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The molecular genetics of *Enhancer of split*, a gene required for embryonic neural development in *Drosophila*

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In *Drosophila*, the very first steps in neurogenesis appear to be controlled by a small group of zygotically acting genes termed the neurogenic loci. Mutations in any of these genes result in a misrouting of epidermal lineages into the neural pathway. Morphological and molecular studies suggest that the correct ectodermal differentiation is mediated by a cell–cell interaction mechanism and that at least some of the neurogenic loci are involved in this mechanism. The molecular analyses of the neurogenic loci *Notch* and *Delta* revealed that the putative gene products are large transmembrane proteins with homology to mammalian epidermal growth factor. We describe here a molecular analysis of *Enhancer of split* [*E(spl)*], a third neurogenic locus, which displays striking genetic interactions with both *Notch* and *Delta*, suggesting a close functional relationship of the respective gene products. We provide evidence for a single genetic complementation group corresponding to a single transcription unit which is necessary for wild-type *E(spl)* function. P-element-mediated transformation indicates that this transcription unit includes functions associated with both the dominant $E(spl)^D$ mutation and the recessive visible allele *groucho*, and is necessary for the correct differentiation of the embryonic nervous system.

Key words: development/*Drosophila*/*Enhancer of split*/molecular genetics/neurogenesis

Introduction

Neurogenesis in the *Drosophila* embryo starts with the differentiation of the ventral ectodermal cell layer into neuroblasts and dermoblasts, the precursors of the nervous system and ventral epidermis respectively. Individual neuroblasts delaminate from this defined region of the ectoderm, termed the 'neurogenic region', after the onset of gastrulation and continue to do so for another 3 h of development (Poulson, 1950; Hartenstein and Campos-Ortega, 1984). Evidence that this ectodermal differentiation is under genetic control first arose 50 years ago when Donald Poulson described the phenotype of embryos mutant for the *Notch* locus (Poulson, 1937). These embryos exhibit an hypertrophied central nervous system and lack ventral and lateral epidermis. This 'neurogenic phenotype' appears to arise due to an abnormal differentiation of the ectoderm such that cells destined to give rise to dermoblasts switch fate and become neuroblasts. A number of other zygotically acting loci have been described whose mutant phenotype is qualitatively similar to *Notch* mutants. These loci, collectively termed the neurogenic loci, are *Notch* (*N*) itself, *big brain (bib)*, *Delta (Dl)*, *Enhancer of split* [*E(spl)*], *mastermind* (*mam*) and *neuralised* (*neu*) (Lehmann *et al.*, 1983; Jürgens *et al.*, 1984; Nüsslein-Volhard *et al.*, 1984). The similarity of phenotype amongst neurogenic mutants suggests they act in a common pathway leading to the definition of this neural/epidermal dichotomy.

The genetic and biochemical analysis of *Notch*, so far the best characterized among the neurogenic loci, has indicated a molecular mechanism for the regulation of early neurogenesis (Artavanis-Tsakonas, 1988). Sequence data has shown that it codes for a 2703 amino acid long transmembrane protein whose extracellular domain shows striking homology to the mammalian epidermal growth factor (Wharton *et al.*, 1985a). The putative intracellular domain contains sequences homologous to certain yeast cell cycle genes (Breeden and Nasmyth, 1987). The structure of the *Notch* protein suggests that it is involved in a cell interaction mechanism mediating the differentiation of the embryonic nervous system (Wharton *et al.*, 1985a). This mechanism may be more ubiquitously applied throughout development since *Notch* is expressed in many different tissue primordia (Hartley *et al.*, 1987; Markopoulou, 1987).

The concept of regulation of early neurogenesis by a cell interaction mechanism is supported by embryological studies both in the grasshopper and in *Drosophila*. Laser ablation of cells in the neural ectoderm of grasshopper embryos (Doe and Goodman, 1985) and transplantation of cells within the ectoderm of *Drosophila* embryos (Technau and Campos-Ortega, 1986) have indicated the dependence of correct ectodermal differentiation on cellular interactions. The implied involvement of *Notch* in a cell interaction mechanism dictates the participation of other gene products in its function. Such gene products might well be the other neurogenic gene products. An involvement of the other neurogenic loci in cell interactions is supported by the finding that *Delta* also codes for a transmembrane protein with homology to the epidermal growth factor (Vässin *et al.*, 1987; M.Muskavitch, personal communication). In addition, as would be expected for individual elements of a given cell interaction mechanism, mutants of at least some of the neurogenic loci interact phenotypically (Campos-Ortega *et al.*, 1984; Vässin *et al.*, 1985). Perhaps the most striking genetic interactions are those seen between alleles of *Notch*, *Enhancer of split* and *Delta*. *E(spl)* was first identified by virtue of the interaction of a dominant allele [$E(spl)^D$] with *split*, a recessive visible mutation of the *Notch* locus. Animals heterozygous for *split* (*spl*/+) are wild-type. However, double heterozygotes of the genotype *spl*/+; $E(spl)^D$/+ display a *spl* phenotype (Welshons, 1956). Revertants of the dominant allele, $E(spl)^R$, are homozygous embryonic lethals and exhibit the characteristic neural hypertrophy of the neurogenic mutants (Lehmann *et al.*, 1983). The require-

ment of correct ectodermal differentiation for both *Notch* and *E(spl)* suggests that they influence the same developmental process. A close relationship of gene function is further implied by the dramatically reduced viability of transheterozygotes of the type $N/+$; $E(spl)^{R}/+$ (Vässin *et al.*, 1985; A.Preiss, unpublished). A similar 'non-complementing' behavior is observed between null alleles of *E(spl)* and *Dl*, but not between *Dl* and *Notch* (Campos-Ortega *et al.*, 1984; Vässin *et al.*, 1985; A.Preiss, unpublished). Of all the neurogenic mutants examined, only *E(spl)* mutants exhibit a cell autonomous phenotype from cell transplantation studies (Technau and Campos-Ortega, 1987). Unfortunately, the only alleles tested in these experiments were large deficiencies involving several cytological bands and so the clone phenotype may not reflect an actual *E(spl)* phenotype. However, cumulatively, the data indicate a pivotal role for the *E(spl)* gene amongst the neurogenic genes in the regulation of neural development.

The genetic data have suggested a special relationship between *Notch* and *E(spl)* which might reflect an underlying biochemical association between the respective gene products. Examination of *Notch* expression in *E(spl)* mutants has indicated that the interaction does not occur at the level of transcription (Hartley *et al.*, 1987). Instead, the DNA sequence of the *split* chromosome shows that it codes for a missense mutation in the *Notch* protein (Hartley *et al.*, 1987; Kelley *et al.*, 1987). This has suggested that this part of the molecule marks a site where the two gene products interact. In order to examine the role of the *E(spl)* gene in neurogenesis and its interaction with *Notch*, we have initiated a genetic and molecular study of the *Enhancer of split* locus. In this paper we describe the isolation and characterization of sequences involved in *E(spl)* function. We provide evidence for a single genetic unit at 96F11-14 which is necessary for wild-type *E(spl)* function. We have cloned DNA encompassing the region and identified a single transcription unit corresponding to this genetic unit. P-element-mediated transformation indicates that this transcription unit includes functions associated with the dominant $E(spl)^{D}$ mutant, with *groucho*, a recessive visible mutant, and is necessary for the correct differentiation of the neurogenic region.

Table I. Cytogenetic analysis of mutants in the 96F/97 region

Mutant	Cytology	Origin	Homozygous[a]	Uncovers neurogenic[b] phenotype	Uncovers *gro*[c] phenotype	ref.
$E(spl)^{D}$	normal	spont.	viable	no	no	1
gro	normal	spont.	viable	no	yes	1
$Df(3R)^{8D06}$	Df(3R) 96E10/12-97A3/4[d]	EMS	em. lethal, ex	yes	yes	2
$E(spl)^{R1}$	Df(3R) 96F2-96F12/14 plus Inv(3R) 96F-99C[d]	X-ray	em. lethal, ex	yes	yes	3
$E(spl)^{R2}$	Df(3R) 96F5-97A9/10[d]	X-ray	em. lethal, ex	yes	yes	3
$E(spl)^{BX21}$	Tr(3/2) 96F10/12 to 2R Centromer	X-ray	em. lethal, variable	yes	yes	4
$E(spl)^{BX22}$	normal	X-ray	em. lethal, int	yes	yes	4
$E(spl)^{BX36}$	Df(3R) 96F1-97B1	X-ray	em. lethal, ex	yes	yes	4
$l(gro)^{X1}$	Df(3R) 96F5/7-96F12/14	X-ray	em. lethal, ex	yes	yes	5
$l(gro)^{X72}$	Df(3R) 96F5/7-97B1	X-ray	em. lethal, ex	yes	yes	5
$l(gro)^{X115}$	normal	X-ray	em. lethal, w^{e}	yes	yes	5
$E(spl)^{E28}$	normal	EMS	lethal, p.l.[e], w^{e}	yes	no	5
$E(spl)^{E48}$	normal	EMS	lethal, w^{e}	yes	yes	5
$E(spl)^{E73}$	normal	EMS	lethal, w^{e}	yes	yes	5
$E(spl)^{E75}$	normal	EMS	lethal, w^{e}	yes	yes	5
$E(spl)^{E77}$	normal	EMS	lethal, w^{e}	yes	yes	5
$E(spl)^{E107}$	normal	EMS	lethal, p.l.[e], w^{e}	yes	no	5
Pr	Inv(3R) 96F11/14-97B2/3[d]	spont.	lethal	no	no	1
Pr^{L}	'add. band' in 96F10/11[d]	spont.	viable	no	yes	1
$Df(3R)ro^{82b}$	Df(3R) 96F11/14-97F3/11[d]	spont.	em. lethal, int	yes	yes	6
Pr^{P9}	Df(3R) 96F11/14-97D8/10	X-ray	em. lethal, int	yes	yes	5
Pr^{rev1}	Df(3R) 96F11/14-97E2/3	X-ray	em. lethal, ex	yes	yes	5
Pr^{rev4}	Df(3R) (96F11/14) 97B	X-ray	lethal	no	no	5
Pr^{rev6}	Inv(3R) 96F11/14-98A	X-ray	lethal	no	no	5
Pr^{rev10}	Inv(3R) 96F11/14-97C1	X-ray	lethal	no	no	5
Vno	Tp(3R) new order: 61-89E|94A1-96F11/14|93F-94A|89E-93F|96F11/14-100F[d]	spont.	lethal	no	no	1
Tl^{5BRXQ}	Df(3R) 96F11/14-97D[d]	X-ray	lethal	no	no	7

[a]Homozygous *viable*: adult fertile flies; *lethal*: no adult flies are observed; *em. lethal*: fully penetrant embryonic lethal; *p.l.*: a low number of homozygous pupae is observed; *ex*: extreme neurogenic phenotype, Figure 1b; *int*: intermediate neurogenic phenotype, Figure 1c; *w*: weak neurogenic phenotype, Figure 1d.

[b]Mutant embryos develop neural hypertrophy (see Figure 1) in *trans* over any other *E(spl)* allele indicated to cause a neurogenic phenotype.

[c]Homozygous *gro* flies have a low penetrant phenotype, which can be enhanced (1), but never reaches 100%; even over deficiencies the penetrance is never complete and varies over different alleles.

[d]No complete penetrance.

[e]This work and P.Lewis, personal communication.

References: (1) Lindsley and Grell (1968), (2) Jürgens *et al.* (1984), (3) Lehmann *et al.* (1983), (4) M.Muskavitch (unpublished), (5) A.Preiss (unpublished), (6) P.Lewis (unpublished), (7) Anderson *et al.* (1985).

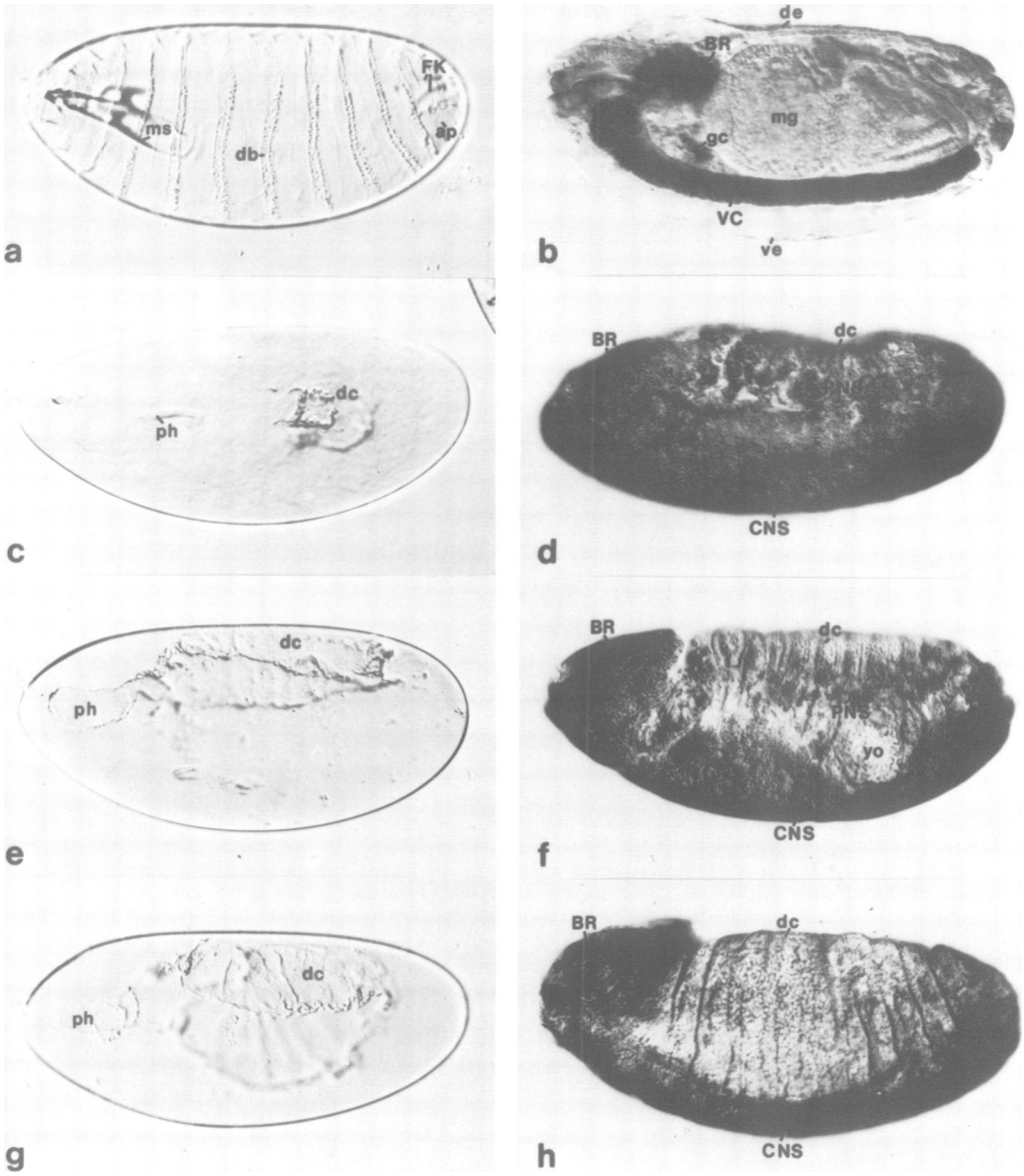

Fig. 1. *E(spl)* neurogenic phenotypes. Pictures on the left show cuticles from embryos (at least 24 h old). The pictures on the right show the embryonic nervous system stained with anti-horseradish peroxidase (anti-HRP) antibodies (Jan and Jan, 1982). Shown is a lateral view, anterior is always to the left, dorsal up. Pictures were taken with Nomarski optics. For a description of *Drosophila* embryogenesis see Campos-Ortega and Hartenstein (1985). (**a**) Ventral–lateral view of a wild-type embryo. The ventral denticle belts (db) are prominent markers of the thoracic and abdominal segments, posteriorly the anal plate (ap) is visible. The interior mouth skeleton (ms) and the Filzkörper (FK) are out of the focal plane. (**b**) The anti-HRP staining of an ~15 h old wild-type embryo clearly shows the ventral cord (VC) and the brain (Br). The garland cells (gc) are also stained with the anti-HRP antibody (Jan and Jan, 1982). Dorsal closure is completed and the epidermis surrounding the embryo is clearly visible (de = dorsal epidermis; ve = ventral epidermis; mg = midgut). (**c**) In homozygous $l(gro)^{X1}$ embryos only patches of the dorsal cuticle (dc) are left, which are usually separated, as in the embryo shown, due to a failure of a complete dorsal closure. Remains of the pharynx (ph) are usually present in these embryos. (**d**) The staining of neuronal cells reveals an extremely hypertrophied central nervous system (CNS). Clusters of peripheral nervous system (PNS) cells are visible below a small patch of remaining dorsal cuticle (dc). This embryo (~15 h or older) seems to have completed dorsal closure. (**e**) In homozygous $E(spl)^{BX22}$ embryos, the dorsal cuticle (dc) is mostly intact—it is segmented, although irregularities due to a failure of normal dorsal closure are frequent. Dorsal cuticular bridges reach from the head region to the roof of the pharynx (ph). The Filzkörper (FK), which come from a dorso-lateral position (Anderson and Nüsslein-Volhard, 1984) are present in these embryos. (**f**) Staining of neurons in $E(spl)^{BX22}$ embryos (15 h or older) reveals the strong hypertrophy of the central nervous system (CNS). The peripheral nervous system (PNS) cells appear in similar clusters, as in the more extreme $l(gro)^{X1}$ homozygotes, below the remains of the dorsal cuticle (dc). In these embryos we often find yolk (yo) or parts of the midgut pushed out through the hypertrophied nervous system. (**g**) Typical cuticle of a homozygous point mutant. The mutant shown is $E(spl)^{E73}$, which develops a similar phenotype in similar frequencies as the other point mutants (Table I; A.Preiss, unpublished). The patch of cuticle extends laterally on both sides with primarily only ventral cuticle missing. In the head region, however, the effect of the mutation seems much stronger: only remains of the cephalic cuticle and the pharynx (ph) are left, connected by cuticle bridges with the dorsal cuticle (dc). (**h**) Anti-HRP staining of the point mutant $E(spl)^{E107}$ clearly reveals a hypertrophy of the central nervous system (CNS), especially in the brain (Br). This phenotype is also typical for the other point mutants in *E(spl)*. The embryo shown has completed dorsal closure. Abbreviations: ap = anal plate; Br = brain; CNS = central nervous system; dc = dorsal cuticle; de = dorsal epidermis; FK = Filzkörper; mg = midgut; ms = mouth skeleton; ph = remains of the pharynx; PNS = peripheral nervous system; VC = ventral cord; ve = ventral epidermis.

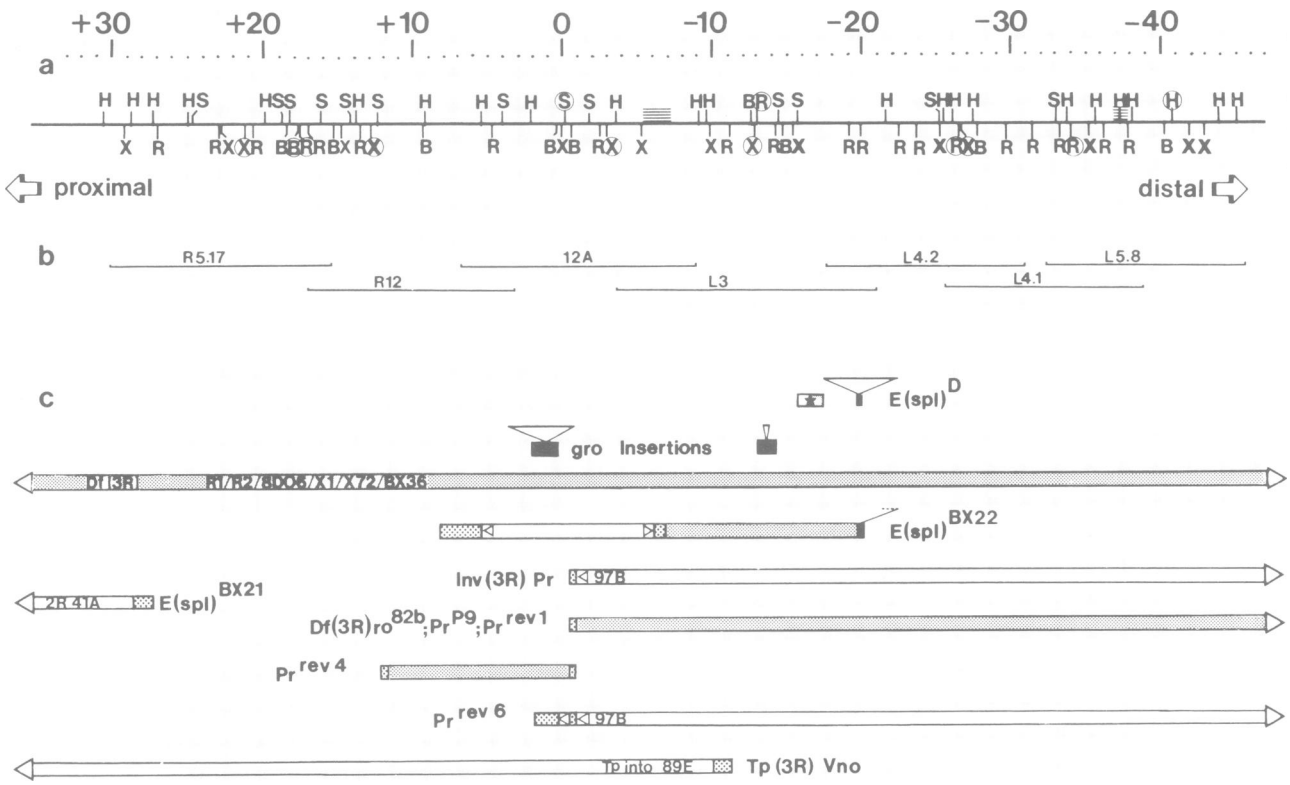

Fig. 2. Physical map of the *E(spl)* region. (**a**) Representation of the physical map of ~80 kbp in the *E(spl)* region. The orientation (proximal/distal) is relative to the right arm of the third chromosome (3R). Depicted restriction sites are: B = *Bam*HI, H = *Hin*dIII, R = *Eco*RI, S = *Sal*I, X = *Xho*I. Encircled restriction sites are polymorphic in some *Drosophila* wild-type strains. The two regions found in the walk with homology to *opa* (Wharton *et al.*, 1987b) are hatched. One unit in the coordinate scale represents 1 kbp. Coordinate 0 is chosen arbitrarily and corresponds to the *Xho*I site next to the adm126D12 clone (M.Wolfner, 1980; see Figure 3d) which was used to initiate the walk. (**b**) Individual phage clones covering the *E(spl)* region. The lambda-clones were isolated from a *Drosophila* Canton S/Charon 4A library (Maniatis *et al.*, 1978). (**c**) Molecular analysis of mutants. All mutants listed in Table I were analyzed in whole genome Southerns for alterations of their genomic DNA in comparison to the parental strain or, where the parental strain was not available, to several *Drosophila* wild-type strains. Alterations found in the cloned region are depicted relative to the physical map. Strains were tested in homozygosity or transheterozygous over deficiency or balancer chromosome. In order to distinguish natural restriction site polymorphisms from mutational events we used at least three different restriction enzymes (*Eco*RI, *Hin*dIII, *Xho*I) and usually five (plus *Bam*HI, *Sal*I) for the analysis. In most cases the smallest restriction fragment defined to overlap the breakpoint of a chromosomal rearrangement (dotted bars) was itself used as a probe to hybridize to the mutant DNA in order to confirm that all the expected breakpoint fragments hybridized. Breakpoints of chromosomal rearrangements [*Pr, Vno, E(spl)*^{BX21}] were confirmed by *in situ* hybridization to polytene chromosomes. Inversions are indicated by small triangles; deletions are lightly shaded. *Pr*^{rev6} carries an inversion on top of the original *Pr* inversion [In(3R) 96F/99C; Table I]. The proximal breakpoint of this second inversion is very close to, but clearly separable from the proximal *Pr* inversion breakpoint. In *E(spl)*^{D} a deletion of ~0.4 kbp is found in the fragment indicated with an asterisk. One insertion is found in *E(spl)*^{D} and two are found in *gro*.

Results

Cytogenetic analysis of E(spl)

In order to define the cytogenetic location of *E(spl)*, several mutagenesis screens were carried out and as a result 15 new alleles were isolated (A.Preiss, unpublished). Such screens were necessary since the few *E(spl)* alleles available (Lehmann *et al.*, 1983; Jürgens *et al.*, 1984) turned out to be large deficiencies (Table I) and thus inadequate for accurate genetic mapping. Table I summarizes the cytogenetic analysis of the *E(spl)* alleles including chromosomal rearrangements which map in the 96F/97 region and were thus useful for the mapping. Genetic complementation analysis of the *E(spl)* alleles revealed that they all fall into a single lethal complementation group (Table I). Embryos which are homozygous or transheterozygous mutant for any of these *E(spl)* alleles eventually die and display the 'neurogenic phenotype': a nervous system hypertrophy varying in degree between different alleles (Figure 1, Table I). The *E(spl)* lethal complementation group also contains the recessive visible mutation *groucho* (*gro*, 3-90.0; Lindsley and Grell, 1968). The details of the screens and the genetic behavior of the various alleles will be described elsewhere.

Cytogenetic examination of deficiencies in the *E(spl)* region positioned the gene at (3R) 96F11/14 by means of its inclusion in the Df(3R) *ro*^{82b} (96F11/14-97F3/11; P.Lewis, personal communication) and its exclusion from the deficiency *Tl*^{5BRXQ} (97A1-97D; Anderson *et al.*, 1985) (Table I).

Chromosomal walk

The chromosomal walk in the *E(spl)* region was initiated with the cDNA clone adm126D12, previously localized to 96F/97A and kindly provided to us by M.Wolfner. The walk comprises ~80 kb of overlapping clones isolated from a *Drosophila* Canton S library (Maniatis *et al.*, 1978) as shown in Figure 2a and b. We found an unusual amount of restriction site polymorphism compared to, for example, the *Notch* and the *Krüppel* region (Grimwade *et al.*, 1985; Preiss *et al.*, 1985) (encircled in Figure 2a). As judged by 'reverse Southern' analysis the region contains few repetitive sequences, mostly located at the very distal part of the walk,

including two *opa* repeats (Wharton *et al.*, 1985b) (Figure 2a, hatched). The orientation of our walk with respect to the chromosome has been verified by *in situ* hybridization. Hybridizing whole lambda-clones of the walk to translocation or inversion chromosomes with a known breakpoint in the cloned region [such as $E(spl)^{BX21}$ or Inv (3R)*Pr*, see Figure 2c and Table I] we found signals either just on the distal or on the proximal side of the breakpoint. The use of a clone overlapping the breakpoint gave signals on both sides (data not shown). We could thus orient the physical with the cytological map.

Mapping of chromosomal breakpoints

In order to define the *E(spl)* locus with respect to the physical map we tested all the mutants listed in Table I for molecular lesions. Genomic DNA prepared from flies homozygous or heterozygous (over wild-type or over deficiency) for a given mutation was compared to DNA from the corresponding parental strain. Strains with unknown parental background [$E(spl)^{D}$, *gro, Vno, Pr*] were compared to several different wild-type strains. In the case of an inversion (*Pr*) or transposition (*Vno*) we verified by *in situ* anlaysis that restriction fragment alterations reflect the chromosomal rearrangement (data not shown). The results of the breakpoint analysis are summarized in Figure 2c, showing locations of alterations found in the various mutants in relation to the physical map. *E(spl)* alleles without detectable alterations are not depicted. We refer to them as point mutants since they are also cytologically normal (Table I).

Besides the large deficiencies that delete the entire cloned region, other deletions uncovering the *E(spl)* neurogenic phenotype affect just the distal half of our walk. Three of these deletions [Df(3R) ro^{82b}, Pr^{rev1}, Pr^{P9}] have a common proximal border which is the proximal breakpoint of the *Pr* inversion. Deletions proximal to this breakpoint (Pr^{rev4}) do not uncover *E(spl)* (Table I). This indicates that *E(spl)* resides distal to the *Pr* breakpoint (coordinate −0.2 of our physical map; see Figure 2). A more precise localization is made possible from the analysis of $E(spl)^{BX22}$ which is associated with a deletion of ~14 kbp and, in addition, an inversion of 14 kbp (see Figure 2c). This cytologically invisible lesion results in a fully penetrant, neurogenic phenotype (Figure 1c). Therefore, sequences essential for *E(spl)* wild-type gene function must be located in the region affected by this mutation. Given the viability of $E(spl)^{BX22}/Pr^{rev4}$ flies, the proximal inversion breakpoint of $E(spl)^{BX22}$ itself seems not to disrupt any essential gene function because it is uncovered by the Pr^{rev4} deletion (see Figure 2c). Furthermore, we find breakpoints of three other chromosomal rearrangements in the cloned region: the distal breakpoint of the transposition *Vein off* (*Vno*, 3−; Lindsley and Grell, 1968) and the proximal breakpoints of both inversions *Pr* and Pr^{rev6}. All three mutations are viable and fertile over deficiencies which uncover these breakpoints (Table I). Therefore, these breakpoints as well seem not to disrupt any essential genes. The translocation breakpoint associated with $E(spl)^{BX21}$ allele occurs proximally to the region deleted in the $E(spl)^{BX22}$ chromosome. Since this translocation is to the centromere of 2R we suspect that the variable neurogenic phenotype of this mutant is caused by position effect variagation.

Besides the gross rearrangements of the *E(spl)* alleles shown in Figure 2 we find minor lesions in *gro* and $E(spl)^{D}$ mutants. Both the $E(spl)^{D}$ and the recessive visible *gro* mutations seem to be associated with insertion elements (Figure 2c). In addition, the $E(spl)^{D}$ chromosome has a small, ~0.4-kbp deletion at −15.7 to −16.9. The significance of these lesions is unclear since the parental background of both mutations is unknown. In fact the large *gro* insertion at 0/+2 can be excluded as causative for the *gro* phenotype since the mutation is not uncovered by an overlapping deletion (Pr^{rev4}; see Figure 2c and Table I). Moreover, it is not complemented by Pr^{L}, which by both molecular and cytological (P.Lewis, personal communication) criteria was found to be a duplication of chromsome material proximal to the *Pr* breakpoint.

In summary, the genetic and molecular analysis allow us to position sequences essential to *E(spl)* function distally to the *Pr* inversion breakpoint (−0.2 on our map) and within the breakpoints of the $E(spl)^{BX22}$ deletion (−5 to −20).

Transcript analysis

The *E(spl)* gene must be active during embryonic development of *Drosophila* since a deletion of the gene leads to an embryonic lethal neurogenic phenotype (Lehmann *et al.*, 1983). It should also be active during oogenesis since, genetically, *E(spl)* exhibits a maternal component (Knust *et al.*, 1987a; A.Preiss, unpublished). The phenotypic interactions of the dominant $E(spl)^{D}$ allele with the eye mutant *split* suggests in addition that the *E(spl)* gene is active during post-embryonic stages. However, this dominant phenotype could be caused by an ectopic expression of the wild-type product. Taking these genetic data into account we expect the wild-type *E(spl)* gene to be active at least during oogenesis (females), in young embryos (due to deposition of maternal transcript), in older embryos (due to the zygotic activity of the gene) and perhaps also in post-embryonic stages.

In order to explore the overall transcriptional activity of the *E(spl)* region we hybridized an embryonic cDNA probe corresponding to poly(A)$^{+}$ RNA of 0−22 h old embryos to restriction fragments of our walk and we found that the entire cloned region is extensively transcribed during embryogenesis (Figure 3b). Subsequent Northern analysis confirmed this initial observation.

In the Northern analysis we searched for transcripts derived from the genomic region distal to the *Pr* breakpoint since this breakpoint genetically defines the proximal border of the *E(spl)* locus. In this area we have detected 14 embryonic transcripts ranging in size from 1 to 5 kb. All of them seem to be transcribed independently as judged by differences in their size and temporal profiles (data not shown). We focused our attention to the area deleted in $E(spl)^{BX22}$ since the genetic and molecular data suggest that it contains sequences necessary for $E(spl)^{+}$ function.

The two largest transcripts from this area, one ~4 kb, the other ~3 kb, appear to derive from the same transcription unit since we cannot detect the 3-kb transcript without also hybridizing to the 4-kb transcript. This transcription unit spans the insertion site of *E(spl)* and includes the $E(spl)^{BX22}$ deletion breakpoint (Figure 3a,c). The $E(spl)^{BX22}$ deletion uncovers at least five additional, smaller transcripts (Figure 3a,c) any of which could account for the *E(spl)* gene product. Figure 3e shows the developmental profiles of the transcripts mapping within the $E(spl)^{BX22}$ deletion. With one exception the various transcripts are not detectable during the first 2 h

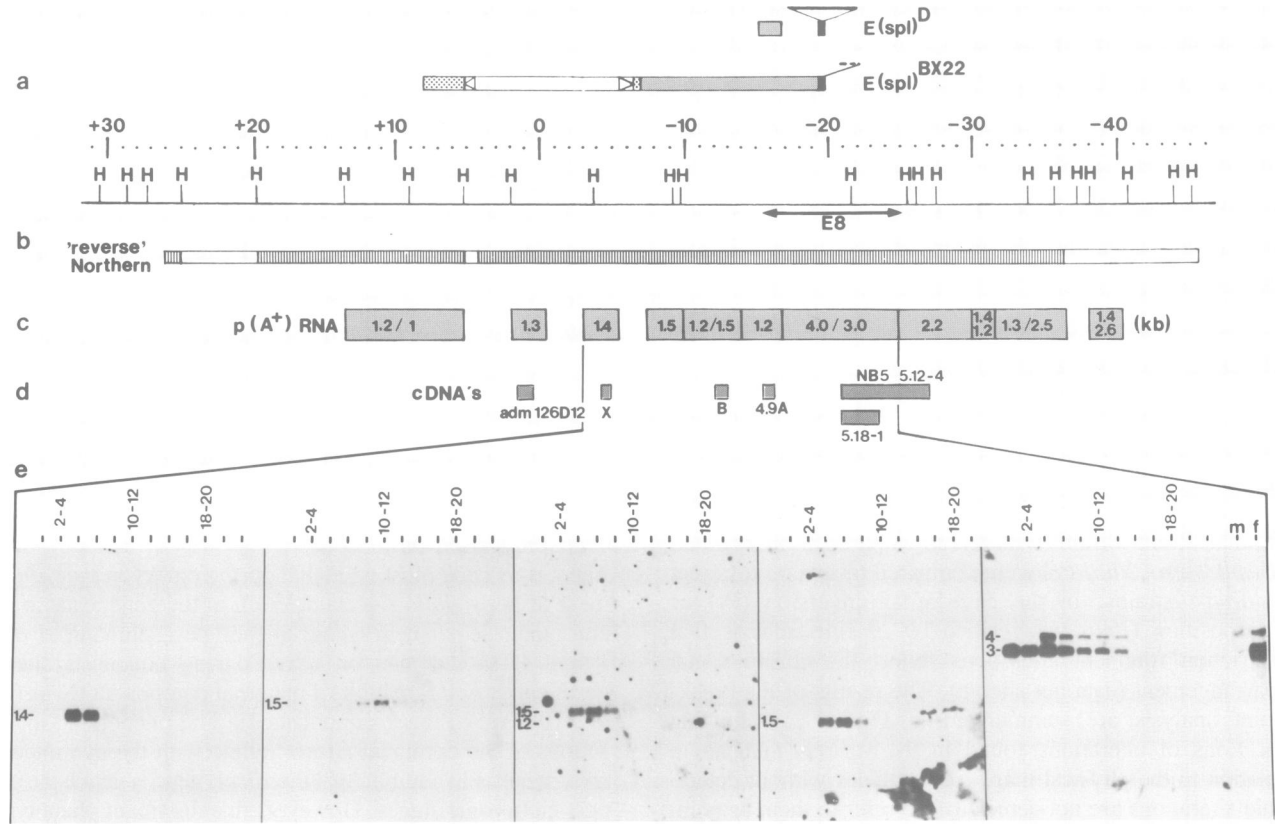

Fig. 3. Transcript analysis of the *E(spl)* region. A 'reverse Northern' analysis shows that the entire cloned region is transcribed during *Drosophila* embryogenesis. Using a cDNA probe corresponding to the poly(A)$^+$ transcripts of 0–22 h old embryos (25°C) restriction fragments transcribed during that period hybridize as shown by hatched bars in (**b**). Embryonic transcripts are depicted underneath the corresponding genomic fragment [light shaded bars in (**c**)] and their approximate length as judged from DNA length standards in the Northern analysis is given in kb. A developmental profile of each of the transcripts affected by the *E(spl)*^{BX22} deletion (**a**) is shown in (**e**). Poly(A)$^+$ RNA (~5 μg) of successively older embryos (0–2; 2–4; 4–6; 6–8; 8–10; 10–12; 12–14; 14–16; 16–18; 18–20; 20–22; 22–24 h at 25°C) was loaded and Northern blots hybridized with either genomic or corresponding cDNA probes. With the exception of the most distal transcription unit which shows maternal expression of the smaller, 3-kb message (present in 0–2 h old embryos and about five times as abundant in females [f] as in males [ml]) all transcripts show a strictly zygotic pattern of expression. Corresponding cDNAs to some transcripts were isolated from the Kauvar library (3–6 h, embryonic; Poole *et al.*, 1985) and (if labelled 'NB') from a 4–8 h embryonic cDNA library (Brown *et al.*, 1988). The cDNA clone adm126D12 was kindly provided by M.Wolfner. (**d**) Only representative cDNAs are shown (dark shaded bars). The arrow (**a**) marks the genomic fragment [E8] which was used for the transformation experiments. Coordinates are the same as in Figure 2; however, only *Hin*dIII (H) sites are depicted in the simplified restriction map. Genomic lesions found in *E(spl)*^{D} and *E(spl)*^{BX22} are shown for orientation (**a**) (compare with Figure 2c).

Table II. Complementation analysis of point mutants in the background of P[ry$^+$ E8]

	l(gro)^{X115}	*E(spl)*^{E28}	*E(spl)*^{E48}	*E(spl)*^{E73}	*E(spl)*^{E75}	*E(spl)*^{E77}	*E(spl)*^{E107}	*E(spl)*^{BX22}
l(gro)^{X115}	+							
E(spl)^{E28}	+	+						
E(spl)^{E48}	+		+					
E(spl)^{E73}	+			+				
E(spl)^{E75}	+				+			
E(spl)^{E77}	+	+	+	+	+	−		
E(spl)^{E107}	+	+	+			+	+	
E(spl)^{BX22}	+	+	+	+	+	+	+	−
E(spl)^{BX21}	+		+	+		+		+
Df(3R)^{8D06}	+	+	+	+	+	+	+	−
l(gro)^{X1}	+	+	+			+	+	

Males mutant in *E(spl)* were mated to virgins homozygous for the P-insertion P[ry$^+$ E8]. Offspring heterozygous for both the P[ry$^+$ E8] insertion and the *E(spl)* mutation were selected and mated *inter se* or with another *E(spl)* allele heterozygous for P[ry$^+$ E8]. The next generation was scored for flies homozygous or transheterozygous mutant in *E(spl)* which could be distinguished from recombinants and from heterozygotes over wild-type by their genetic markers.

+ = fertile homozygous mutant flies or transheterozygotes for the indicated combination were observed and established as a line. The lines were subsequently tested by Southern analysis for the integrity of the P-insertion and the expected *E(spl)* mutation (see Figure 5).

− = no homozygous offspring or transheterozygotes of the indicated combination were observed. *E(spl)*^{E77} is viable *in trans* over deficiencies like Df(3R)^{8D06} or *l(gro)*^{X1} but homozygotes do not survive most likely due to a second lethal mutation.

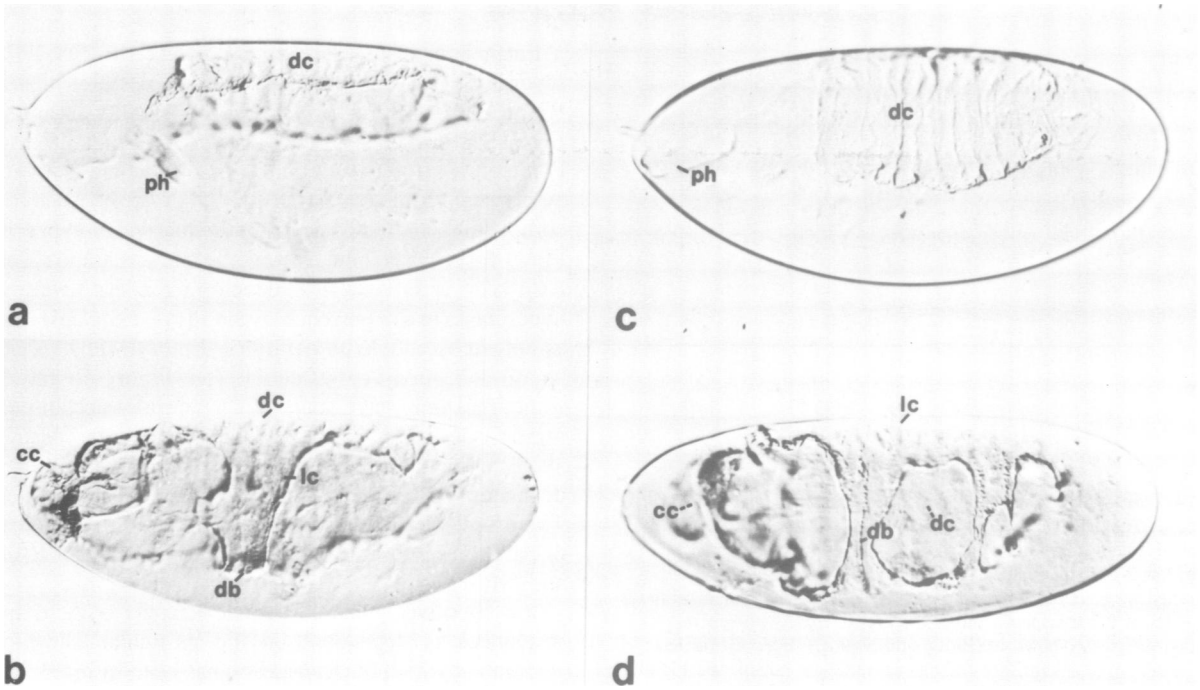

Fig. 4. Rescue of *E(spl)*^{BX22}. A transformed line with an insertion of the P[ry$^+$ E8] transposon in 13B on the X-chromosome was crossed into the *E(spl)*^{BX22} stock and tested for its ability to rescue the neural hypertrophy of homozygous mutant embryos. Homozygous *E(spl)*^{BX22} embryos in general display little variation in the mutant phenotype. (**a**) Lateral view and (**c**) view from ventral onto the dorsal surface. Only the dorsal cuticle (dc) and some remains of the pharynx (ph) connected by dorsal bridges are left in these embryos (see also Figure 1c). In the background of the P[ry$^+$ E8] transposon, however, homozygous mutant embryos show a less severe phenotype as judged by the appearance of lateral (lc) and even ventrical cuticle with the characteristic ventral denticle belts (dc). In addition, as judged by the apparent cephalic cuticle (cc), the head defects in these rescued embryos are dramatically reduced. (**b**) Lateral and (**d**) a ventral view of a rescued embryo with the genotype P[ry$^+$ E8]/P[ry$^+$ E8] or X or Y; *E(spl)*^{BX22}/*E(spl)*^{BX22}.

of embryogenesis. On the other hand, RNA from the transcription unit defined by the 3-kb and 4-kb transcripts is detectable both in very early embryos as well as in adult females (Figure 3e) indicating a maternal component. These transcripts are rather abundant (at least 10% of the level of actin 5B message; Fyrberg *et al.*, 1983). In later stages they are present at a low level but remain detectable until adulthood (data not shown). Females in comparison to males show at least a 5-fold increase in the smaller message (Figure 3e). As this transcriptional unit is the only one with a maternal component affected by the *E(spl)*^{BX22} mutation, it is the most likely candidate for the *E(spl)* gene.

Transformation

The transcriptional and molecular genetic analyses of the *E(spl)* region suggest that the transcription unit which gives rise to the 3-kb and 4-kb poly(A)$^+$ transcripts and is disrupted by the BX22 deletion is a likely candidate for encoding *E(spl)* functions. In order to confirm that this transcription unit encodes *E(spl)* wild-type information, we transformed *Drosophila* embryos with cloned DNA from this region (Rubin and Spradling, 1982), and tested the ability of the introduced DNA to complement *E(spl)* mutations.

For transformation, a genomic 10.4-kb *Xho*I fragment, encompassing the region from −15.2 to −25.6 was used (Figure 3a). This fragment appears to contain the entire genetic unit from which the two large transcripts derive (see Figure 3a,c). In addition, it includes part of the adjacent transcripts on either side, as judged by Northern and sequence analysis (data not shown). We subcloned this fragment into the Carnegie 20 vector, which includes the wild-type *rosy* gene as a selectable marker (Rubin and Spradling, 1983). Injections of this DNA (P[ry$^+$ E8]) into *rosy* mutant embryos yielded seven independent lines. Six of the seven lines carry the P[ry$^+$ E8] insertion on the third chromosome (cytological positions: 75C1/2; 77B1−4; 85C; 89B1−7/; 91F7/8; 99E; data not shown). The seventh P[ry$^+$ E8] insertion occurred on the X chromosome at 13B (data not shown). This line was chosen to test the ability of the P[ry$^+$ E8] insertion to rescue *E(spl)* mutations. The results of the various crosses are outlined in Table II.

In the background of one or two copies of the insertion P[ry$^+$ E8](13B) all seven point mutations are viable and fertile in homozygosity as well as in hemizygosity (Table II). The presence of the transposon was verified in the transformed lines by Southern analysis taking advantage of the restriction site polymorphisms characteristic of the mutants and the transposed sequences (Figure 5).

The complete rescue of the point mutations by P[ry$^+$ E8](13B) suggested a complete rescue of *gro* as well, since *gro* falls into the same lethal complementation group (Table I). Homozygous *gro* stocks usually express the *gro* phenotype with a rather low penetrance (Lindsley and Grell, 1968) and therefore we tested the mutation in the background of an *E(spl)* deficiency [Df(3R)^{8D06}] which enhances the *gro* phenotype and raises the penetrance to >80% (A.Preiss, unpublished). A cross of Df(3R)^{8D06} females in the background of the insertion to *gro* males yielded only wild-type offspring. Therefore, the line P[ry$^+$ E8](13B) com-

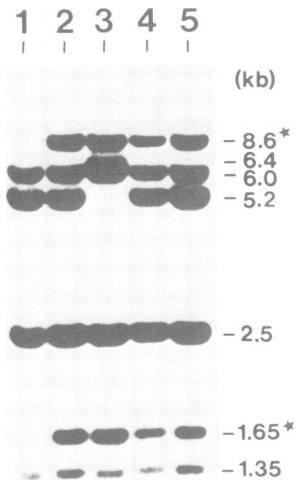

Fig. 5. Southern analysis of transformed lines. The presence of the transposon P[ry$^+$ E8] in all transformed lines and in all subsequently established homo- and heterozygous mutant lines was confirmed by Southern analysis. Genomic DNA from *rosy*^{506} flies (**lane 1**), transformed lines (**lanes 2, 4** and **5**) and a homozygous mutant in the background of the transposon (**lane 3**) were digested with several restriction enzymes (shown is *Eco*RI) and Southern blots hybridized with a genomic 10.4-kbp *Xho*I fragment (−15.2 to −25.6; see Figure 2) which corresponds to the fragment cloned in the construct P[ry$^+$ E8]. In transformed lines [three different lines with insertions in 13B (**lane 2**), 85C (**lane 4**) and 77B (**lane 5**) are shown] the presence of P[ry$^+$ E8] can be monitored by the appearance of two extra fragments (denoted by an asterisk) which correspond to the ends of the *Xho*I insert plus adjacent parts of the Carnegie 20 vector and can be calculated for *Eco*RI to be 8.6 kbp and 1.65 kbp in length respectively. In the parental strain these ends correspond to a 5.6-kbp *Eco*RI fragment on the proximal and a 6-kbp fragment on the distal side (compare with Figure 2). Both adjacent *Eco*RI sites are polymorphic in several wild-type strains. The proximal site at −14 is missing in the e^4 *tx* strain which is the parental strain to the EMS induced point mutants (see Table I; A.Preiss, unpublished). Homozygous point mutants in the background of the P[ry$^+$ E8] transposon therefore show a single 6.4-kbp *Eco*RI fragments instead of the 5.6-kbp of the *rosy*^{506} strain plus the 8.6-kbp and the 1.65-kbp fragments indicating a complete transposon. The homozygous point mutant shown is P[ry$^+$ E8]/P[ry$^+$ E8] or +; *E(spl)*^{E48}/*E(spl)*^{E48} (**lane 3**).

pletely rescues the *gro* mutation even in the background of *E(spl)* deficiencies (see cross [A], Table II).

The *E(spl)*^{D} mutation exhibits the dominant interaction with *split* and is lethal or semilethal when combined with other *E(spl)* mutations. The dominant phenotype, the enhancement of *split* by *E(spl)*^{D} is not altered in the background of P[ry$^+$ E8](13B). This result is not surprising even assuming that the P[ry$^+$ E8] transposon includes the entire *E(spl)* gene in view of the fact that a duplication of the entire *E(spl)* chromosomal region itself in an *E(spl)*^{D} background results in only a very subtle enhancement of the *split* phenotype (Knust *et al.*, 1987a). The lethality of certain combinations of the *E(spl)*^{D} with other *E(spl)* mutations, on the other hand, is a phenotype which can be scored unambiguously: a cross of *E(spl)*^{D} males to females with *E(spl)* deletions gives no transheterozygous offspring and a cross to females carrying any of the point mutations results in a reduced number of transheterozygotes (A.Preiss, unpublished). However, if we cross an *E(spl)* mutant female in the presence of the P[ry$^+$ E8] insertion to *E(spl)*^{D} males, transheterozygous offspring are observed in high numbers, no matter whether we use large deficiencies involving several polytene bands such as Df(3R)^{8D06}, the smaller deficiency *E(spl)*^{BX22} or point mutations (see cross [B], Table II). This result demonstrates that we can rescue the lethality observed in combinations of *E(spl)*^{D} with other *E(spl)* mutations.

Further investigations of the effect of P[ry$^+$ E8] on several deletions in the *E(spl)* region revealed that P[ry$^+$ E8] does not rescue the embryonic lethality in any of the *E(spl)* deficiencies tested. Moreover, mutant embryos homozygous for two deficiencies that delete at least eight cytological visible bands [Df(3R)^{8D06}; *l (gro)*^{X1}; Table I] are completely unaffected in their mutant phenotypes by the P[ry$^+$ E8] transposon. However, embryos homozygous for *E(spl)*^{BX22} or Df(3R) *ro*^{82b} develop a far less severe neural hyperplasia in the presence of the P[ry$^+$ E8](13B) insertion as judged by cuticle preparations. Embryos always develop lateral and often ventral cuticle (see Figure 4b and d) and sometimes only have some holes ventrally and in the head region. Generally, homozygous mutant embryos of these alleles show little variance in their mutant phenotypes independent of the genetic background (see Figure 4a and c). Therefore, the sequences present in P[ry$^+$ E8] are capable of partially rescuing the neurogenic phenotype of *E(spl)*^{BX22} and Df(3R) *ro*^{82b}.

It is conceivable that the genetic behavior of the transposed sequences is dependent on the particular chromosomal location in which the transposon has been inserted. In order to investigate such possibility we tested two 3rd chromosome lines (P[ry$^+$ E8](85C) and P[ry$^+$ E8](89B)) in *cis* with *E(spl)* mutations for their rescuing ability. These lines were used to recombine the P[ry$^+$ E8] insertion onto an *E(spl)* mutant chromosome [*l (gro)*^{X1} and *l (gro)*^{X115} respectively; Table I]. The successful recombination was monitored on whole genome Southerns and by *in situ* hybridization to polytene chromosomes respectively (data not shown) We find that the genetic behavior of the P[ry$^+$ E8], including the incomplete rescue of the neurogenic phenotype, is independent of the chromosomal insertion site. Hence, the incomplete rescue of the neurogenic phenotype of, for example, the BX22 deletion could be due to the absence of either coding or regulatory *E(spl)* sequences from the transforming transposon.

In summary, the transformation analysis has shown that the sequences residing between coordinates −15.2 and −25.6 which include the transcription unit giving rise to the 3-kb and 4-kb poly(A)$^+$ RNAs are capable of rescuing, in some cases partly and in others completely, *E(spl)* phenotypes. We therefore conclude that these sequences belong to the *E(spl)* locus.

Discussion

Our current understanding of the cellular and molecular mechanisms underlying the very first steps of *Drosophila* neurogenesis has been prompted by the molecular anlaysis of the *Notch* locus in combination with the existing genetic and embryological studies. These analyses lead us to propose the involvement of *Notch* and some or all other neurogenic loci in a cell interaction mechanism which mediates the correct differentiation of certain tissues including the neurogenic region of the embryonic ectoderm (Wharton *et al.*, 1985a; Hartley *et al.*, 1987; Artavanis-Tsakonas, 1988). Part of the rationale behind this hypothesis is the fact that several of the neurogenic loci have been documented to interact genetically. Given the apparently specific interaction of *spl*, a point mutation in the *Notch* locus, with the dominant mutation *E(spl)*^{D}, the *E(spl)* gene is of particular interest in

any attempt to elucidate the molecular components of the postulated cell interaction mechanism. Apart from the dominant $E(spl)^{D}$ mutation, a relationship between that gene and *Notch* has been suggested by the examination of revertants of the dominant phenotype. Such revertants are embryonic lethal and exhibit a neural hypertrophy, implying that *E(spl)* itself is required for normal neurogenesis. Combinations of the $E(spl)^{D}$ with these mutants are lethal, suggesting that both mutations affect the same locus. However, since these revertants turned out to involve large, cytologically visible deletions, one cannot necessarily conclude that both the dominant and the neurogenic phenotype reflect mutations in the same gene.

In several screens for *E(spl)* alleles we isolated an additional 15 mutants which all dislay the same complementation behavior. They are all lethal in homozygosity and in *trans* over each other and therefore represent a single lethal complementation group. Furthermore, all the *E(spl)* alleles are lethal or semilethal in combination with the dominant $E(spl)^{D}$ (A.Preiss, unpublished). This complementation group also contains the recessive visible allele *gro* (Table I). In homozygosity, the lethal *E(spl)* mutations result in a hypertrophy of varying degree of the embryonic nervous system. We find only a weak, not fully penetrant neural hyperplasia in point mutants while large deletions result in a severe hypertrophy of the nervous system. As is discussed in more detail later, the simplest hypothesis regarding the variability of the neurogenic phenotype is that the severe neural hyperplasia is in fact a synthetic phenotype resulting from the loss of several unrelated gene functions rather than the amorphic phenotype of the *E(spl)* gene. Consistent with this hypothesis is that genetic screens for lethal complementation groups uncovered by a cytologically visible deficiency, revealed but a single lethal with the neurogenic phenotype and these mutants only exhibit a weak neural hyperplasia (Ziemer *et al.*, 1988). Since this complementation group appears to correspond to our point mutants which also result in a less severe phenotype, this phenotype may be more informative to an understanding of *E(spl)* function rather than the more severe phenotype of the deficiencies.

We cloned the *E(spl)* region in a chromosomal walk and the molecular analysis of the region revealed a plethora of embryonic transcripts. Even the smallest deletion, $E(spl)^{BX22}$, uncovers at least six transcription units. Only one of them has a maternal component which is consistent with the maternal effect of *E(spl)* revealed by genetic analysis (Knust *et al.*, 1987a; A.Preiss, unpublished). Since this transcription unit is the best candidate to code for *E(spl)* wild-type function, we used an overlapping genomic fragment that contains all the coding sequences for transformation experiments. We found that we can rescue all aspects of *E(spl)* phenotypes. First, we completely rescue the neural hypertrophy as well as the lethality of point mutants, producing normal, fertile adults. The recessive visible *gro* mutation, which falls into the same complementation group, is also completely rescued. We are unable, however, to rescue the lethality of any of the *E(spl)* deletions tested, all of which involve more than one transcription unit. The most extreme neural hypertrophy of large deficiencies like $Df(3R)^{8D06}$ and $l\ (gro)^{X1}$ is not affected by the transforming DNA. However, these deficiencies involve at least eight cytologically visible bands in the chromosome and therefore probably several distinct genetic loci. We have observed that deletions involving essentially the same parts of the chromosome can result in varying degrees of neural hyperplasia (compare, for example Pr^{rev1} and Df(3R) ro^{82b} respectively, Table I), suggesting that varying the genetic background is likely to vary the neurogenic phenotype. Since we cannot address this issue by transformation, any effects of our fragment on the phenotype of these deficiencies might pass undetected. In contrast, we succeed in partially rescuing the neurogenic phenotype of both deletions $E(spl)^{BX22}$ and Df(3R) ro^{82b} respectively, which shows that we provide at least a part of the wild-type neurogenic function. The transforming sequences also rescue the lethality displayed by heterozygous combinations of the dominant $E(spl)^{D}$ mutation with the other *E(spl)* alleles. This lethality is rescued in all tested combinations, no matter whether we use point mutants, smaller deletions like BX22 or very large deletions like $Df(3R)^{8D06}$. These data strongly suggest that the $E(spl)^{D}$ is in fact a mutation in the same gene product as the point mutants. However, our data do not allow us to rule out the possibility that the dominant interaction of $E(spl)^{D}$ with *split* is caused by a closely linked, independently mutated gene. We are currently addressing this by transforming with DNA fragments isolated from $E(spl)^{D}$ mutants into a wild-type background in an attempt to define unambiguously the genomic region responsible for the enhancement of the *spl* phenotype.

Although the transformation analysis indicates that the sequences coded by the transforming transposon are necessary for wild-type *E(spl)* function, the lack of complete rescue of the neurogenic phenotype of the deletion $E(spl)^{BX22}$ seems to argue that these sequences are not sufficient for complete *E(spl)* wild-type activity. Of course this argument assumes that the lethal complementation group defined by the point mutations, which display a weaker neurogenic phenotype and are completely rescued by the transforming transposon, do not represent the amorphic but only a hypomorphic state of the locus. Sequence data show that the transposon most likely contains all the coding sequences of the transcription unit defined by the 3-kb and 4-kb mRNAs (Hartley *et al.*, 1988). On the other hand we do not yet know if all the non transcribed controlling elements are present, nor can we be certain that we are not missing an exonic region even though the transforming fragment partially overlaps with adjacent transcripts. Any one of these simple possibilities could account for the lack of complete rescue.

An alternative explanation for our partial rescue of the severe phenotype might infer a complexity of the *E(spl)* locus in the sense that the gene is defined by several transcription units. This hypothesis has been recently proposed by Knust *et al.* (1987b) since they find that only a deletion involving at least 11 transcripts leads to a fully penetrant, severe neurogenic phenotype. The authors consider the possibility that even more transcripts are involved in *E(spl)* gene function since larger deficiencies result in an even more severe neural hyperplasia. Given the extraordinarily large number of transcripts included by this assertion as part of a single genetic unit, such definition of the *E(spl)* locus is certainly not the simplest one. Our data do not conclusively show that *E(spl)* is functionally defined by the transformed fragment, and therefore we cannot rigorously exclude this possibility although our data do not invoke such a complicated hypothesis. For example the $E(spl)^{BX22}$ deletion, completely included within this deletion of 11 transcripts, still gives a severe neural hypertrophy with full penetrance

even though it affects just five of the transcripts they consider necessary for *E(spl)* gene function (we have detected a sixth embryonic transcript from this region). From within this deficiency we have been able to define only a single lethal complementation group, mutations in which display the neurogenic phenotype. This suggests that only one of the six transcription units is essential for embryonic neural development. Consistent with this hypothesis, we are able to rescue this complementation group, which is the only one with a neurogenic phenotype in the entire *E(spl)* region (Ziemer *et al.*, 1988), with a genomic fragment which encompasses a single transcription unit. The same fragment complements other aspects of *E(spl)* phenotypes, including the recessive visible *groucho* and, importantly, the lethality of combinations of the dominant $E(spl)^{D}$ with other *E(spl)* alleles. Consequently, we prefer the hypothesis that the absence of regulatory sequence in our construct, rather than the absence of other transcription units, dictates our inability to rescue fully the $E(spl)^{BX22}$ neurogenic phenotype.

However the different classes of *E(spl)* phenotypes arise, their existence will aid our understanding of the function of the gene product. In a similar fashion, there are a number of classes of mutant phenotype associated with lesions in the *Notch* locus. Their DNA sequence has helped define functional domains of the gene product. Similarly, the analysis of *E(spl)* mutants may aid our understanding of the function of the *E(spl)* gene product. Recently we have shown that the putative *E(spl)* sequences code for a gene product with striking homology to the beta subunit of transducin, the signal transducing G-protein of the photo-transduction cascade (Gilman, 1987; Hartley *et al.*, 1988). This observation is particularly intriguing since it provides plausible hypotheses regarding the involvement of *E(spl)* in a cell interaction mechanism and its ability to interact with other neurogenic genes such as *Notch* or *Delta*.

Materials and methods

Fly strains and nomenclature

A description of markers and balancer chromosomes is found in Lindsley and Grell (1968). This includes the mutation *Vno, Pr, Pr^{L}, gro* and $E(spl)^{D}$. In order to distinguish the dominant *E(spl)* allele from lack of function alleles we use the abbreviation $E(spl)^{D}$. The *Toll* revertants Tl^{5BRXP} and Tl^{5BRXQ} were kindly provided by K.V.Anderson; the $E(spl)^{D}$ revertants $E(spl)^{R1}$ and $E(spl)^{R2}$ by J.A.Campos-Ortega, the $Df(3R)^{8D06}$ by C.Nüsslein-Volhard and $Df(3R)ro^{82b}$ by P.Lewis. We are very grateful to M.Muskavitch for the X-ray induced revertants of $E(spl)^{D}$ BX21, BX22 and BX36. The *E(spl)* alleles *l (gro)* X1, X72 and X115 are X-ray induced in rucuca and were isolated as lethal alleles of *groucho*; *Pr* revertants (rev1; rev4; rev6; rev10 and P9) are also X-ray induced. Point mutants in *E(spl)* (E28, E48, E73, E75, E77, E107) were isolated from an $e^{4}tx$ strain after treatment with EMS (protocol according to Lewis and Bacher, 1968).

Analysis of embryonic phenotypes

Cuticle preparation from embryos at least 24 h old (25°C) was performed according to the protocol of Wieschaus and Nüsslein-Volhard (1985).

Staining of the embryonic nervous system wtih anti-horseradish peroxidase (anti-HRP) antibodies (Jan and Jan, 1982) was according to a protocol of H.Steller. Embryos were fixed as described (Steller *et al.*, 1987) and vitelline membranes removed by the heptane/methanol method (Mitchison and Sedat, 1983). After slowly rehydrating in PBS, embryos were preincubated for 1 h at room temperature in PBS plus 3% normal goat serum and 0.2% saponin (PSS). Rabbit anti-HRP antiserum (Cappel) was added to a dilution of 1:400 and incubated overnight at 4°C. After washing the embryos for several hours in a few changes of PSS, the secondary antibody (HRP-conjugated goat anti-rabbit; BioRad) was added to a dilution of 1:400. Incubation was overnight at 4°C, followed by several washes in PBS. A standard DAB precipitation was carried out in a solution of PBS plus 0.5 mg/ml 3,3′-diaminobenzidine and 0.003% hydrogen peroxide. Embryos were subsequently washed with PBS and mounted in 80% glycerol.

Cloning protocols, DNA and RNA procedures

Basic cloning protocols were as described in Maniatis *et al.* (1982).

Preparation of genomic fly DNA involved the homogenization of the flies in 80 mM EDTA, 160 mM sucrose, 100 mM Tris−HCl, pH 8. After adding 0.5% SDS and 170 μg/ml proteinase K and incubating for 1 h at 68°C the homogenate was extracted with phenol and chloroform. SDS was precipitated with 0.53 M potassium acetate for 2 h on ice. DNA was precipitated with 1 vol of ethanol for 10 min at room temperature. The DNA was treated with RNase prior or after restriction digests. RNA was extracted as described in Yedvobnik *et al.* (1984). Poly(A)$^{+}$ RNA was prepared using a Hybond-mAP paper (Amersham) according to the instructions of the manufacturer. Transfer of either DNA or RNA was on nitrocellulose (Schleicher and Schüll). Random priming was used to label probes as described in Feinberg and Vogelstein (1984). Preparation of radioactively labeled cDNA was as described in Krug and Berger (1987) with minor modifications. We used 1 μg of poly(A)$^{+}$ RNA from 0−22 h old embryos (25°C) in the labeling reaction (45 min at 42°C) which included actinomycin D (0.1 mg/ml), dNTPs at 1 mM, cold dCTP at 0.5 mM and 50 μCi of [^{32}P]dCTP (400 Ci/mmol); pyrophosphate and spermidine were omitted. The reaction mixture was alkali treated, neutralized and fractionated on a Bio-Gel P60 column (BioRad). Subsequently, the separated probe was used to hybridize to restriction fragments representing the entire walk ('reverse Northern').

Transformation

Transformation procedures were basically as described in Spradling (1985). For transformation we used a 10.4-kb *Xho*I fragment subcloned into the *Sal*I site of the Carnegie 20 vector (Rubin and Spradling, 1983). After injection of the DNA (Santamaria, 1985) embryos were allowed to develop to adult flies which were pairmated to ry^{506} males and virgin females, as appropriate. Their offspring was scored for wild-type eyed flies which were used to establish separate lines. The lines were monitored by *in situ* hybridization of the 10.4-kb *Xho*I fragment to polytene chromosomes for the presence and location of an insertion of P[ry^{+} E8]. As a control for the entity of P[ry^{+} E8] genomic DNA from all separate lines was monitored in whole genome Southerns to contain the proper restriction fragments.

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A P[ry^{+} E8]/P[ry^{+} E8]; $Df(3R)^{8D06}$ *st* e/e^{4} $E(spl)^{E77}$ *tx* ♀ × ♂ *gro/gro*
→ gives only wild-type offspring

B P[ry^{+} E8]/P[ry^{+} E8]; $Df(3R)^{8D06}$ *st* e/e^{4} $E(spl)^{E77}$ *tx* ♀ × ♂ $E(spl)^{D}$ *tx*/$E(spl)^{D}$ *tx*
→ gives viable transheterozygotes:

84 P[ry^{+} E8]/X or Y; $Df(3R)^{8D06}$ *st* $e/E(spl)^{D}$ *tx* : 141 P[ry^{+} E8]/X or Y; e^{4} $E(spl)^{E77}$ *tx*/$E(spl)^{D}$ *tx*

P[ry^{+} E8]/P[ry^{+} E8]; $E(spl)^{BX22}$ *tx*/$E(spl)^{BX21}$ *tx* ♀ × ♂ $E(spl)^{D}$ *tx*/$E(spl)^{D}$ *tx*
→ gives P[ry^{+} E8]/X or Y; $E(spl)^{BX22}$ *tx* or $E(spl)^{BX21}$ *tx*/$E(spl)^{D}$ *tx*

Crosses. Virgins from the established lines (see Table II) homozygous for the P-insertion P[ry^{+} E8] and transheterozygous for two different *E(spl)* alleles (as indicated) were crossed to *gro* and $E(spl)^{D}$ males respectively. Offspring of cross [A] were scored for the *groucho* phenotype, offspring of cross [B] were scored for transheterozygotes of $E(spl)^{D}$ over the other *E(spl)* mutant alleles.

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