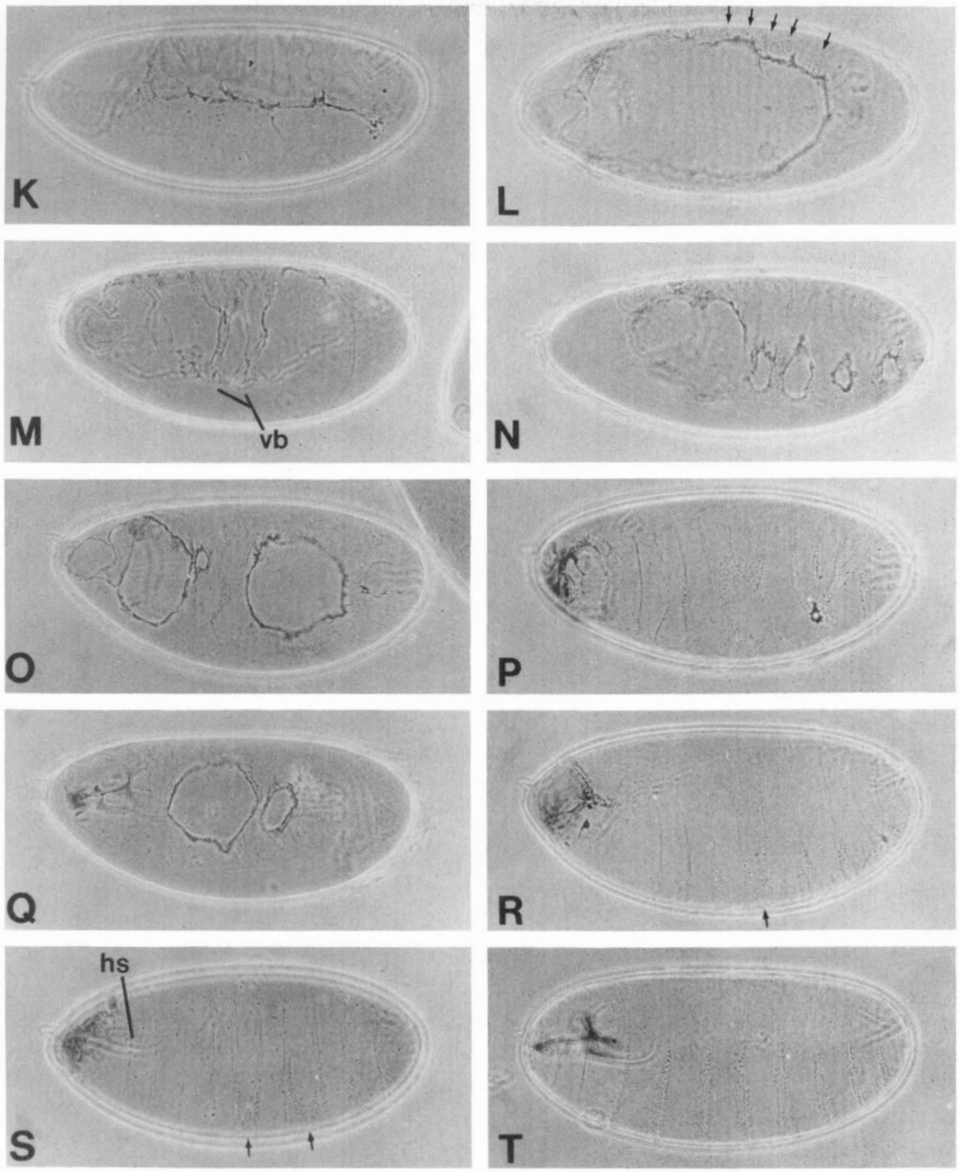

FIGURE 2.—Part 2. The embryo in **K** has a laterally extended dorsal shield, but as it does not contain any denticles, it was placed in this category rather than in the following one (weak-intermediate). **L-M**: weak-intermediate phenotypes (wint). Extended dorsal shields that show some denticles (small arrows on **L**) are included here; similar embryos that contain ventral bridges (vb) are also included (**M**). **N–R**: weak phenotypes (w) are rather variable. The common characteristic is that they must contain a substantial amount of ventral cuticle. **N**, **O** and **P** are examples of embryos with head and ventral holes; the ventral hole in **P** is only tiny. **Q** has only ventral holes with only a slight defect in the head skeleton. **R** has only a head hole with small defects in the organization of the denticle bands (small arrows). Embryos like the one in **S** were not scored as a separate "no hole" category, although the continuity of their phenotype with the previous neurogenic embryos is evident. The example in **S** has similar denticle band fusion defects (small arrows) like the embryo in **R**, but instead of a cephalic hole it has a grossly disorganized head with an arm of the head skeleton still evident (hs). **T** is a wild-type embryo. The actual genotypes of these embryos are: **A**: *Df(3R)8D06* homozygote; **B**, **C**: *C2.8/C2.8* or *C2.8/Y*; *Df(3R)8D06/Df(3R)8D06*; **D**: *Df(3R)8D06* (pat)/*Df(3R)boss*^{15}(mat); **E**: *Df(3R)boss*^{15} homozygote; **F**: *Df(3R)boss*^{14} homozygote; **G**, **H**, **J**: *E(spl)*^{BX22} homozygotes; **I**: embryo from *E8/Y ; +/E(spl)*^{BX22} × *+/E(spl)*^{BX22}; **K**: *Ep/Ep ; Df(3R)8D06/Df(3R)8D06*; **L**, **N**: *C2.8/C2.8 ; E(spl)*^{BX22}*/E(spl)*^{BX22}; **M**: *E8/E8 ; E(spl)*^{BX22}*/E(spl)*^{BX22}; **O**: *E25R*(pat)/+ *; Df(3R)8D06/Df(3R)8D06*; **P**, **R**: *E25R*(mat)/+ *; Df(3R)8D06/Df(3R)8D06*; **Q**: *E25R*(pat)/+ *; Df(3R)boss*^{14}*/Df(3R)boss*^{14}; **S**: *Df(3R)P709* homozygote.

Df(3R)P709 the weakest defects. This asymmetric behavior of *Df(3R)boss* can be attributed to its carrying a functional *m9/10* gene, which we know to be maternally expressed (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988): higher maternal *m9/10* levels result in a milder phenotype. In fact, *Df(3R)boss/Df(3R)P709* fertile adults can be obtained, albeit at a reduced frequency (about 25% of expectation compared to sibling progeny classes), only when *Df(3R)boss* is maternally supplied. This is not surprising, given that *Df(3R)boss* and *Df(3R)P709* delete complementary portions of the *E(spl)* region, although *m7* should be affected by both alleles. Two different point mutant alleles of *E(spl) m9/10*, *E(spl)*^{E48} and *E(spl)*^{E73}, were also tested against the deficiencies. The detailed results will be described in a later section, but one striking

TABLE 1

Complementation among *E(spl)* alleles

Paternal chromosome	Maternal chromosome					
	E48	*E73*	*8D06*	*BX22*	*P709*	*boss*
E48	int[a]	NT	w wint	lar[b]	lar[b]	+[b]
E73	NT	w[c]	w	lar	NT	+[b]
8D06	w wint	w[c] wint	ex	mex int	int	mex
BX22	lar[b]	w[c]	mex int	int	wint w	int wint
P709	lar[b]	NT	int	wint w	w[d]	wint[d] +
boss	+[b]	w[c] +	ex mex	int	wint w	mex

Viability of heterozygotes to adulthood is indicated by +, while viability to hatching first instar larva and subsequent death before pupation is indicated by lar. In the remaining cases the relevant progeny class is embryonic lethal with complete penetrance and the table indicates the severity of the neurogenic phenotype: ex: extreme; mex: moderate-extreme; int: intermediate; wint: weak-intermediate; w: weak. When an embryo population spans two phenotypic classes, the most predominant one is written on top. The phenotypic classes are described in the text and examples are shown in Figure 2. NT stands for cases where the embryonic phenotype was not tested. All these combinations are nonetheless lethal, as no adults were ever obtained.

[a] This intermediate phenotype is probably due to a second site mutation on the $E(spl)^{E48}$ chromosome, since the phenotype is improved over deficiencies.

[b] In these crosses there was a small percentage of lethal embryos (≤10%; see Table 3).

[c] When $E(spl)^{E73}$/+ is the mother, the neurogenic progeny exceeds the expected 25%, therefore $E(spl)^{+}$ progeny classes must also be affected. An additional 18–38% of the total progeny is embryonic lethal with head and ventral cuticle defects (see Table 3). Due to this increased embryonic lethality it is impossible to determine whether a small percentage of the $E(spl)^{E73}/Df$ embryos hatch (as in the case of $E(spl)^{E48}$).

[d] From 38 to 43% of the lethal embryos in these classes exhibit no cuticle holes, rather just segmentation, dorsal closure or head abnormalities. Additionally, in the case of *Df(3R)P709*/+ (father) × *Df(3R)boss*/+ (mother), adults were obtained at a frequency of 25% of expected if fully viable, while the majority of the dead embryos were weakly neuralized.

observation was central to our functional analysis of the locus: although these alleles had been shown to be lethal over all previously tested *E(spl)* deficiencies, and indeed were lethal over *Df(3R)8D06*, $E(spl)^{BX22}$ and *Df(3R)P709*, they were fully viable over the *Df(3R)boss* alleles. This confirmed the fact that each *Df(3R)boss* chromosome contains a fully functional copy of *E(spl) m9/10*, and that its neurogenic defect must be attributed to deletion of (a) gene(s) proximal to *m9/10*.

These complementation data suggest that the zygotic component of *E(spl)* active in neurogenesis is distinct from the *m9/10* gene, since this zygotic component must be virtually absent in the strongly hypomorphic yet $m9/10^{+}$ *Df(3R)boss* chromosomes. Conversely, it must be largely present in the weakly hypomorphic yet *m9/10* null (deleted) *Df(3R)P709*. Furthermore, a substantial contribution to the zygotic component of *E(spl)* must reside proximally to *m9/10*, since *Df(3R)P709* consistently yields a milder phenotype than $E(spl)^{BX22}$, despite the fact that *Df(3R)P709* deletes a large region distal to *m9/10*. We therefore conclude that *E(spl)* function is mediated by a group of genes, whose members are located both proximally and distally to the *m7* breakpoint common to *Df(3R)boss* and *Df(3R)P709*, but most likely do not extend further distally than *m9/10*. These data have not been able to determine which of the many genes located within this region are responsible for the different defects associated with *E(spl)* mutations. This question was addressed by the analysis of transformed fragments of the locus.

***P* element constructs:** A variety of *P* element constructs from the *E(spl)* region were generated: some contain single HLH genes, while others include a number of transcription units, as diagrammed in Figure 1. A 2.8-kb *Cla*I fragment containing just the *m8* gene and a 5.6-kb *Xho*I fragment containing a functional copy of only *m7* were subcloned into Carnegie 20 (RUBIN and SPRADLING 1983) to generate P[ry^{+},*C2.8*] and P[ry^{+}, *X5.6*], respectively. P[ry^{+}, *E25R*] contains 25 kb of genomic DNA encompassing transcripts *m5* through *m9/10*. P[ry^{+}, *Ep*] has the same proximal end as P[ry^{+}, *E25R*] but extends for only 14.8 kb ending at an *Xho*I site in the coding region of *m8*, thus containing functional copies of transcription units *m5* through *m7*. Both constructs were made in the pDM30 vector (D. MISMER and G. RUBIN, personal communication) as described in MATERIALS AND METHODS. P[ry^{+}, *Ep*] and the previously described *E(spl) m9/10*$^{+}$ transposon P[ry^{+}, *E8*] (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988) in combination encompass the same genomic region as P[*ry*+, *E25R*], although they are not functionally equivalent, since neither of the smaller constructs contains a functional copy of *m8*. These five constructs were used to analyse the relative contributions of the different transcription units to $E(spl)^{+}$ function. Henceforth we shall refer to them as *E8*, *Ep*, *E25R*, *C2.8* and *X5.6* for brevity.

Transformed lines were obtained by microinjection of the constructs along with the helper plasmid pπ25.7wc (KARESS and RUBIN 1984) into embryos from a ry^{506} or a *cn;* ry^{506} strain. Four independent lines of *Ep*, two of *E25R*, and several of *C2.8* and *X5.6* were recovered. We chose to limit our analysis to a subset of the obtained transformed lines: *Ep-B1* (*X* chromosome) and *Ep-B2* (chromosome *II*); *E25R-A2* (*II*) and *E25R-A3* (*III*); *C2.8–32* (*X*); *X5.6–108* (*II*). The two *E25R* lines are recessive lethal, whereas all other lines are homozygous viable. We also used the homozygous viable line *E8–13B*, inserted into the *X* chromosome.

***E25R* and *Ep* constructs express the relevant genes:** *E25R* carries all transcription units affected by

the $E(spl)^{BX22}$ deficiency, the smallest *E(spl)* deficiency available. Accordingly it was shown that one copy of *E25R*, whether paternally or maternally contributed, is sufficient to rescue $E(spl)^{BX22}$ to adulthood (Table 2d). Both *E25R* lines were able to supply the function(s) responsible for this rescue (the recombinant *E25R-A3* $E(spl)^{BX22}/E(spl)^{BX22}$ lines were tested by DNA blot to verify the absence of endogenous $E(spl)^{+}$ DNA; data not shown). In addition to proving the ability of *E25R* to express its resident genes, this result strengthens the conclusion of PREISS, HARTLEY and ARTAVANIS-TSAKONAS (1988) that the proximal breakpoint of the small inversion also present in $E(spl)^{BX22}$, but not covered by *E25R* (see Figure 1), does not disrupt any vital genetic functions.

We did not expect *Ep* to similarly rescue $E(spl)^{BX22}$, since it does not supply the *m9/10* vital function. It was, however, able to rescue the heterozygote $E(spl)^{BX22}/Df(3R)boss^{15}$ to adulthood (Table 2e), since in this combination the *m9/10* (as well as the *m8*) activity is supplied by the $Df(3R)boss^{15}$ chromosome. Single copies of either *Ep-B1* or *Ep-B2* were capable of rescue.

It has previously been shown that *E8* expresses *m9/10*, since it is capable of rescuing the point mutations to adulthood (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). Similar proof of activity is lacking for constructs *C2.8* and *X5.6*, but their ability to improve the neurogenic phenotype of deficiencies (see next section) suggests that they at least partially express the respective genes.

Phenotypic rescue by transformed constructs: We combined the different transformed constructs with various *E(spl)* deficiency alleles and, where appropriate, analyzed the cuticular phenotype of the dead embryos as described in MATERIALS AND METHODS. The results are summarized in Table 2. For each combination tested, Table 2 lists the genotype analytically for *m9/10*, maternal *m9/10*, each of the HLH genes, *m5*, *m7* and *m8*, as well as any putative undetermined additional functional members of *E(spl)*, collectively referred to as "*prox*," since from the complementation analysis presented above any additional *E(spl)* component should lie proximally to the already characterized region. In assigning this analytical genotype we have assumed that the *Df(3R)boss* chromosomes do not express the *m7* gene, since the insertion element seems to be within the promoter region of the gene, less than 200 bp upstream of the putative transcription start site (data not shown).

Table 2a shows the results of the complementation tests discussed above in this analytical format. Table 2b presents the effect of the various constructs on the null *E(spl)* phenotype generated by homozygous *Df(3R)8D06*. Neither *E8* nor *C2.8*, alone or in combination, have any effect on the extreme null phenotype. In contrast, both *Ep* and *E25R* have significant effects in a copy-sensitive manner, as they produce better rescue when present in two copies than in one. Furthermore, the rescue of *Df(3R)8DO6* effected by *E25R* is significantly better than that produced by *Ep* at the same copy number (and this also holds for all other *E(spl)* alleles tested). The ability of *Ep* and *E25R* to improve the phenotype of *Df(3R)8D06* suggests a role for the HLH genes in neurogenesis, although both of these constructs carry additional transcription units. A more direct proof for the involvement of HLH gene *E(spl) m8* is the improvement of the phenotype of *Ep/+ ; Df(3R)8D06/Df(3R)8D06* effected by *C2.8*. With respect to *E(spl) m9/10*, we note that one copy of *E8* improves the *Ep/+ ; Df(3R)8D06/Df(3R)8D06* phenotype only when *E8* is maternally contributed, and that the degree of rescue by one copy of *E25R* depends on the cross direction, both observations consistent with the hypothesis that high maternal *E(spl) m9/10* expression facilitates neurogenesis. However, in the absence of the zygotic *E(spl)* HLH components, *E8* alone seems unable to produce any phenotypic improvement.

Like *Df(3R)8D06*, the degree of rescue of *Df(3R)boss* increases in the order *E8/E8* (no rescue) < *Ep/+* < *Ep/+ & C2.8/+* < *Ep/Ep* < *E25R/+* < *E25R/E25R* (Table 2c). In each case, the phenotype is better than that of the same transformant combination with *Df(3R)8D06*. Additionally, two copies of *X5.6* result in rescue, although to a lesser extent than *Ep/+*, while two copies of *C2.8* result in only marginal improvement, with many embryos exhibiting anteriorly extended narrow dorsal patches and better Filzkörper. Most interestingly, *E25R/E25R ; Df(3R)boss/Df(3R)boss* animals complete embryogenesis and hatch, but die as first instar larvae. This suggests that the genes carried by *E25R* are sufficient for *E(spl)* activity, or, stated differently, the proposed *E(spl)* proximal function, if any, can be largely substituted by genes on *E25R*.

The rescue characteristics of $E(spl)^{BX22}$ (Table 2d) confirm the order of activity of the different transformants determined by the rescue of *Df(3R)8D06* or *Df(3R)boss*. The effects of *C2.8* and *X5.6* are more pronounced with the $E(spl)^{BX22}$ allele. Indeed, for any transformant combination, $E(spl)^{BX22}$ produces a better phenotype than *Df(3R)8D06* or *Df(3R)boss*. Since *m5* through *m9/10* (inclusive) are inactivated by $E(spl)^{BX22}$, the residual zygotic activity in this chromosome supports the existence of the putative *E(spl)* proximal gene(s). The rescue to hatching first instar larvae by one or two copies of *Ep* supports the hypothesis that the zygotic component of *m9/10* is not required for neurogenesis, but is required for postembryonic development. None of the *Ep/+* or *Ep/Ep* ; $E(spl)^{BX22}/E(spl)^{BX22}$ larvae pupate, unless zygotic *E(spl) m9/10* is provided by adding *E8*, in which case fertile

TABLE 2

Transformant rescues of *E(spl)* deficiencies

Genotype	*prox*	*m5*	*m7*	*m8*	*m9*	mat *m9*	Phenotype
a. Allele combinations							
8D06/8D06	−/−	−/−	−/−	−/−	−/−	+/−	ex
boss/boss	−/−	−/−	−/−	+/+	+/+	+/+	mex
boss(m)/*8D06*(p)	−/−	−/−	−/−	+/−	+/−	+/+	mex[a]
8D06(m)/*boss*(p)	−/−	−/−	−/−	+/−	+/−	+/−	ex-mex
BX22/8D06	+/−	−/−	−/−	−/−	−/−	+/−	mex-int
BX22/BX22	+/+	−/−	−/−	−/−	−/−	+/−	int
P709/8D06	+/−	+/−	−/−	−/−	−/−	+/−	int[b]
P709/P709	+/+	+/+	−/−	−/−	−/−	+/−	weak-nh[c]
BX22(m)/*boss*(p)	+/−	−/−	−/−	+/−	+/−	+/−	int
boss(m)/*BX22*(p)	+/−	−/−	−/−	+/−	+/−	+/+	int-wint[d]
P709(m)/*boss*(p)	+/−	+/−	−/−	+/−	+/−	+/−	wint-weak
boss(m)/*P709*(p)	+/−	+/−	−/−	+/−	+/−	+/+	wint-adults[e]
BX22/P709	+/+	+/−	−/−	−/−	−/−	+/−	wint-weak
b. *8D06/8D06*							
E8/E8	−/−	−/−	−/−	−/−	+/+	+/+/+	ex-mex[f]
C2.8/C2.8	−/−	−/−	−/−	+/+	−/−	+/−	ex-mex[f]
C2.8(p)/*E8*(m)	−/−	−/−	−/−	+/−	+/−	+/+/+	ex-mex[f]
Ep/+	−/−	+/−	+/−	−/−	−/−	+/−	mex-int[g]
C2.8/+;Ep/+	−/−	+/−	+/−	+/−	−/−	+/−	mex-int[g]
E8(p)/+;Ep/+	−/−	+/−	+/−	−/−	+/−	+/−	mex-int[g]
E8(m)/+, *Y;Ep/+*	−/−	+/−	+/−	−/−	+/−	+/+/+	int-mex[g]
Ep/Ep	−/−	+/+	+/+	−/−	−/−	+/−	int-wint[d]
Ep E8/Ep E8	−/−	+/+	+/+	−/−	+/+	+/+/+	weak-wint
E25R(p)/+	−/−	+/−	+/−	+/−	+/−	+/−	weak-wint
E25R(m)/+	−/−	+/−	+/−	+/−	+/−	+/+	weak
E25R/E25R	−/−	+/+	+/+	+/+	+/+	+/+	nh-weak[h]
c. *boss/boss*							
E8/E8	−/−	−/−	−/−	+/+	+/+/+/+	+/+/+/+	mex
C2.8/C2.8	−/−	−/−	−/−	+/+/+/+	+/+	+/+	mex-int
X5.6/X5.6	−/−	−/−	+/+	+/+	+/+	+/+	mex-int
Ep/+	−/−	+/−	+/−	+/+	+/+	+/+	int-wink[i]
C2.8/+;Ep/+	−/−	+/−	+/−	+/+/+	+/+	+/+	int-weak[i]
Ep/Ep	−/−	+/+	+/+	+/+	+/+	+/+	weak
E25R(p)/+	−/−	+/−	+/−	+/+/+	+/+/+	+/+	weak-nh
E25R(m)/+	−/−	+/−	+/−	+/+/+	+/+/+	+/+/+	weak-nh
E25R/E25R	−/−	+/+	+/+	+/+/+/+	+/+/+/+	+/+/+	nh-larvae[j]
d. *BX22/BX22*							
(*E25R*/+ father)	+/+	−/−	−/−	−/−	−/−	+/−	int
(*E25R*/+ mother)	+/+	−/−	−/−	−/−	−/−	+/+	int[k]
E8(p)/+	+/+	−/−	−/−	−/−	+/−	+/−	int
E8(m)/+, *Y*	+/+	−/−	−/−	−/−	+/−	+/+/+	wint-int
E8/E8	+/+	−/−	−/−	−/−	+/+	+/+/+	wint-int
C2.8/+	+/+	−/−	−/−	+/−	−/−	+/−	int-wint[l]
C2.8/C2.8	+/+	−/−	−/−	+/+	−/−	+/−	wint-int[l]
E8(p)/*C2.8*(m)	+/+	−/−	−/−	+/−	+/−	+/−	int-wint
C2.8(pP/*E8*(m)	+/+	−/−	−/−	+/−	+/−	+/+/+	weak-nh
X5.6/X5.6	+/+	−/−	+/+	−/−	−/−	+/−	wint
Ep/+	+/+	+/−	+/−	−/−	−/−	+/−	larvae-weak[m]
Ep/Ep	+/+	+/+	+/+	−/−	−/−	+/−	larvae[m]
E8(p)/+;*Ep/+*	+/+	+/−	+/−	−/−	+/−	+/−	adults
E25R(p or m)/+	+/+	+/−	+/−	+/−	+/−	+/− or +	adults
E25R/E35R	+/+	+/+	+/+	+/+	+/+	+/+	adults
e. Various combinations							
BX22/8D06							
E8(m)/+, *Y*	+/−	−/−	−/−	−/−	+/−	+/+/+	int
C2.8/+	+/−	−/−	−/−	+/−	+/−	+/−	int-mex[n]
Ep/+	+/−	+/−	+/−	−/−	−/−	+/−	weak-wint
C2.8/+;Ep/+	+/−	+/−	+/−	+/−	−/−	+/−	weak-nh[p]
E8(m)/+, *Y;Ep/+*	+/−	+/−	+/−	−/−	+/−	+/+/+	adults
E25R(p or m)/+	+/−	+/−	+/−	+/−	+/−	+/−	adults

Genotype	*prox*	*m5*	*m7*	*m8*	*m9*	mat *m9*	Phenotype
BX22/boss							
Ep/+	+/−	+/−	+/−	+/−	+/−	+/−	adults
BX22/P709							
E8(p)/+	+/+	+/−	−/−	−/−	+/−	+/−	wint-weak
E8(m)/+	+/+	+/−	−/−	−/−	+/−	+/+/+	adults
P709/P709							
E8/E8	+/+	+/+	−/−	−/−	+/+	+/+/+	nh-weak[c]
boss/P709							
E8(m)/+	+/−	+/−	−/−	+/−	+/+	+/+/+	adults[q]
Ep/+	+/−	+/+	+/−	+/−	+/−	+/−	adults

Different chromosome combinations tested are listed. Their genotypes are presented analytically for the presence (+) or absence (−) of each gene implicated in *E(spl)* function. "*prox*" refers collectively to additional members of the *E(spl)* HLH family. The maternal genotype for *E(spl) m9/10* is also given. Their phenotypes are categorized according to the classes described in the text and Figure 2. nh: embryos exhibit no cuticle holes, just head and/or ventral cuticle defects. The range of obtained phenotypes for each given chromosome combination is shown, the predominant one listed first; in cases where a certain phenotypic class was represented by less than 10% of the lethal progeny, that class is omitted. In a, *E(spl)* allele combinations are listed in the absence of transformed constructs. The phenotypes of embryos containing transformed constructs in the background of homozygous *E(spl)* deficiencies are shown in [*Df(3R)8D06*], c [*Df(3R)boss*], and d [$E(spl)^{BX22}$]. Table 2e lists various other combinations of transformed constructs and *E(spl)* deficiencies. In the following notes, bold letters refer to examples of neurogenic phenotypes from Figure 2.

[a] Most embryonic phenotypes are shifted to a shorter dorsal shield (D) than in the case of *Df(3R)boss* homozygotes (mostly E).

[b] There is quite a high incidence of laterally extended dorsal shields (K) without any denticles (range of phenotypes: H–K).

[c] 43% of the *Df(3R)P709* homozygous progeny have no cuticle holes, just head, segmentation or dorsal closure defects. The no-hole progeny is increased to 82% by addition of *E8/E8*; embryos of this class have no head defects either, just thin or disorganized ventral denticles, probably a defect not associated with *E(spl)*.

[d] Phenotypes range from H to L.

[e] The most predominant phenotypic class is weak; 38% of the lethal progeny exhibit no cuticle holes.

[f] Percentage of moderate-extreme embryos, all of the most severe class (D), is low in all cases (12–15%). Such embryos are also encountered among *Df(3R)8D06* homozygotes at a frequency of about 4% and the slight increase produced by the transformed constructs does not warrant the conclusion that these transformants result in any significant rescue.

[g] Only a low frequency (12%) of intermediate embryos is encountered in the cases of *Ep/+* and *E8*(p)/+;*Ep/+*. Intermediates are increased to 50% by the addition of *C2.8/+* and to 77% by the addition of maternal *E8*. In all cases the majority of intermediate embryos lack cephalic connections (G).

[h] Approximately equal numbers of embryos with only slight segmentation defects (local fusions between neighboring denticle bands) and one with small holes in the dorsal cephalic region.

[i] *Ep/+* phenotypes range from H to L, while those of *C2.8/+;Ep/+* range from I to O; the latter genotype produces mostly weak-intermediate embryos.

[j] Die as first instar larvae soon after hatching.

[k] 89% of these embryos exhibit the I/J phenotype, also weak-intermediate phenotypes are seen with a frequency of 7%.

[l] *C2.8/+* phenotype ranges from H to L, while that of *C2.8/C2.8* ranges from I to M.

[m] 70% of the *Ep/+* individuals hatch; 96% in the case of *Ep/Ep*.

[n] Only 10% of long moderate-extreme dorsal shields (E).

[o] Only 10% of no-hole embryos.

[q] Adults obtained at reduced frequency. Embryonic phenotype not tested.

adults are obtained. This result further demonstrates that *m8* is dispensable, although the rescue of $E(spl)^{BX22}$ by *C2.8* unambiguously shows that *m8* participates in the process of neural-epidermal partitioning. In contrast to *Df(3R)8D06*, the phenotype of $E(spl)^{BX22}$ is significantly improved by *E8* alone to a weak-intermediate level, in agreement with the hypothesis that increased maternal dosage of *E(spl) m9/10* facilitates neurogenesis only in the presence of zygotic *E(spl)* activity.

From the above results (see also rescues of $E(spl)^{BX22}$/*Df(3R)8D06*, Table 2e) we propose that the members of the *E(spl)* HLH family provide the zygotic neurogenic function of *E(spl)*. Each *E(spl)* HLH gene provides only partial function, as evidenced by the graded, copy-dependent rescue ability of the different transformation constructs. Nonetheless, the full wild-type complement of *E(spl)* HLH genes is not needed for complete activity, since one member (at least *m8*) can be inactivated without deleterious effects, suggesting some degree of functional overlap and explaining the inability to isolate point lethal mutations in these genes. According to this model, *Df(3R)boss* deficiencies exhibit somewhat milder phenotypes than the null *Df(3R)8D06* by virtue of containing functional copies of *E(spl) m8* and *m9/10*. Under the assumption that *E(spl)* function requires no additional classes of genes other than the HLH family and *m9/10*, we must invoke the existence of additional HLH genes (the "*prox*" genes) in order to account for the significantly better phenotype of $E(spl)^{BX22}$ compared to that of *Df(3R)8D06*. In fact, a gene with strong sequence homology to the *E(spl)* HLH genes, especially to *m7*, has been characterized approximately 20 kb proxi-

mally to *m5* (R. REINKE, personal communication) and using transformants kindly provided by R. REINKE (Albert Einstein College of Medicine) we have shown that this gene improves the phenotype of *E(spl)* deficiencies (results not shown). We have recently identified three more HLH genes in this proximal region using low stringency DNA blot hybridization with the other *E(spl)* HLH genes as probes (results not shown). It is then possible that "*prox*" represents as many as four (or more) additional HLH *E(spl)* genes. These, like *m5*, *m7* and *m8*, must also be redundant, since the neurogenic defects of *Df(3R)8D06* and *Df(3R)boss* homozygotes are almost fully rescued by *E25R*, which extends proximally no further than *m5*.

The results of Table 2, upon which the model for *E(spl)* function is based, contain a few apparent discrepancies, when one closely compares different embryonic genotype-phenotype combinations. Although the genotypes of *E25R/E25R ; Df(3R)8D06/Df(3R)8D06* and *Ep/Ep ; Df(3R)boss/Df(3R)boss* are formally identical, the former has a much better phenotype (small cephalic holes, or just head and/or segment abnormalities; Figure 2, R–T) compared to the weak phenotype of the latter (head and ventral holes; Figure 2, N–R). The same holds for *Df(3R)boss* (maternal)/*Df(3R)8D06* (paternal) compared to *C2.8/E8* (maternal) ; *Df(3R)8D06/Df(3R)8D06*, with the former exhibiting a moderate-extreme (Figure 2, D-E) and the latter an extreme phenotype (Figure 2, A–D). These differences can be accounted for by positing that the expression of the different transformed genes is not quantitatively equal to that of the endogenous genes, and that the larger the construct the higher the expression of the genes contained. If this were true, *E25R* would approach wild-type expresssion, while *C2.8* would exhibit an appreciable degree of underexpression. This assumption is not unreasonable, since it is known for other genes that larger constructs better approximate wild type expression either by virtue of containing quantitative enhancer elements or by buffering the gene in question from negative position effects from the neighboring insertion site sequences (KELLUM and SCHEDL 1991). We have, however, no quantitative data on the expression levels of our constructs. An indication that *Ep* somewhat underexpresses the *m5* and *m7* genes is the fact that the frequency of rescue of $E(spl)^{BX22}/Df(3R)boss^{15}$ to adulthood is dependent on maternal *m9/10*: when compared to the numbers of sibling balancer progeny, *Ep/+* ; $E(spl)^{BX22}/Df(3R)boss^{15}$ *trans*-heterozygotes are obtained at the expected frequency (107% viability) when $TM3/Df(3R)boss^{15}$ is the mother, but at only 57% of expectation when $TM3/E(spl)^{BX22}$ is the mother. In contrast, *Df(3R)8D06/+*, which carries exactly the same *E(spl)* genes as *Ep/+ ;* $E(spl)^{BX22}/Df(3R)boss$, has little, if any, reduction in viability when *TM3/Df(3R)8D06* is the mother. The same is true for the equivalent genotype *E25R/+ ;* $E(spl)^{BX22}/Df(3R)8D06$; maternally or paternally contributed *E25R* gives normal viability. The latter result confirms our assumption that *E25R* has expression levels comparable to wild type.

Evidence for a maternal role of *E(spl) m9/10*: We have consistently observed that a given *E(spl)* zygotic genotype results in more severely defective embryos when the mother is heterozygous for a deficiency of *m9/10* than when it is wild-type or carries a duplication for *m9/10* (*e.g.*, *E8*). Many such examples can be found in Table 2: all cases of complementation of *Df(3R)boss* (Table 2a) and most cases of rescue by *E8* or *E25R* (Table 2, b, d, e). For example, $Df(3R)boss/E(spl)^{BX22}$ (Table 2a) gives lateralized dorsal shields with cephalic connections and frequently also with denticles (Figure 2, H-L), when the mother is *Df(3R)boss/+*, whereas it gives intermediate dorsal shields with mostly no pharyngeal connections (Figure 2, G-H), when the mother is $E(spl)^{BX22}/+$. Similarly, whenever *E8* has improved the phenotype of a given allelic combination, it has been supplied by the mother, whereas paternal contribution of *E8* seems to have no effects (Table 2, d, e). The only cases where maternal *E8* shows no phenotypic improvement are *Df(3R)8D06* and *Df(3R)boss* homozygotes (Table 2, b, c). In the first case, we attribute the lack of improvement to the complete absence of the *E(spl)* HLH genes. In the case of *Df(3R)boss*, the mothers already carry two wild type *m9/10* copies and it seems that increasing the copy number to four makes no further improvement. For the same reason, no asymmetry was observed between maternal and paternal rescue of *Df(3R)boss* homozygotes by *E25R* (Table 2c).

Whenever we tested the effects of maternally contributed *E8*, the progeny inevitably received one copy of $m9/10^{+}$ from the *E8* homozygous mother. In the following two crosses we tested the maternal effect of *m9/10* in the absence of any zygotic wild-type copies: *E25R/+* ; $E(spl)^{BX22}/TM3$ mothers × $E(spl)^{BX22}/TM3$ fathers and $E(spl)^{BX22}/TM3$ mothers × *E25R/+* ; $E(spl)^{BX22}/TM3$ fathers. All neurogenic embryos from these crosses are $E(spl)^{BX22}$ homozygotes, since any embryo receiving *E25R* will survive to adulthood. Although in the latter cross (*E25R* from father), the neurogenic embryos looked identical to $E(spl)^{BX22}$ homozygotes from $E(spl)^{BX22}/+$ *inter se*, the neurogenic embryos from the former cross were significantly better: virtually all of them had more extensive cuticle shields with two pharyngeal connections and a small percentage even exhibited denticles (see Figure 3; Table 2d)). Thus, it seems that increased maternal levels of $m9/10^{+}$ alone can improve a neurogenic phenotype, although the improvement is more pronounced when zygotic $m9/10^{+}$ is also present.

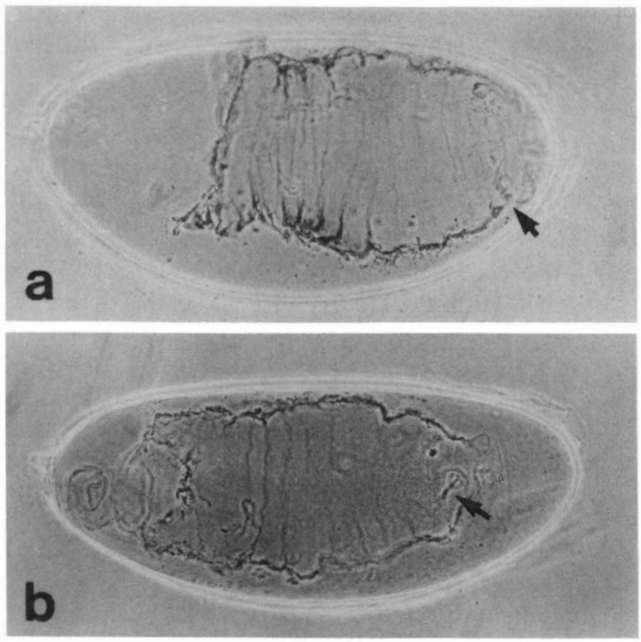

FIGURE 3.—An example of maternal $m9/10^{+}$ rescue of the $E(spl)^{BX22}$ phenotype. **a** is an $E(spl)^{BX22}/E(spl)^{BX22}$ embryo from a *TM3*/$E(spl)^{BX22}$ mother and an *E25R*/+ ; *TM3*/$E(spl)^{BX22}$ father. Its phenotype is identical to that of $E(spl)^{BX22}$, as expected since paternal *E25R* should have no effect on the phenotype. **b** is an $E(spl)^{BX22}/E(spl)^{BX22}$ embryo from the reciprocal cross, and shows a significantly improved, albeit still intermediate, neurogenic phenotype. Note the improved cephalic region including two cephalic bridges in the rescued embryo in **b**. Also note that the Filzkörper in **a** are at the edge of the cuticle shield and not as well formed as the ones in **b** (arrows).

A number of lethal *E(spl)* allele combinations can actually be rescued to adulthood by *E8* alone. The two genotypes $E(spl)^{BX22}/Df(3R)P709$ (Table 2a) and *Ep*/+ ; $E(spl)^{BX22}/Df(3R)8D06$ (Table 2e) have essentially the same phenotype: lateralized/ventralized (denticles and ventral strips) dorsal shields or more extensive ventral cuticle with holes (Figure 2, L–P). One of these, $E(spl)^{BX22}/Df(3R)P709$, was tested with an added, paternally derived, copy of *E8* and no phenotypic improvement was seen (Table 2e). However, when one copy of maternally derived *E8* is added to either genotype (from a mother homozygous for *E8*), fertile adult *trans*-heterozygotes are obtained at a normal frequency (118% for *E8*/+ or *Y* ; $E(spl)^{BX22}/Df(3R)P709$ and 113% for *E8*/+ or *Y* ; *Ep*/+ ; $E(spl)^{BX22}/Df(3R)8D06$, compared to the numbers of their sibling *TM3* progeny). Note that adults of these genotypes can rarely be obtained (at a frequency of 2–3% of expectation) when *E8* is paternally contributed, suggesting that zygotic *m9/10* product does have a weak rescuing ability. The viability of these genotypes strengthens the conclusion that members of the *E(spl)* HLH family are functionally redundant, since *E8*/+ ; *Ep*/+ ; $E(spl)^{BX22}/Df(3R)8D06$ and *E8*/+ ; $E(spl)^{BX22}/Df(3R)P709$ contain no functional copies of *m8* or of *m7* plus *m8*, respectively.

TABLE 3

Lethality associated with $E(spl)^{E73}$ and $E(spl)^{E48}$

Male	×	Female	Neuro	Major	Minor	Hatch	*N*
E73/+	×	*E73*/+	34	13	25	28	380
+/+	×	*E73*/+	14	19	18	49	490
E73/+	×	*8D06*/+	14	0	0	86	721
8D06/+	×	*E73*/+	56	6	12	25	329
E73/+	×	*BX22*/+	0	0	0	100	196
BX22/+	×	*E73*/+	43	14	15	27	467
E73/+	×	*boss*/+	2	1	0	97	808
boss/+	×	*E73*/+	35	12	13	40	478
E48/+	×	*8D06*/+	20	0	0	80	1020
8D06/+	×	*E48*/+	30	0	0	70	472
E48/+	×	*BX22*/+	5	0	2	94	987
BX22/+	×	*E48*/+	4	0	0	96	3644
E48/+	×	*boss*/+	4	1	2	94	719
boss/+	×	*E48*/+	9	0	0	90	1503
E48/+	×	*P709*/+	6	0	4	90	1063
P709/+	×	*E48*/+	3	1	1	95	1571

$E(spl)^{E73}/TM6B$, $E(spl)^{E48}/TM3$ and Df/*TM3* males were outcrossed to *cn* ; ry^{506} females and the *E(spl)*/*ry* flies mated in the stated crosses. Approximately 200 eggs were collected from an overnight egglay and their development was followed for 48 hr at 25° to determine the percentage of hatching (Hatch), after correcting for unfertilized eggs. From a larger initial egg collection we mounted only the embryos unhatched after 48 hr and scored the different phenotypes (again unfertilized eggs were ignored), to obtain the percentages of the following different classes: Neuro: includes all embryos showing cuticle holes; the neurogenic phenotypes seen were always weak, except for approximately half the dead embryos of the genotype $E(spl)^{E48}/Df(3R)8D06$ (irrespective of cross direction) and about 15% of the dead $E(spl)^{E73}$(mat)/*Df(3R)8D06*(pat) embryos, all of which exhibited weak-intermediate phenotypes. Major: includes embryos with severe head defects and/or severe denticle bands fusions (such as one wide denticle band). The head defects included here are absence of head skeleton or small amounts of head skeleton material present in a disorganized form. Minor: includes embryos with denticle bands fusions that do not alter the basic pattern too severely and/or slight reduction in size/malformation of the head skeleton. Some of the late hatching embryos exhibit such a phenotype but die as first instar larvae (these were included in the hatching category). *N* stands for the total embryo population (including embryos that hatch) from which the above phenotypic percentages were obtained. *N* was calculated from the total number of dead embryos scored (60–350) and the percentage of hataching calculated earlier from the smaller embryo sample.

In contrast to these genotypes, for which survival to adulthood is absolutely dependent on maternal *m9/10* product, *E8*/+ ; *Ep*/+ ; $E(spl)^{BX22}/E(spl)^{BX22}$ adults are found at high frequency whether the *E8* copy is paternally (85% viability) or maternally (80% viability) contributed. This is not surprising, as *Ep*/+ ; $E(spl)^{BX22}/E(spl)^{BX22}$ does not have a neurogenic defect, and *E8* is therefore only needed to provide later *m9/10* function(s).

The complementation behavior of *E(spl) m9/10* point mutations also lends support to a primarily maternal contribution of this gene in neurogenesis. Two alleles, $E(spl)^{E48}$ and $E(spl)^{E73}$, were tested against various deficiencies, and the results are presented on Table 3 and summarized on Table 1. $E(spl)^{E48}$ was primarily larval lethal over $E(spl)^{BX22}$ and *Df(3R)P709* and viable over *Df(3R)boss*, with a small percentage of

embryonic lethality in all these crosses. The dead embryos consisted of weakly neuralized ones as well as ones with no cuticle holes, rather head and/or denticle bands defects of varying degrees. The converse was the case for $E(spl)^{E48}/Df(3R)8D06$, with most embryos dying with weak-intermediate or weak neurogenic defects, but also a small percentage of larval escapers. The only dependence on cross direction was found with *Df(3R)boss*, where the frequency and severity of lethal neurogenic embryos were somewhat increased when $E(spl)^{E48}/+$ was the mother. It appears then that $E(spl)^{E48}$ is a null allele of *E(spl) m9/10*, since it is larval lethal over most deficiencies of *m9/10* and we have shown this indeed to be the phenotype for a zygotic *m9/10* null mutation (see *Ep/Ep ;* $E(spl)^{BX22}/E(spl)^{BX22}$, Table 2d). The embryonic lethality over *Df(3R)8D06* is probably a combined result of halving the *E(spl)* HLH gene copy number and absence of zygotic *m9/10*. Indeed, weakly neuralized embryos are also seen, albeit at very low frequencies (<10%), among the following two genotypic classes: (a) *Df(3R)8D06/+* individuals, which express $m9/10^{+}$ zygotically, but carry half the wild type *E(spl)* HLH copy number, and (b) $E(spl)^{E48}/E(spl)^{BX22}$ (or $E(spl)^{E48}/Df(3R)P709$) individuals, which have a less severe *E(spl)* HLH copy number reduction (not to half the wild-type copy number), but lack zygotic *m9/10*.

$E(spl)^{E73}$ gives quite a different picture. Although it behaves essentially identically to $E(spl)^{E48}$ when contributed paternally, it results in a high frequency of embryonic lethality when contributed maternally (Table 3). This frequency exceeds the expected 25% and must thus affect progeny classes in addition to $E(spl)^{E73}/Df$. This fact was confirmed by the incidence of embryonic lethality in the cross +/+ (father) × $E(spl)^{E73}/+$ (mother), in which 51% of the total progeny were embryonic lethal. The embryonic phenotype of the different genotypic classes ($E(spl)^{E73}/+$, $E(spl)^{E73}/Df$ and maybe even +/+) was indistinguishable and consisted of weak neurogenic and head/denticle band defects. The dominant maternal-effect embryonic lethality of this allele suggests that it encodes an antimorphic product, the maternal production of which is sufficient to disrupt early neurogenesis even in the presence of a wild type complement of *E(spl)* HLH genes.

Interactions between *E(spl)* and *Dl*: The fact that maternal *m9/10* modulates the phenotype of *E(spl)* allele combinations does not necessarily imply that the *E(spl)* HLH proteins interact directly with the m9/10 protein. The most parsimonious interpretation of our data is that the HLH proteins and m9/10 are simply both members of the cell signalling pathway for lateral inhibition during embryonic neurogenesis. If this is so, maternal *m9/10* levels could affect the phenotypes associated with lesions in other neurogenic genes as well. As a test of this possibility, we assayed the phenotype of Dl^{6B37} homozygotes from different maternal backgrounds.

Dl^{6B37} homozygotes grown at 25° exhibit a weak-intermediate phenotype, with lateralized dorsal shields with cephalic connections, as well as the occasional appearance of ventral cuticle. We performed the following crosses (mothers listed on the left):

$+/Dl^{6B37} \times +/Dl^{6B37}$ (to obtain the control phenotype described above)

$+/Dl^{6B37} \times +$ *Df(3R)P709*$/Dl^{6B37} +$

$+$ *Df(3R)P709*$/Dl^{6B37} + \times +/Dl^{6B37}$

E25R-A3$/Dl^{6B37} \times +/Dl^{6B37}$ (see *P element constructs* section).

In each of these crosses, the only dead embryos should be the Dl^{6B37} homozygotes, which was verified in each case by collecting approximately 200 embryos and making sure that only one quarter of them fail to hatch. The Dl^{6B37} homozygotes look identical to control in all cases, except for the ones produced by *Df(3R)P709*$/Dl^{6B37}$ mothers. In this case the phenotype is shifted toward intermediate; no embryo exhibits any ventral cuticle (denticles) and many have lost one or both of their anterior cephalic connections (Figure 4). Thus, it seems that reduction in *m9/10* maternal copy number exacerbates the phenotype associated with reduced *Dl* function. As in the case of *E(spl)* alleles, increase of maternal *m9/10* beyond two copies does not seem to afford any improvements to the Dl^{6B37} phenotype (Figure 4c).

The interaction detected between *E(spl)m9/10* and *Dl* raises the question as to which of the two neurogenic functions within the *E(spl)* locus is responsible for the *trans*-heterozygous lethality of *E(spl)* deficiencies with strong *Dl* alleles, which is reportedly due to weak neurogenic defects in embryos (VÄSSIN, VIELMETTER and CAMPOS-ORTEGA 1985). We tested for *trans*-heterozygote adult viability of the strong allele Dl^{9P39} over a variety of *E(spl)* allele/transformant combinations. The viability of *trans*-heterozygotes with respect to their sibling *Dl* or *E(spl)* progeny is shown on Table 4. No *Df(3R)8D06*$/Dl^{9P39}$ adults are obtained when *Ep/Ep ; TM3/Df(3R)8D06* is the mother, even though partial function of *E(spl)* has been restored by the *Ep* construct. However, when *E8/E8 ; TM3/Df(3R)8D06* is the mother, a significant fraction of *trans*-heterozygotes survive. Such a rescue is not achieved by *E8* paternally, although a low percentage of escapers is produced anyway when *TM3*$/Dl^{9P39}$ is the mother. In this direction, however, addition of *Ep* increases the frequency of *trans*-heterozygotes. These data seem to implicate both components of the *E(spl)* locus in the lethality of *Dl-E(spl) trans*-heterozygotes. Indeed, both the low HLH copy-number/$m9/10^{+}$ $Df(3R)boss^{14}$ allele and the *m9/10* null/high HLH copy number *Df(3R)P709* allele exhibit good viability over

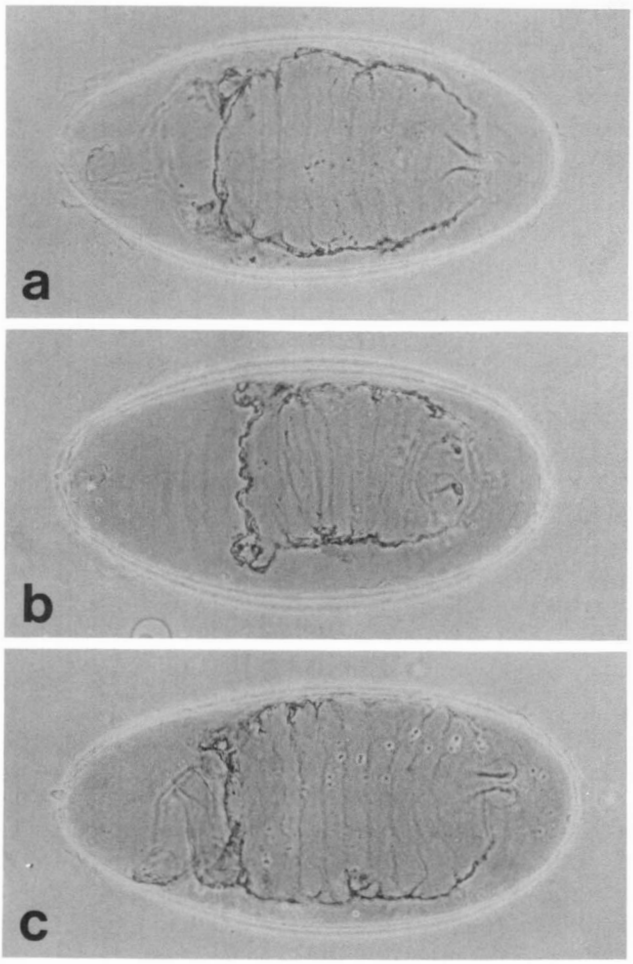

FIGURE 4.—An example of the interaction of Dl^{6B37} with deletions of *E(spl)m9/10*. **a** shows a homozygous Dl^{6B37} embryo exhibiting a head-connected dorsal cuticular shield. **b** and **c** are also Dl^{6B37} homozygous embryos laid by a mother heterozygous for *Df(3R)P709* (**b**), or for E35R-A3 (**c**). Although the embryo in **c** is indistinguishable from that in **a**, the one in **b** shows a much reduced amount of cuticle especially in the cephalic region.

TABLE 4

Rescue of Dl^{9P39} +/+ *Df[E(spl)]* transheterozygotes

Male × Female	Percent viability of $E(spl)/Dl^{9P39}$ wrt *TM3/Dl*	wrt *TM3/E(spl)*	*N*
TM3/Dl × *Ep/Ep; TM3/Df (3R) 8D06*	0	0	100
TM3/Dl × *E8/E8; TM3/Df(3R)8D06*	54	42	99
TM3/Df(3R)8D06 × *TM3/Dl*	15	6	121
Ep/Ep;TM3/Df(3R)8D06 × *TM3/Dl*	74	45	105
E8/Y;TM3/Df(3R)8D06 × *TM3/Dl*	8	4	152
TM3/Dl × *TM3/Df(3R)boss*^{14}	93	83	257
TM3/Df(3R)boss^{14} × *TM3/Dl*	63	32	109
TM3/Dl × *TM3/Df(3R)P709*	43	32	244
TM3/Df(3R)P709 × *TM3/Dl*	167	104	248

The crosses shown were scored for adults from the different progeny classes: *Dl/E(spl)*, *TM3/E(spl)* and *TM3/Dl*. The percent of the first class (the transheterozygotes between *Dl* and *E(spl)*) with respect to the numbers of each of the other two classes is presented. The total number of flies scored is listed under *N*. Dl^{9P39} was used in all crosses.

Dl^{9P39} in both cross directions, although the frequency of *trans*-heterozygotes with *Df(3R)P709* does show the expected maternal effect. We can thus conclude that the lethality of the *Dl* +/+ *Df[E(spl)]* genotype is due to the concomitant reduction in the levels of activity of three classes of genes: *Dl*, the *E(spl)* HLH genes and maternal *m9/10*, again implicating the close association of all three in a common pathway. Restoration of either one of these functions results in improved viability.

Immunocytochemical localization of m9/10: The foregoing genetic analysis unambiguously suggests a role for the E(spl) HLH proteins in neurogenesis and implies that E(spl) m9/10 has a much more generalized function, which is reflected in pleiotropic effects of *m9/10* reduction of function. Different mutant alleles may yield embryonic, larval or pupal (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988) lethality or recessive visible defects. How widely distributed is the E(spl) m9/10 protein? What is its subcellular localization? Answers to these questions can provide us with insights on whether the *E(spl) m9/10* product is a G-protein subunit and on how it interacts with *Notch* and other neurogenic genes. Toward that end we raised rabbit polyclonal as well as mouse polyclonal and monoclonal antibodies against four different fusion proteins of m9/10 segments as outlined on Figure 5. The details on antibody production will be presented elsewhere; we will limit ourselves here to an outline of the immunocytochemical studies performed and the information they have provided us on the nature of the E(spl) protein. Histochemically, all antisera and monoclonal supernatants gave identical staining patterns. Surprisingly, embryos homozygous for *E(spl)* deficiencies were not devoid of antigen (data not shown). However, the monoclonals were shown on Western blots to recognize a single protein of approximately 80 kD which corresponds to the predicted molecular weight of the E(spl) m9/10 protein. Given that three different monoclonal antibodies and several polyclonals gave similar results histochemically as well as on Western blots, we assume that the staining detectable in the deficiency embryos persisting for more than 15 hr after oviposition reflects the contribution of the maternal *m9/10* mRNA and a stable protein product rather than nonspecific staining by the antibodies. In fact, perdurance of the maternal *m9/10* product until the end of embryogenesis is in agreement with the larval lethality observed for *E(spl) m9/10* zygotically null individuals, despite the requirement of the m9/10 product in neurogenesis and possibly other embryonic functions as well.

We find that the m9/10 protein is expressed throughout development, and the expression pattern during embryogenesis is summarized in Figure 6. The most striking feature of m9/10 expression, in addition

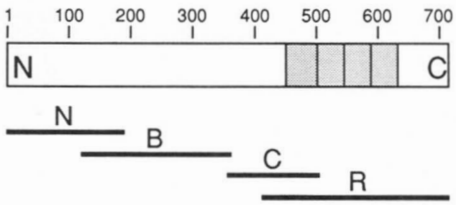

FIGURE 5.—A schematic representation of the m9/10 protein is shown with the amino (N) and carboxy (C) termini indicated. The coordinates correspond to number of amino acid residue. The stippled boxes correspond to the approximately 42 amino acid long β-transducin-like repeats. The four segments indicated below the protein map (N, B, C and R) are the ones used for generation of fusion proteins. Fragments N, B and C were fused to glutathione-*S*-transferase (SMITH and JOHNSON 1988) and the purified fusion proteins were used to immunize mice. Monoclonal antibodies were produced from one of the mice immunized with fusion protein B. Fragment R was fused to trpE (using the pATH1 vector; DIECKMANN and TZAGOLOFF 1985) and was used for rabbit immunizations.

to its being ubiquitous, is the obvious nuclear localization of the protein. Besides embryos, we have detected nuclear m9/10 protein in Kc and S2 cell lines, as well as imaginal disks, larval CNS and adult ovaries (data not shown). It is worth noting that the staining pattern we observe is not homogeneous but has a rather punctate appearance, the significance of which remains unknown. Comparing the nuclear staining pattern obtained with the DNA markers DAPI or propidium iodide (not shown) with that obtained with the anti-m9/10 antibodies, it is clear that the two patterns are not identical, indicating that the antigen is not tightly associated with the DNA. In large nuclei such as the nuclei of the cellular blastoderm or those of ovarian follicle and nurse cells, it is clear that portions of the nuclei are unstained. The unstained regions may correspond to nucleoli but until we have double stained with nucleolar specific markers this is only a hypothesis. The nuclear localization is consistent throughout development except where mitotic figures are found. In these cells, the antibodies stain diffusely throughout the cell (*e.g.*, see Figure 6b). This is particularly evident during the later divisions of the syncytial blastoderm.

We have used our antibodies to stain mutants for the zygotic neurogenic genes *Notch*, *big brain*, *mastermind*, *neuralized* and *Delta*. In none of these cases do we observe absence of E(spl) m9/10 protein or abnormal distribution. It must be emphasized however that in all these experiments, the maternal *E(spl) m9/10* contribution was not eliminated. However, we consider these results valid since the zygotic mutant condition is expected to be sufficient to produce abnormal E(spl) m9/10 distribution if such existed. We have also stained *Df(3R)boss* embryos, that are deficient for all *E(spl)* HLH genes except *m8*, while retaining *m9/10*. The localization of the m9/10 protein again remained normal. It thus appears that the nuclear localization of m9/10 is not a regulatory step in the function of this protein in neurogenesis.

DISCUSSION

The *E(spl)* chromosomal region contains a large number of transcription units with a variety of temporal expression profiles (KNUST, TIETZE and CAMPOS-ORTEGA 1987; PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). We have used *P* element transformation to show that two distinct classes of genes from this locus play a role in embryonic neurogenesis. One class, which corresponds to the zygotic component of *E(spl)*, comprises several homologous, functionally redundant genes that encode proteins with a HLH motif (KLÄMBT *et al.* 1989). The second class corresponds to the maternal function of *E(spl)* and is provided by the single copy gene *E(spl) m9/10* (HARTLEY, PREISS and ARTAVANIS-TSAKONAS 1988), which is also zygotically expressed and affects processes in addition to embryonic neurogenesis. Whereas these two components act in concert in the process of neuroblast-dermoblast segregation, they may also exhibit separable functions evidenced by distinct adult phenotypes: the enhancement of *spl* is attributed to a neomorphic allele of *E(spl) m8* (of the HLH family), while the *groucho* phenotype and the enhancement of *nd* and *Ax* are attributed to different alleles of *E(spl) m9/10* (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988; KLÄMBT *et al.* 1989; XU *et al.*, 1990).

Although the involvement of the HLH genes and *m9/10* in embryonic neurogenesis has been unequivocally established in the present study from constructs expressing no additional genes, *i.e.*, *C2.8*, *X5.6* and *E8*, we have not conclusively excluded a function in neurogenesis for genes *m6* and *mX*, since both of our large constructs contain these transcription units. However, two lines of evidence point toward a different function for these two genes. First, they should not have a vital role, since no lethal point mutations were recovered within $E(spl)^{BX22}$ other than the *m9/10* ones (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988), unless they too are members of a redundant gene family, for which no evidence exists. Second, their temporal (and, for *m6*, spatial) patterns of expression (KNUST, TIETZE and CAMPOS-ORTEGA 1987; PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988) imply functions during embryonic stages after neuroblast segregation has been completed. Even *m4* is unlikely to participate in *E(spl)* function despite the coincidence of its expression with the neurogenic region at the time of neuroblast segregation (KNUST, TIETZE and CAMPOS-ORTEGA 1987). We base this claim on the viability of individuals deficient for *m4* (Pr^{rev4}/*Df(3R)8D06*; A. PREISS, unpublished results) and the virtually complete rescue of the neurogenic defects of *Df(3R)8D06* and $Df(3R)boss^{14}$ by *E25R*,

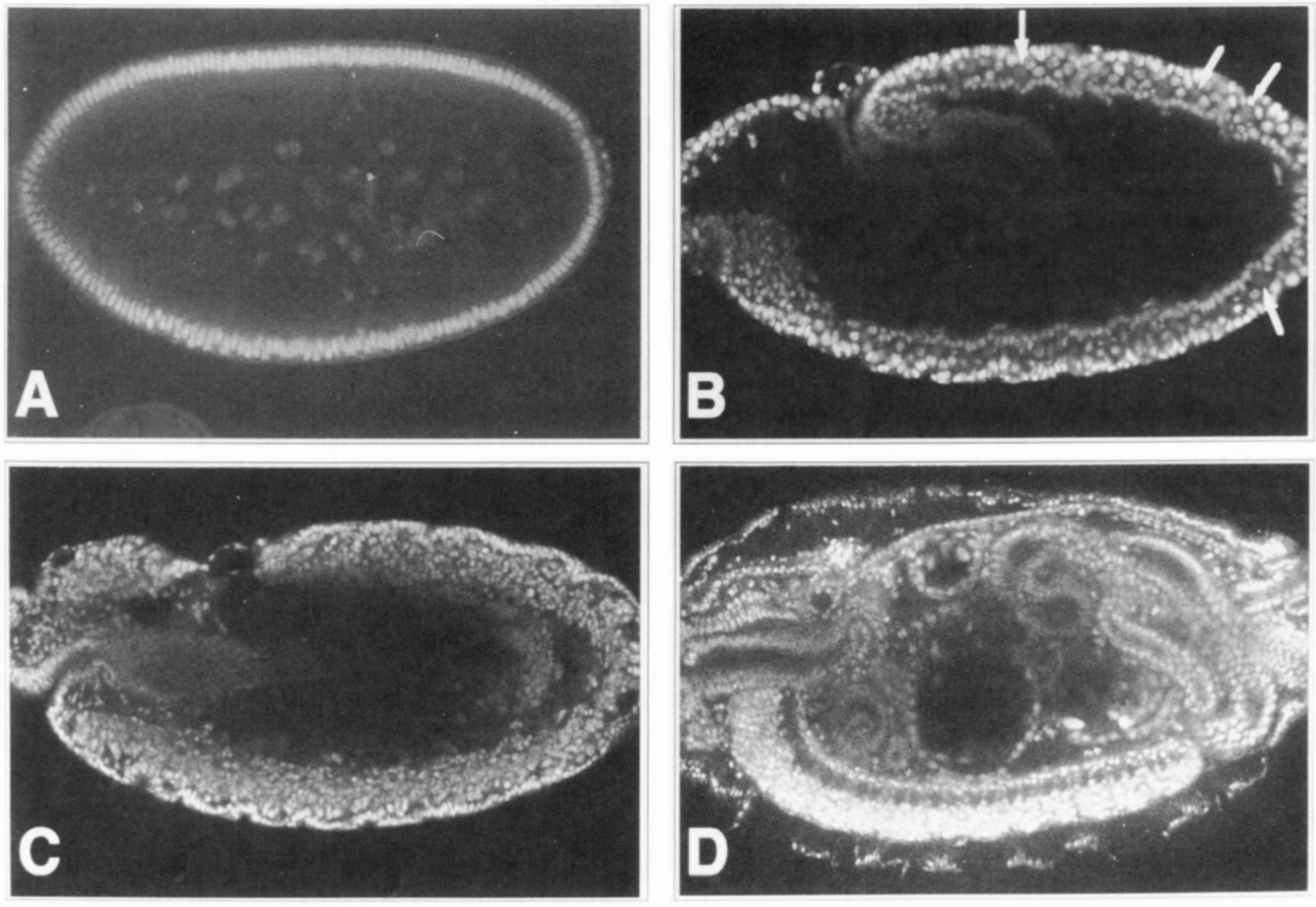

FIGURE 6.—Wild-type embryos of various developmental ages are shown stained with an anti-m9/10 monoclonal antibody and visualized by fluorescence using laser scanning confocal microscopy. All panels show sagittal sections with the anterior to the left and dorsal to the top. **A** is an embryo in the process of cellularization. Staining is observed in the peripheral somatic blastoderm nuclei, the nuclei of the pole cells (at the posterior pole) and the internal polyploid nuclei of the vitellophages. **B** is a stage 9 embryo (stages according to CAMPOS-ORTEGA and HARTENSTEIN 1985). Large segregating neuroblasts are seen between the epidermis and the mesoderm. Arrows point to neuroblasts displaying diffuse cytoplasmic staining. These were shown to contain mitotic chromosome figures upon observation of their propidium iodide staining pattern (not shown). **C** and **D** are at stages 12 and 16, respectively. Nuclei in all tissues appear to be staining.

which does not contain *m4*, or any gene homologous to it. As these rescued individuals do not develop into wild-type larvae, we cannot exclude a minor role for *m4*. Alternatively, some other gene included in these large deficiencies could be responsible for the slight defects of the *E25R/E25R ; Df(3R)boss/Df(3R)boss* individuals.

E(spl) m9/10 and the *E(spl)* HLH genes exhibit a dosage-dependent interaction, as the level of maternal *m9/10* product seems to influence the activity of the HLH gene products: from any genotype with a given zygotic HLH gene copy deficit, two different degrees of phenotypic severity can result according to whether the maternal copy number of *m9/10* is 1 (more severe) or 2 or more (less severe). In the cases where the more severe version of the phenotype is weak-intermediate or weak, increasing the maternal *m9/10* level leads to the survival of adult flies. On the other hand, a wild-type zygotic HLH genotype can produce neurogenic embryos when the maternal *m9/10* pool is poisoned by the presence of one antimorphic allele. As *E(spl)m9/10* exhibits a similar dosage- dependent interaction with Dl^{6B37}, we believe that *m9/10* does not participate in a more intimate interaction with the *E(spl)* HLH genes than it does with other neurogenic genes. Consequently, we currently favor the view that *m9/10* and the HLH genes are fortuitously juxtaposed since they do not share structural similarity or common expression patterns. Although we have determined the phenotype of embryos devoid of E(spl) HLH proteins to be extreme neurogenic (*E8/E8 ; Df(3R)8D06/Df(3R)8D06*), the zygotic (larval) lethality of *m9/10* null flies coupled with the maternal expression of *m9/10* has prohibited us from determining the phenotype of embryos devoid of m9/10 protein. We believe, however, that *m9/10* is also a necessary component of the neurogenic signalling mechanism, consistent with its participation in a tripartite dosage interaction with the *E(spl)* HLH genes and *Dl* (this work).

The members of the *E(spl)* HLH gene family exhibit functional redundancy, shown by the viability of flies deficient for *E(spl) m7* and *m8*. We have not shown, however, that the function of each member is entirely equivalent to that of the others. Our study offers no suggestion of functional specialization for any of the *E(spl)* HLH genes. However, it is still possible that each of these genes is expressed in a different subdomain, or subset of proneural clusters (as proposed by SIMPSON 1990), of the developing neuroectoderm and that there is sufficient overlap in these subdomains to allow the loss of some of the HLH genes to be tolerated. The answer to this question will come from a detailed analysis of the domains of expression of these genes by *in situ* hybridization and immunolocalization of their products.

At least one of the *E(spl)* HLH genes has been shown to affect imaginal development (*m8* in the eye disk, KLÄMBT *et al.* 1989; CAMPOS-ORTEGA and KNUST 1990), and it is highly likely that other *E(spl)* HLH genes will also show such pleiotropy. Is it possible that these genes will show a greater degree of nonoverlap in terms of their imaginal functions? One precedent for such a situation is provided by another closely related cluster of HLH genes in Drosophila, the *achaete-scute* complex (ASC). There are four members in this group, which play a central role in defining the proneural clusters, ectodermal domains from which central and peripheral neuroblasts will differentiate (see ROMANI *et al.* 1989; JIMÉNEZ and CAMPOS-ORTEGA 1990). While the functions of the individual members of the ASC show considerable overlap in the embryonic central nervous system (CNS), their embryonic and adult peripheral nervous system (PNS) domains of action are more distinctly defined. Different genes are expressed in different subdomains of sensory organs and consequently exhibiting distinct mutant phenotypes (GHYSEN and DAMBLY-CHAUDIÈRE 1988). It is as yet unclear whether members of the *E(spl)* HLH complex also have different imaginal domains of action, since no visible mutations mapping to individual *E(spl)* HLH genes have been isolated. Indeed, the *m7*- and *m8*-deficient flies constructed by combinations of deficiencies and transformed constructs appear wild-type. The only exception is the $E(spl)^D$ defect, which maps to *m8*; however, it is possible that a similar alteration in another *E(spl)* HLH gene would cause a similar phenotype. Indeed, the *m8* terminal deletion in the spontaneous $E(spl)^D$ allele must have been quite a serendipitous occurrence, since in screens for more second-site mutations enhancing the *spl* phenotype, no other allele of *E(spl)* was recovered (BRAND and CAMPOS-ORTEGA 1990). It is thus possible that the members of the *E(spl)* HLH complex perform largely overlapping functions even during postembryonic development.

Whether their equivalence is partial or complete, it is clear from the present analysis that a minimum copy number of these genes must be present in order for neurogenesis to proceed normally. Adding an increasing number of *E(spl)* HLH genes to a given deficiency genotype results in a graded improvement of its neurogenic defect, sometimes leading to complete rescue. It appears that the minimum level for normal *E(spl)* function is the hemizygous copy number, since *Df(3R)8D06/+* embryos are generally wild type, although weakly neurogenic lethal embryos are observed at a low frequency. Further reductions in HLH gene copy number result in fully penetrant embryonic lethality, *e.g.*, *Df(3R)P709/Df(3R)boss* from a *Df(3R)P709/+* mother, which should contain exactly the hemizygous complement of *E(spl)* HLH genes except for *m7*, and we know that *m7* itself is dispensable. This example also illustrates the interplay between the *E(spl)* HLH genes and *E(spl) m9/10*, since the same zygotic genotype from the reciprocal cross, *i.e.*, from a *Df(3R)boss/+* mother overlaps wild type with a viability of approximately 25% of expectation. This suggests that a lower level of *E(spl)* HLH activity can be tolerated when the maternal *m9/10* activity is increased, an interaction documented in various mutant combinations in this study.

The apparent requirement of *E(spl)* HLH genes for the expression of *m9/10*'s function suggests that the two members of the *E(spl)* complex act within the same pathway, although this does not in itself predict whether they act in sequence or simultaneously. The fact that the zygotic *m9/10* product is much less capable of rescue than the maternal one may suggest the existence of such a hierarchy, although it could simply reflect a difference in their molecular nature. However, we have no indication for distinct protein products from the *m9/10* gene, rather the two alternative *m9/10* transcripts seem to differ only in their 3′ untranslated regions (HARTLEY, PREISS and ARTAVANIS-TSAKONAS 1988). We therefore propose the following model for the involvement of primarily the maternal *m9/10* product in embryonic neurogenesis. Neuroblast segregation takes place during stages 9–11 of embryogenesis, corresponding to 3.5–7.5-hr postfertilization at 25° (CAMPOS-ORTEGA and HARTENSTEIN 1985). This corresponds with the period of peak transcription of the *E(spl)* HLH genes as well as of zygotic *m9/10* (PREISS, HARTLEY and ARTAVANIS-TSAKONAS 1988). We propose that *m9/10* product may be required before, or be instrumental in, the transcriptional activation of the *E(spl)* HLH genes; the nuclear localization of m9/10 protein is consistent with such a hypothesis. However, little or no zygotic *m9/10* product should be present at this early stage, explaining the observed requirement for maternal *m9/10*. The subsequent stimulation of zygotic *m9/10*

transcription upon the activation of the *E(spl)* HLH genes may also be dependent on the early (maternal) activity of *m9/10*. This possible autoregulatory step may be necessary for the normal functioning of the neurogenic signaling mechanism. Our data are consistent with such a hypothesis, since rescue mediated by the simultaneous provision of maternal and zygotic *E8* is significantly better than that mediated solely by provision of maternal *m9/10*$^+$ only.

The biochemical function of E(spl) m9/10 is still unclear, since its nuclear localization makes it an unlikely candidate for a G-protein subunit. We can, however, speculate that the E(spl)HLH proteins may be transcription factors required to switch on the epidermal differentiation program, by analogy to other HLH proteins. Since disruption of the neurogenic signalling mechanism results in inability to switch on the epidermal pathway, the dermoblasts must be the cells that require a gene regulation event as an outcome of receiving the lateral inhibition signal to initiate and maintain their epidermal nature. This regulatory event could be mediated by the *E(spl)* HLH gene products. In fact, DE LA CONCHA *et al.* (1988) proposed that *E(spl)* is the last link in the transduction of the lateral inhibition signal on the basis of dosage interactions among different neurogenic genes. Such a role is consistent with the observed restriction of the expression domains of *m5*, *m7* and *m8* to the epidermis after neuroblast delamination (KNUST, TIETZE and CAMPOS-ORTEGA 1987).

Two models regarding the biochemical function of the E(spl) HLH proteins could account for their role as regulators of the epidermal fate. One could envisage that these proteins interfere with the ASC proteins already expressed within the neurogenic regions in a way similar to that proposed for *extramacrochaetae* (emc) and *hairy* (h) (ELLIS, SPANN and POSAKONY 1990; GARELL and MODOLELL 1990; PARKHURST, BOPP and ISH-HOROWICZ 1990). These proteins, also of the HLH superfamily, are involved in adult PNS development probably not as transcriptional activators, but rather as negative regulators of the activity of ASC proteins. emc or h are proposed to bind to ASC proteins and either prevent them from binding to their DNA targets (emc) or render them transcription activation incompetent (h). Indeed, the E(spl) HLH proteins share a much higher similarity with hairy than with any other member of the HLH superfamily (except themselves). As biochemical data are lacking even in the cases of emc and h, alternative models are also possible. For example, *E(spl)* HLH proteins could directly bind DNA sites involved in switching on epidermal specific genes. In this case, we would expect that they would need a partner, since HLH proteins have been shown to bind DNA as dimers or oligomers (MURRE *et al.* 1989). Such a partner, when mutated, should also most likely exhibit a neurogenic phenotype, and thus we would expect it to be among the neurogenic genes (most probably a maternal neurogenic gene, since no other zygotic neurogenic gene seems to encode a HLH protein). Of course, the possibility also exists that *E(spl)* HLH proteins bind DNA as homodimers or dimers with other *E(spl)* HLH members. These different hypotheses can serve as guides upon which to base the biochemical analysis of the *E(spl)* HLH proteins.

Elucidation of the neurogenic signaling mechanism must await further biochemical characterization of all the participating components. At present, two transmembrane molecules are known to be involved (Notch, Delta) as well as several nuclear components, *i.e.*, mastermind (SMOLLER *et al.* 1990), E(spl)m9/10 and the E(spl) HLH proteins, the latter only presumed nuclear by analogy to other HLH proteins. The question arises as to how the lateral inhibition signal is transmitted between the cell membrane and the nucleus. Consideration of genetic interactions raises an interesting possibility. Given that allele-specific interactions occur between *Notch* and both *E(spl) m8* (*spl* enhancement) and *E(spl) m9/10* (*nd* and *Ax* enhancement), might there be a direct interaction between Notch and these two *E(spl)* products? We are currently investigating the possibility that either the *E(spl)* products are transiently present in the cytoplasm or, alternatively, that Notch, in whole or in part, may be found to reside in the nucleus. In this respect, it should be mentioned that *nd* alleles, mutations affecting the intracellular domain of the Notch protein (XU *et al.* 1990), display dramatic interactions with *mastermind*, which also appears to encode a nuclear protein. If indeed such direct interactions take place, we may have identified the link between extracellular signals and the nucleus without having to invoke additional cytoplasmic intermediaries. More experimentation is needed to validate or refute the different hypotheses, and we now have the biochemical reagents necessary to address these questions directly.

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