The EMBO Journal vol.6 no.11 pp.3407–3417, 1987

The embryonic expression of the *Notch* locus of *Drosophila melanogaster* and the implications of point mutations in the extracellular EGF-like domain of the predicted protein

David A.Hartley, Tian Xu and Spyros Artavanis-Tsakonas

Department of Biology, Yale University, New Haven, CT, USA

Communicated by W.Gehring

The *Notch* locus of *Drosophila melanogaster* is one of a small number of zygotically acting 'neurogenic' genes necessary for the correct segregation of neural from epidermal lineages during embryogenesis. The predicted gene product is implicated in a cell interaction mechanism required to achieve this ectodermal differentiation. We have examined wild-type *Notch* expression by *in situ* hybridization and find it to be expressed in more cells than we would have predicted given a sole function in regulating neurogenesis. We conclude from these data that *Notch* plays a more general role in development. In order to assess the dependence of *Notch* expression on other neurogenic gene function we have hybridized *Notch* probes to *Enhancer of split* mutants which are known to interfere with expression of *Notch* phenotypes. We intimate that the nature of interaction between these genes is not at the level of transcription. Instead, the DNA sequence of *split*, which is a missense mutation in the EGF-like extracellular domain of the *Notch* protein, suggests a direct biochemical interaction between *Notch* and *E(spl)* proteins. The similar site of a second point mutation, Ax^{E2}, implies that protein interactions also occur between *Notch* proteins. Finally we discuss the general implications of our findings with a view to the models and mechanisms of *Notch* action in regulating individual cellular interactions during development.

Key words: Notch locus/*Drosophila melanogaster*/wild-type/mutation/EGF-like domain

Introduction

Neurogenesis in *Drosophila* is initiated by the individual segregation of neural precursors, neuroblasts, from undifferentiated ectoderm, commencing at ~4 h of development and lasting another 3 or so hours. Evidence for a genetic basis for the control of early ectodermal differentiation was first provided by Donald Poulson 50 years ago when he described the hemizygous embryonic phenotype for a deficiency of a portion of the X chromosome as a 3-fold enlargement of the CNS at the expense of epidermal structures (Poulson, 1937). This phenotype, attributable to mutations at the *Notch* (*N*) locus, appears to result from a developmental misrouting of cells which normally give rise to the epidermis. Five other zygotic and a number of maternal effect loci effecting a qualitatively similar 'neurogenic' phenotype have been described (Shannon, 1973; Lehmann *et al.*, 1983; Perrimon *et al.*, 1984). The similarity of phenotype deriving from mutations at the zygotic neurogenic loci, *Notch* (*N*), *big brain* (*bib*), *mastermind (mam), Delta (Dl), Enhancer of split* [*E(spl)*] and *neuralised (neu)*, prompts the suggestion that they act in concert to define the neural/epidermal dichotomy.

The accumulated data on the structure and expression of *Notch* prompt the view that it regulates ectodermal differentiation by a mechanism involving cellular interaction. The predicted gene product, derived from nucleic acid sequence, exhibits a striking structure consisting of a 36-fold tandemly repeated array of a cysteine-rich 40 amino acid sequence (Wharton *et al.*, 1985b). This arrangement shares sequence homology with a group of mammalian proteins implicated in cell growth and differentiation, or extracellular biochemical reactions. The repeated epidermal growth factor (EGF)-like structure accounts for nearly one-half of the protein and is followed some 300 amino acids later by a hydrophobic sequence typical of a transmembrane domain. Further towards the carboxy terminus there are sequences reminiscent of a nucleotide binding site and the polyglutamine *opa* repeat (Wharton *et al.*, 1985a). The sequence data strongly implicate the *Notch* gene product in the role of a signal transducer acting at the cell surface, which presumably functions to segregate epidermal from neural lineages by a cell interaction mechanism. This notion is consistent with the results of experimental ablation of cells in the analogous neurogenic region of the grasshopper (Doe and Goodman, 1985) and cell transplantation data in the fly (Technau and Campos-Ortega, 1986) which demonstrate the dependence of neurogenesis on cell interactions.

The *Notch* locus is defined by a linear array of non-complementing embryonic lethal *N* alleles which display a heterozygous phenotype consisting of variably notched wings, thickened wing veins and bristle abnormalities (Welshons, 1965). A number of recessive mutations have been mapped within the confines of the 0.14 map units encompassing all known *N* mutations (Welshons, 1965). Their mutual interactions are manifold and complex. They include a number of recessive lethals which do not exhibit the dominant notched wing phenotype and a group of recessive visible mutations. The recessive visibles subdivide into two groups — those which cause scarring or roughening of the eye [*facet* (*fa*) mutations and *split* (*spl*)] and those which affect wing morphology [*facet-notchoid* (fa^{no}), *notchoid* (*nd*) and *notchoid*^{2} (nd^2)]. All except fa^{no} produce viable mutant adults when heterozygous with *N* alleles. In addition, the eye mutants all complement the wing mutants whilst individual members of each group do not complement each other, with the exception of *spl* which complements all others (Welshons, 1965). Another class of dominant mutations at the *N* locus, termed *Abruptex* (*Ax*), exhibit gapped wing veins and bristle abnormalities (Welshons, 1971; Foster, 1975; Portin, 1975). There are two types of *Ax* alleles: recessive viables and recessive lethals. The viable mutants subdivide into two classes based on their interactions with *N* alleles and each other. *Notch* mutations suppress all *Ax* mutations, but one class of *Ax* mutants suppresses the *N* phenotype whilst the other enhances it. Amongst these viable *Abruptex* alleles, heterozygotes within each subclass are viable whilst those between subclasses are inviable — a phenomenon known as negative complementation (Foster, 1975; Portin, 1975). The extraordinary complexity of mutant interactions at this locus might belie the notion of a unitary function in regulating the neural/epidermal

© IRL Press Limited, Oxford, England

dichotomy during embryonic CNS formation. Certainly the existence of all these mutants greatly aids a molecular dissection of *Notch* function, by defining the perturbations at the locus responsible for different phenotypes.

In addition to interactions between mutants at the *N* locus, interactions between the zygotic neurogenic loci are manifold. The initial definition of the *E(spl)* locus was as a presumably gain of function allele which enhances the *spl* phenotype at the *N* locus such that the severity of phenotype increases from [*spl*/+ ; +/+] < [*spl*/+ ; *E(spl)*/+] ≤ [*spl*/*spl* ; +/+] ≈ [*spl*/− ; +/+] < [*spl*/*spl* ; *E(spl)*/+] ≈ [*spl*/− ; *E(spl)*/+] (Welshons, 1956). Revertants of this *E(spl)* allele are recessive, embryonic lethal and exhibit the characteristic neural hypertrophy of the 'neurogenic' phenotype (Lehmann *et al.*, 1983). Furthermore, doubly heterozygote animals of the genotype *N*/+ ; $E(spl)^R$/+ exhibit a dramatically reduced viability (Vässin *et al.*, 1985; A. Preiss, unpublished results). In contrast, increasing the wild-type dosage of *E(spl)* using duplications, suppresses the dominant notched wing phenotype in *N*/+ females (Vässin *et al.*, 1985).

Models of *Notch* function elucidated from analysis of the mutant phenotype predict a role in discriminating between neural and epidermal precursors intermingled in the neurogenic ectoderm or in repression of the neural fate of peripheral cells once the neuroblasts have segregated into the interior (Wright, 1970; Hartenstein and Campos-Ortega, 1984). An analysis of *Notch* transcript distribution by *in situ* hybridization, indicating the spatial limits of its expression, might discriminate between these possibilities. The simplest model would predict the limited expression of *Notch* in neural or epidermal precursors, thus delineating one lineage from the other. In addition to wild-type, we have examined *Notch* expression in relationship to *E(spl)*, by *in situ* hybridization to amorphic *E(spl)* mutants, since perturbation of *N* transcription in *E(spl)* mutants might explain the neurogenic phenotype in these mutants. The data suggest a more ubiquitous distribution of *Notch* transcript in both wild-type and mutant embryos than these models have anticipated. An alternative approach to unravelling the nature of interactions between these loci is to determine the nature of the mutant products which exhibit phenotypic interactions. We present here the sequence of the *spl* mutant allele of *Notch* and argue that our results suggest that the wild-type *N* and *E(spl)* gene products interact directly at the protein level. We also demonstrate that the Ax^{E2} arises from a single amino acid change raising the possibility that wild-type *Notch* gene products exhibit homotypic interactions given the negative complementation phenomenon which occurs between some *Ax* alleles.

Results

Wild-type Notch expression

We have examined *Notch* embryonic expression by *in situ* hybridization to tissue sections with two different probes complementary to different parts of the 10.2-kb transcript. The first is a cDNA clone — pTN1 — which extends from the end of the first exon (A) to the beginning of the largest exon (F) (see Figure 1). This clone overlaps sequence which codes for the EGF-like cysteine-rich repeats. We and other groups have found that this sequence has homology to other *Drosophila* genes (Knust *et al.*, 1987)

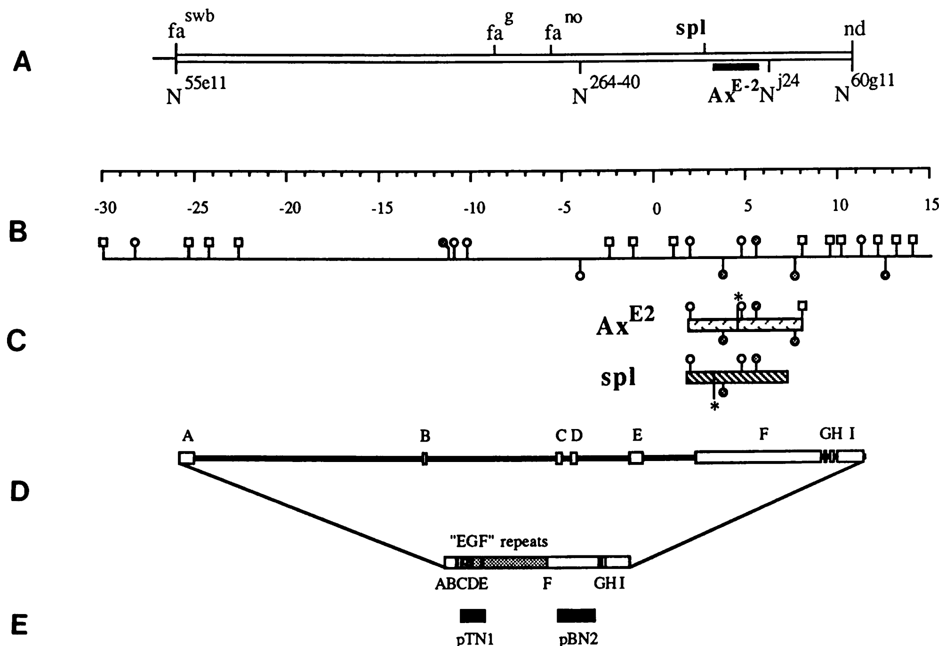

Fig. 1. The *Notch* locus of *Drosophila melanogaster* — a correlation of the genetic and physical maps in relation to the transcript. **A**, The genetic map of the locus indicating the position of relevant alleles as mapped by recombination. The mapped position of the *split* and $Abruptex^{E2}$ mutants are indicated in bold type. **B**, The physical map of the locus. A partial restriction map of 45 kb encompassing the locus is indicated with a scale in kb above. The coordinates on the scale are as defined in Artavanis-Tsakonas *et al.* (1983). The symbols for the restriction sites are as follows: ⚲, *Eco*RI; ⚲, *Bam*HI; ⚲, *Bgl*II. **C**, The regions of DNA from the corresponding part of the *split* and Ax^{E2} chromosomes which have been sequenced in this work. In both cases this amounts to most of the large exon, F, and includes part of the intervening sequence preceding it. The sites of changes which give rise to an altered amino acid sequence are marked with an asterisk. **D**, The structure of the primary and mature transcripts aligned to the restriction map of the genomic region from which they derive. The nine exons A−I are indicated as boxes whilst the introns are diagrammed as solid lines. The portion of the mature transcript which codes for the EGF-like domain of the predicted *Notch* protein is indicated by shading. **E**, The subclones used as probes for *in situ* hybridization aligned to the 10.2-kb transcript from which they derive. The 5′ probe, pTN1, is a cDNA which starts at the 3′ end of exon A and extends to the 5′ end of the large exon, F, subcloned into pT3T7-18. The second probe, pBN2, is a 2-kb *Bam*HI fragment derived from the 3′ end of exon F. The former clone contains sequence which codes for the EGF-like domain of the *Notch* product whilst the latter does not.

and so to ensure that hybridization is specific to *Notch* we have also hybridized a probe devoid of EGF-like homology, pBN2, corresponding to the inferred 'intracellular' portion of the gene product (Figure 1). Both of these probes reveal a qualitatively and quantitatively similar profile of hybridization. We detect *N* RNA throughout embryogenesis; its presence in nuclear cleavage stage embryos is indicative of maternal product, confirming Northern blot and germ line mosaic data (Jimenez and Campos-Ortega, 1982; Kidd *et al.*, 1983; Yedvobnik *et al.*, 1985). Zygotic transcription appears to start at the cellular blastoderm judging from the observation that grains first appear over nuclei at this stage — previously they are only seen over cytoplasm (data not shown). From analysing serial sections of the same embryo and from cross-sections of different embryos it appears that *Notch* is transcribed throughout the blastoderm and its transcript is not confined to the anlage of neurogenic ectoderm. Figures 2a and 3a show sagittal- and cross-sections respectively through cellular blastoderms, and indicate the dispersed nature of *Notch* transcript at this time. Following gastrulation, grains are still evident over mesoderm and ectoderm, as shown in the cross-section in Figure 3b and can be seen throughout the germ band during germ band extension, as illustrated in sagittal-section (Figure 2b) and cross-section (Figure 3c,d). Figure 3c shows the constitution of the histologically defined neurogenic region (Poulson, 1950) in cross-section, consisting of an eight-cell wide strip of enlarged cells on each side of the ventral midline. These cells are the progenitors of both neural and epidermal tissue (Hartenstein and Campos-Ortega, 1984) and, as the figure shows, the distribution of *Notch* RNA shows no bias within or outside this region. Although the limits of our histology do not permit a cell-by-cell identification, it appears that *Notch* is transcribed in both neural and epidermal precursors from the toplogy of grain distribution during neuroblast segregation. For example, Figure 3d depicts an ~5-h-old embryo (germ band fully extended) cross-section where most of the neuroblasts have segregated, yet all the cells appear to be labelled.

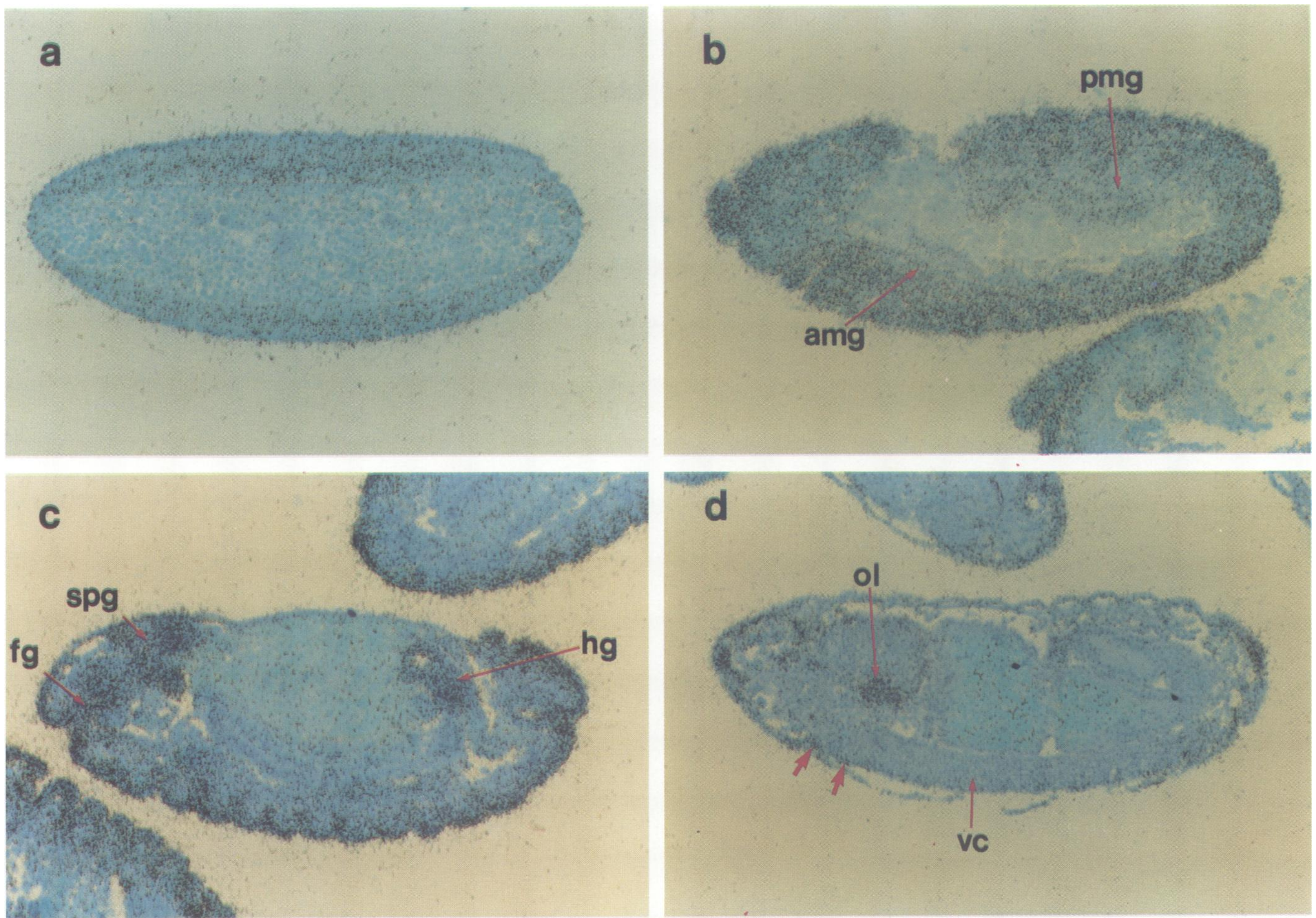

Fig. 2. *Notch* transcription during embryogenesis. Brightfield autoradiographs of increasingly older embryos in sagittal section: anterior is to the left and posterior to the right in all cases. The probes are pTN1 in (**a**) and (**c**–**d**) and pBN2 in (**b**). Exposures are two days at 4°C except (**b**) which is overnight. Blastoderm expression is depicted in (**a**), the grains are distributed indiscriminately around the embryo but show an intracellular preference for internal cytoplasm. Ubiquitous grain distribution continues to the extended germ band stage at ~6 h of development as shown in (**b**), when a decreased density over the midgut primorida (amg, pmg) becomes apparent. By this time, the majority of neuroblasts have segregated into the embryo (Hartenstein and Campos-Ortega, 1984) yet *Notch* is expressed in cells both at the periphery and interior. Further restrictions on *Notch* expression become evident after germ band shortening; (**c**) shows an ~10-h-old embryo which is clearly segmented. Within each segment individual neuromeres are separated by infolding epidermis of the segmental grooves. At this time, *Notch* expression is most abundant at the embryonic periphery stretching from the hindgut (hg) and continuing anteriorly along the segmented ventral surface, into the foregut (fg) and in procephalic epidermis covering the supraoesophageal ganglia (spg). Grains are less evident over developing nervous system immediately below the ventral and anterior surfaces and in the midgut and developing musculature. The peripheral nature of *Notch* labelling appears more obvious upon retraction of epidermis (ep) from CNS when the neuromeres fuse to form the ventral cord (vc). Grains are over both epidermis and the base of the ventral cord where the neuroblasts remain. The final domains of embryonic *Notch* expression are at the periphery of the CNS, in the foregut and, in particular, the optic lobe primordia (ol) at the back of the supraoesophageal ganglia as shown in the 17-h-old embryo in (**d**).

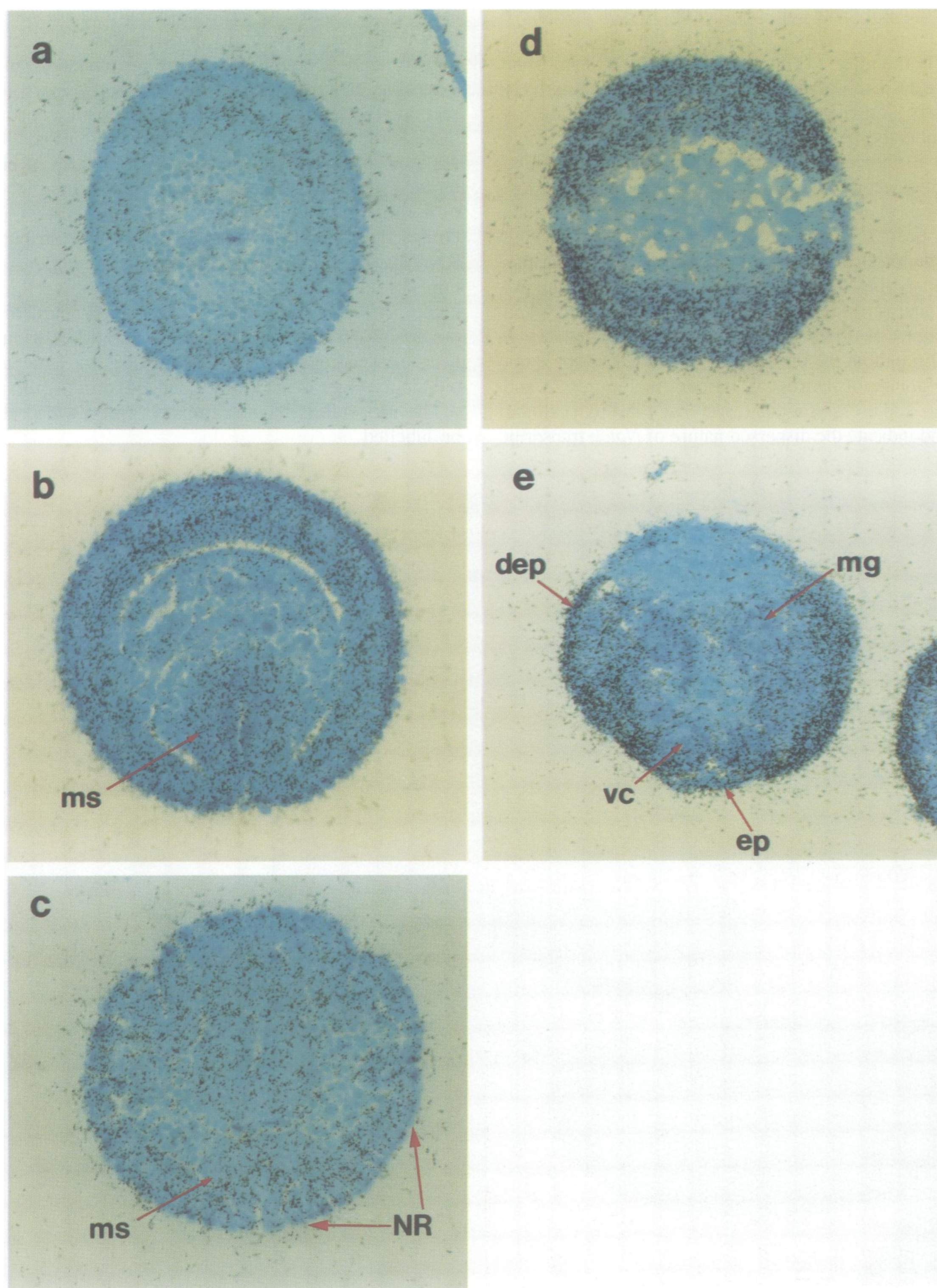

Fig. 3. *Notch* transcription during embryogenesis. Brightfield autoradiographs of increasingly older embryos in cross-section: dorsal is up and ventral down. In all cases the probe is ^{35}S-anti-sense transcript derived from pTN1 and the exposures are overnight (**a**–**b**) and 2 days (**c**–**f**). Cellular blastoderm cross-sections (**a**) show grains throughout the circumference indicating that *Notch* expression is not confined to the anlage of ventral ectoderm from which CNS and epidermis will differentiate. At ~3 h of development the mesoderm (ms) invaginates along the ventral midline as shown in (**b**) — grains are evident throughout ectoderm and mesoderm. The constitution of the neurogenic region is illustrated in (**c**) which shows the eight-cell wide strip of cells on either side of the ventral midline upon invagination and flattening of the mesoderm layer (ms). The arrows indicate the limits of the neurogenic region (NR) in one half of the embryo — neural and epidermal precursors are intermingled in this layer (Poulson, 1950; Hartenstein and Campos-Ortega, 1984). *Notch* transcription at this stage is detected in all cells both within and outside the neurogenic region including the invaginating posterior midgut in the centre of the section. *Notch* expression at the extended germ band stage, ~5 h of development, continues to be ubiquitous throughout the germ band as shown in (**d**). The dorsal half of the embryo shows a posterior segment and the ventral half a more anterior segment. Upon completion of germ band shortening and during dorsal closure, grains become restricted to a more defined portion of the embryo. The ~11- to 12-h-old embryo in (**e**) shows that *Notch* expression is primarily in the epidermis, both ventrally (ep) and dorsally (dep) and at the base of the ventral cord of the CNS (vc). In contrast, there are significantly fewer grains over the midgut (mg).

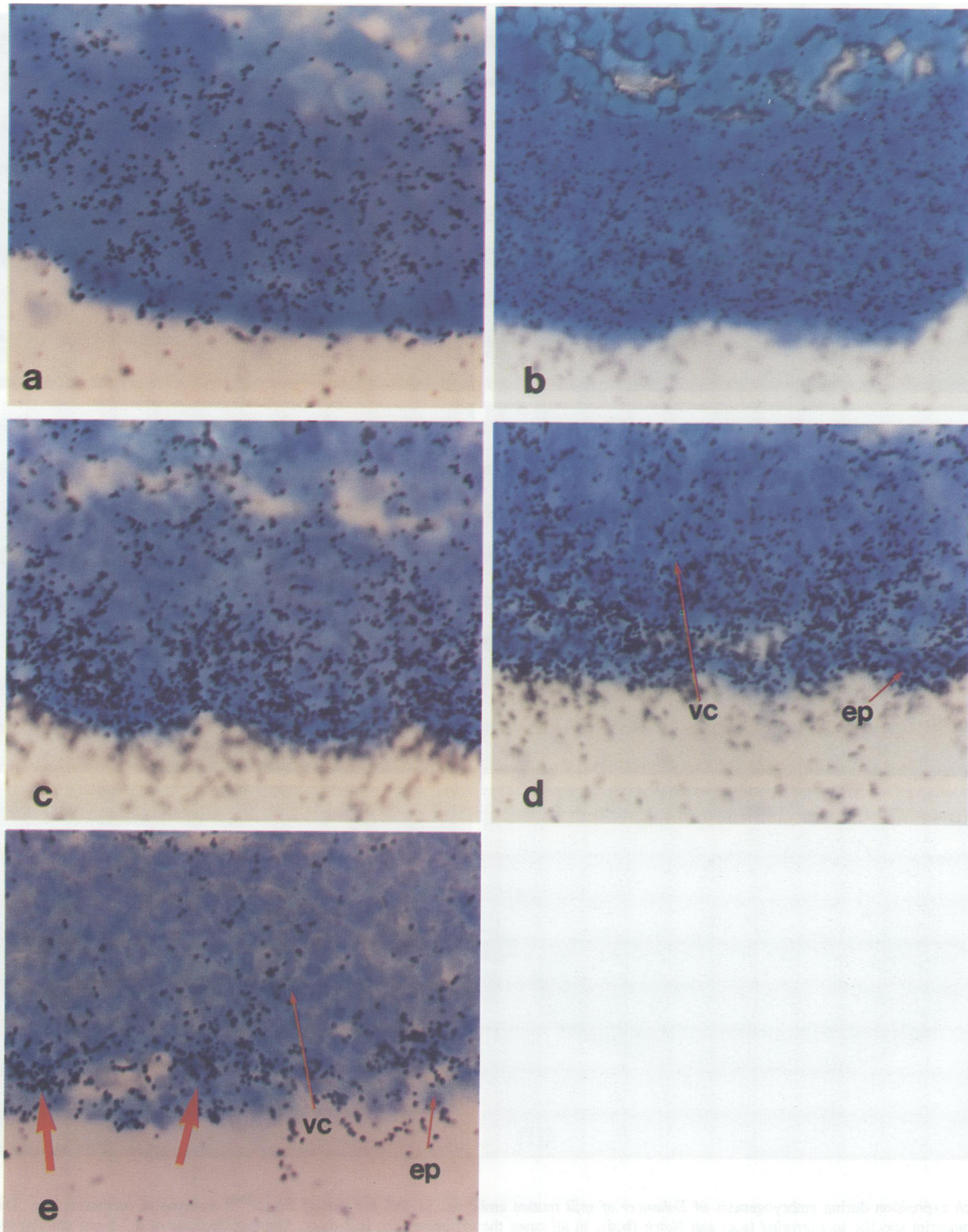

Fig. 4. *Notch* transcription in the germ band, during embryogenesis. Close-up pictures of autoradiographs of increasingly older embryo sections showing just the ventral germ band and the developing nerve cord. (**a**) The germ band of an ~5-h-old embryo, grains cover the width of the germ band. (**b**) The germ band of an 8-h-old embryo, prior to germ band shortening, grains still appear to label all cells, except perhaps the mesoderm layer which is most internal. (**c**) At ~10 h the segmented embryo shows expression at the periphery, over epidermis along the edge and extending into the segmental grooves and covering the periphery of the CNS. (**d**) Fusion of the neuromeres to form the ventral cord (vc) makes the peripheral labelling more apparent. (**e**) A 16-h-old embryo exhibits few grains, principally at the base of the ventral cord (vc) in a periodic fashion over single cells or small clusters (arrowed) and in the epidermis (ep). In all cases the probe is ^{35}S-anti-sense transcript derived from pTN1 and the exposure was overnight.

The first signs of any changes in grain density become apparent during stage 11 of Campos-Ortega and Hartenstein (1985), after the appearance of the parasegmental furrows. There is an initial decrease in labelling in the midgut primordia and the mesoderm as depicted in Figure 2b. The onset of germ band retraction leads to a pronounced lower labelling over the developing nervous system within the germ band which becomes most apparent once the germ band has fully shortened. Figure 2c shows such an

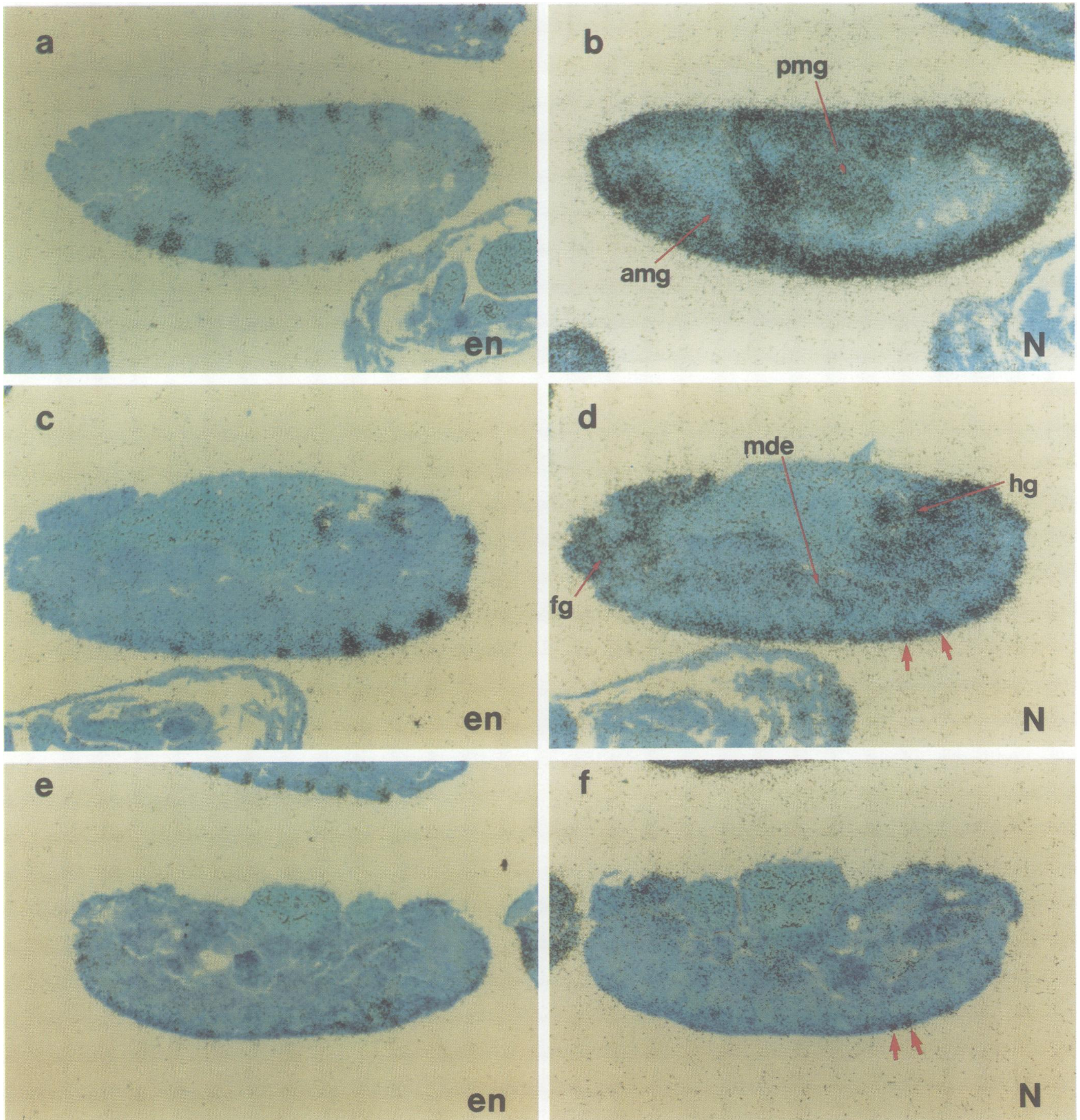

Fig. 5. *Notch* expression during embryogenesis of *Enhancer of split* mutant embryos. Sagittal sections of $E(spl)^{8D06}$ embryos of increasing age. The probes are anti-sense transcript specific to *engrailed* (**a,c**) and *Notch* (**b,d**). In all cases the exposure time is 3 days. Alternate sections of a 7-h-old embryo hybridized to *en* (**a**) and *N* (**b**) show little difference from wild-type: *Notch* is expressed throughout the germ band but at decreased levels in the midgut primordia (amg, pmg) and mesoderm. Upon completion of germ band shortening, the mutant phenotype appears obvious (**c,d**) in comparison with a similarly aged wild-type embryo (see Figure 2c). *Notch* expression is very abundant in the foregut (fg) and hindgut (hg) and along the periphery. The periodic pattern of grains arrowed in (**d**) presumably reflects supernumary neuroblasts. In contrast to the wild-type, there is an additional layer of hybridization in the *E(spl)* mutant above the hypertrophied CNS, termed as mesodermal derivatives in Lehmann *et al.* (1983) (mde). The disruption in the *engrailed* pattern shown in (**c**) may simply reflect the distortion effected by the enlarged CNS. The older mutant embryos (~15–16 h) shown in (**f**) and (**g**) exhibit little *en* (**f**) or *N* (**g**) expression. Wild-type embryos at this time retain more overall *Notch* expression. One common feature between remaining wild-type and mutant *Notch* labelling is the small clusters of grains (arrows) at the base of both the hypertrophied mutant (**g**) and the wild-type ventral cords (see Figure 2d).

embryo (~10 h old) in sagittal section — grains are still evident in epidermis, at the base (over neuroblasts?) of the nervous system and in particular in the hindgut and the foregut. At the onset of dorsal closure, individual neuromeres of the ventral cord fuse and become separate from epidermis — *Notch* transcription can be seen at the ventral and lateral (base) periphery of neuromeres and in epidermis as shown in the cross-section (Figure 3e). At this time the stomodeal (pharynx, oesophagus but not proventri-

culus) and proctodeal *Notch* expression is still very much apparent. Later in development the ventral cord condenses and labelling in the nervous system is confined to a few cells at the base of the ventral cord, around the periphery of the brain (supraoesophageal ganglia) and in addition the optic lobe primordia in the posterior portion of the supraoesophageal ganglia continue to display *Notch* RNA as depicted in the sagittal-section in Figure 2d. Epidermal labelling decays up to hatching, but the neural and anterior labelling endures.

The decay of *Notch* transcription over the developing ventral nervous system is shown more clearly in the higher magnification autoradiographs in Figure 4 which show part of the ventral germ band of increasingly older embryos in sagittal-section. The dispersed signal over germ band extended embryos (3–8 h at 25°C) is illustrated in Figure 4a and b and the polarization of signal after germ band shortening is evident in the segmented embryo section of Figure 4c which shows grains at the base of the neuromeres and in epidermis extending into the segmental grooves. The labelling at the base of the CNS becomes evident upon fusion of the neuromeres shown in the ~14-h-old embryo section (Figure 4d). The decay of this labelling leads to the remnant grains over a few cells periodically repeated along the base of the almost mature CNS shown in Figure 4e. The site of these grains appears to coincide with the reported location of histologically distinguishable neuroblasts earlier in development. We cannot, however, conclude that these cells are remaining neuroblasts since the resolution of our sections does not permit it and since such cells cannot be distinguished by cytological criteria.

Notch expression in E(spl) mutants

The phenotypic interactions between *Notch* and *E(spl)* are evident, but the molecular basis underlying these interactions is unknown. In the absence of data concerning the structure and expression of the *E(spl)* gene we have chosen to assess the dependence of *Notch* transcription on *E(spl)* gene activity. Regulatory interactions between genes involved in pattern formation often appear to be mediated by transcriptional control (for example, Ingham *et al.*, 1985) — we have examined whether the same is true of the *E(spl)* and *N* genetic interaction by hybridizing *Notch* probes to loss of function *E(spl)* mutant embryos. The mutant phenotype becomes apparent in sectioned material of embryos older than ~8 h, but younger mutant embryos become increasingly harder to identify. We have used a molecular approach to identifying younger mutant embryos by hybridizing a cDNA clone, derived from a walk we have initiated in the *E(spl)* region, to alternate sections of deficient mutant embryos from a stock of $E(spl)^{8D06}$/*TM6B*. Embryos which fail to label with this probe are homozygous for the deficiency; since three-quarters of the embryos label normally, we presume these are heterozygotes and homozygous balancer embryos. The next sections were hybridized to *Notch* probes. The neurogenic phenotype associated with deficient embryos results in a considerable distortion of morphology beyond the primary defect of epidermal hypoplasia and neural hyperplasia. Consequently, *engrailed* hybridization was used as a control for defects in the overall embryonic pattern. Alternative sections were hybridized to an engrailed cDNA probe (Kuner *et al.*, 1985) and the *Notch* 5′ cDNA probe. We have noted perturbations in *engrailed* expression in this severe mutant — the significance of this observation is unknown, particularly given that this deficiency deletes ~17 bands.

The results of our *in situ* hybridization analysis indicate that the pattern of *Notch* RNA distribution in *E(spl)* mutants is similar to wild-type. For example, the entire germ band is labelled up until germ band shortening, as is seen in wild-type embryo sections — Figure 5a,b shows serial sagittal-sections through germ band extended embryos hybridized to *engrailed* (Figure 5a) and *Notch* (Figure 5b). Consistent with the labelling of wild-type embryos of this age (~7 h), grains are seen throughout the germ band with decreased amounts over mesoderm and midgut. The mutant labelling after germ band shortening similarly recalls the wild-type, when grains become more peripherally distributed as is shown in Figure 5c,d. A comparison between mutant (Figure 5d) and wild-type (Figure 2c) expression in similarly aged embryos, illustrates both the similarity and differences in labelling. The similarity is the overall peripheral bias of the grain distribution and the difference is the periodic nature of the peripheral grains over the mutant in contrast to the continuous distribution of grains over the wild-type periphery. This difference presumably reflects the lack of epidermis in the mutant which is strongly labelled in the wild-type. The expression along the base of the expanded CNS in these mutants is more extensive than in the wild-type ventral cord which presumably reflects supernumary neuroblasts. In addition, in the extreme mutant $E(spl)^{8D06}$, amorphous tissue above the hyperplasic CNS, identified as mesodermal derivatives by Lehmann *et al.* (1983), continues to be labelled in germ band retracted embryos as shown in Figure 5d. This late expression in presumptive undifferentiated mesoderm does not coincide with wild-type. It is thought that mesoderm fails to differentiate in neurogenic embryos (Poulson, 1945) due to the absence of adjacent epidermis which it normally requires for induction (Bock, 1942; Haget, 1953). The ectopic expression of *Notch* in this tissue is the only transcriptional abnormality we detect — pronounced labelling of foregut and hindgut is as in the wild-type.

The nature of the mutant split product

Our *in situ* data suggest that transcriptional control does not account for interactions between the *E(spl)* and *Notch* loci. In order to investigate further the nature of this interaction we have analysed the molecular lesion associated with *split*, the *Notch* recessive visible mutation known to interact with the dominant *E(spl)* allele. We have not detected rearrangement within *split* chromosomes by restriction analysis bar a *Sal*I polymorphism at a map position of +3.1 (using the coordinates described in Artavanis-Tsakonas *et al.*, 1983; data not shown) and consequently we have used DNA sequencing to determine the nature of the mutant product. Our sequence data are weakened by the absence of the parental chromosome on which the *spl* mutation arose, but the approximate position of the *spl* mutation can be delineated given the extensive meiotic recombination mapping data extant.

Figure 1 shows the genetic map of the *Notch* locus aligned to the restriction map of genomic DNA and the mature 10.2-kb transcript. We have previously shown from correlating the physical position of breakpoints and insertions within the locus to their position on the meiotic map that 0.01 map units consistently correspond to 2.75–2.9 kb of DNA throughout the 40 kb mapped (Grimwade *et al.*, 1984). The *spl* mutation has been mapped 0.04 cM proximally to *facet-glossy* (fa^g) (Welshons, 1965). fa^g is an insertion, mapping to coordinates −8.4 to −9.2 (Artavanis-Tsakonas *et al.*, 1984; Kidd and Young, 1986). Assuming the correspondence of 2.75–2.9 kb to 0.01 cM, this would place *spl* within a *Bgl*II fragment at +2 to +3.8. In addition, this position corresponds to an ~2-kb deletion, N^{81l3}, which does not appear to recombine with *split* (0 recombinants in 39 300 chromosomes recovered) (Grimwade *et al.*, 1984). We have sequenced 5.1 kbp overspanning this region from the *split* chromosome (which by our estimate corresponds to ~0.02 cM), as shown in Figure 1 and find 19 basepair changes from our

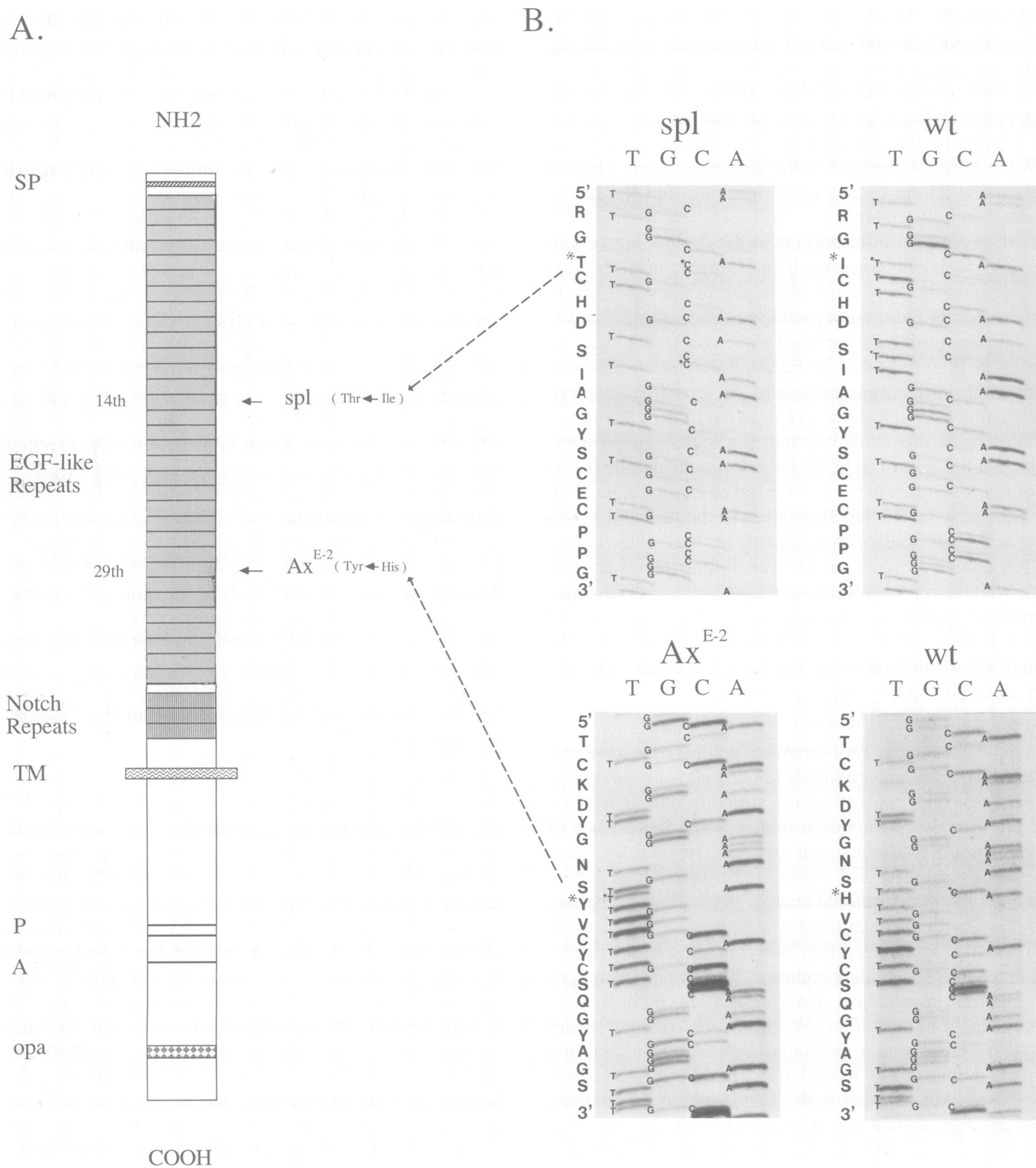

Fig. 6. **(A)** A diagrammatic representation of the 2703 amino acid putative *Notch* protein. The position of the ***split*** (***spl***) mutation, a thymine to cytosine transition which causes the isoleucine at amino acid 578 in the 14th EGF-like repeat to change to a threonine, and the position of the ***Abruptex***^{E2} (***Ax***^{E2}) mutation, a cytosine to thymine transition causing a histidine (1167 in the 29th EGF-like repeat) to change to a tyrosine are indicated. SP, signal peptide; TM, transmembrane domain; P, A, nucleotide phosphate binding sequence homology; opa, opa repeat; EGF-like repeats, the epidermal growth factor-like repeats; Notch Repeats, cysteine-rich repeat present in *Notch* and lin-12. **(B)** Autoradiographs of sequencing gels showing part of the ***spl*** chromosome sequence, the ***Ax***^{E2} chromosome sequence and the corresponding wild-type (wt) chromosome sequence. (*) indicates the nucleotides or amino acids which differ between the mutant and wild-type genes. The autoradiograph of the ***spl*** sequence shows the nucleotide change responsible for the single amino acid alteration we detect and in addition a silent T−C transition. The single nucleotide change between 6 kb of wild-type and ***Ax***^{E2} sequence is shown in the autoradiograph to the right. The differences between the ***spl*** and wild-type sequence we have determined (Wharton *et al.*, 1985b) are as follows: 2474 (T−C), 2487 (T−C), 2889 (C−G), 2901 (T−C), 3447 (C−G), 4932 (C−T), 5307 (C−T), 5322 (C−T), 5454 (C−T), 5493 (A−G), 5712 (G−A), 5890 (C−A), 6000 (C−A), 6045 (A−G), 6096 (G−A), 6132 (C−T), 6453 (T−G), 6468 (T−A), 6489 (G−A). As a result of re-examining the autoradiographs of wild-type sequence corresponding to this region, we have discovered a number of errors in our published sequence (Wharton *et al.*, 1985b). They are at nucleotides 2811, 3360, 3616, 6654 and 6655. They change respectively an A to C, A to C, A to G, C to G and C to G. The latter four change the published amino acid sequence at 873 (Arg to Ser), 959 (Arg to Gly), 1971 (Phe to Leu) and 1972 (His to Asp).

previously determined wild-type sequence (Wharton *et al.*, 1985b). Eighteen of these changes do not alter the deduced amino acid sequence (one results in the *Sal*I polymorphism we had previously discovered). The 19th changes an isoleucine to a threonine at amino acid position 578, within the estimated limits of the *spl* mutation in the coding region. Figure 6 shows the

position of this mutation on a sequencing gel of cloned *spl* DNA: the T−C transition illustrated results in the Ile−Thr change. In addition this same autoradiograph illustrates a silent site polymorphism, also a T−C transition, which does not alter the amino acid sequence. The Ile−Thr mutation occurs in what we have defined as the extracellular EGF-homologous region of the *Notch* product, in the 14th cysteine-rich repeat (Wharton *et al.*, 1985b). We have determined that the splice junction at the 5′ end of exon F (see Figure 1) is conserved between the two sequences and in addition restriction analysis suggests that rearrangements have not occurred in the preceding intron (data not shown). The relative lack of amino acid polymorphism between the wild-type and *split* chromosomes and the position of the single amino acid change makes us confident that this mutation is responsible for the *spl* phenotype. The formal possibility exists that our prediction of the position of the *spl* lesion from the excellent recombination mapping data (Welshons, 1965) is inaccurate due to effects of the *spl* lesion on recombination in this region. Although we consider such a possibility unlikely, rigorous proof awaits germ line transformation using *in vitro* mutagenized constructs. An alternative explanation for the phenotype would be that the accumulated polymorphisms we have discovered result in reduced gene activity due to unfavourable codon usage or changes in nucleic acid secondary structure. However, since these polymorphisms extend over all of the region we have sequenced and yet genetically *spl* maps as a single site, we do not favour these explanations.

The dramatic phenotypic consequences of the interaction between *split* and the dominant *E(spl)* allele makes the nature of the *spl* lesion, a single amino acid substitution in the proposed extracellular region of the *Notch* product, quite surprising. The data suggest that the site of the *spl* mutation defines a domain of the *Notch* protein required for intermolecular interaction between *Notch* and *E(spl)* gene products. Another type of phenotypic interaction between mutant neurogenic alleles which may also reflect such intermolecular interaction is provided by the unusual complementation patterns between some dominant *Abruptex* alleles.

The Abruptex^{E2} mutant product

The *Abruptex* mutations at the *Notch* locus exhibit intriguing complementation patterns with each other and with loss of function *N* alleles (Foster, 1975; Portin, 1975), the most unusual being the negative complementation displayed by certain heteroallelic combinations of *Ax* alleles. It has been recognized that these genetic interactions might be explained by *Abruptex* heteropolymeric gene products having different activities from the homopolymer (Foster, 1975). We have sought to investigate this possibility and the extended implication that at least during some time in development a homopolymeric wild-type *Notch* product has functional significance. To this end, we have cloned and sequenced a representative of the *Abruptex* mutant alleles, Ax^{E2}, one of the 'Enhancer of *Notch*' alleles — so named because in heterozygous N/Ax^{E2} females the extent and frequency of wing nicking is higher than in *N*/+ (Foster, 1975; Portin, 1975). We have again used our estimate of the correlation between physical and genetic distances to predict the site of the Ax^{E2} lesion based on its meiotic position relative to w^a, fa^{no}, *spl* and *rb* amongst 35 000 chromosomes (Foster, 1975). These data place Ax^{E2} 0.01 cM to the right of *spl* although unlike the *split* mutation, Ax^{E2} has not been mapped relative to proximal markers within the *Notch* locus. We have sequenced 6.0 kb encompassing this region which corresponds by our estimate to ~0.021 cM (or 5.0 kb proximally to *split*, ~0.02 cM) and find a single amino acid change at 1167 which changes a histidine residue to a tyrosine. The C−T transition giving rise to this change is illustrated in the sequencing gel shown in Figure 6. This change also occurs in the repeated EGF-like 'extracellular' domain in the 29th cysteine-rich repeat. Remarkably, the C−T transition is the only nucleotide change we have found in the 6.0 kb, making us confident that the His−Tyr mutation is solely responsible for the *Abruptex* phenotype.

Discussion

We have examined the expression of the *Notch* gene during embryogenesis by *in situ* hybridization in order to test the simplest hypothesis regarding its role in embryonic development. This hypothesis, derived from the evidence that the absence of *Notch* gene activity results in the misrouting of epidermal precursors into a neural pathway, predicts the limited expression of *Notch* in epidermal or neural precursors or at least its confinement to the neurogenic region. However, we find that none of these postulates are accurate. Firstly, early on, *Notch* is expressed throughout the embryo and is not limited to neurogenic ectoderm. Secondly, during neurogenesis when neuroblasts segregate from medial ectoderm and throughout subsequent development *Notch* is expressed in both neural and epidermal precursors. Thirdly, the final limits on *Notch* expression overlap the structures absent in the mutant and extend to structures whose mutant morphology appears normal: hindgut, pharynx, oesophagus and dorsal epidermis. The implication from these data is that the *Notch* product has a more pleiotropic role in development than merely regulating the neural/epidermal dichotomy and its role in neurogenesis is context dependent — i.e. it is not the expression of *Notch per se* which influences cell fate but the context of that expression. The existence of *Notch* alleles which affect later post-embryonic development, the pleiotropic nature of these non-lethal *Notch* mutations and the manifold nature of interactions between *Notch* mutations and other neurogenic mutants agree with these conclusions. Certainly the absence of long range non-autonomy in *Notch* mosaics (Dietrich and Campos-Ortega, 1985; Hoppe and Greenspan, 1986) argues against diffusion-based models for *Notch* function and suggests that the pattern of expression we observe is not insignificant.

The complex chronological and topological distribution of *Notch* transcripts does not correlate with a particular region nor a particular tissue type of the embryo. It is possible, given the limited resolution of this analysis, that small groups of *Notch*-expressing cells may have escaped our detection and it is also possible that the distribution of the protein does not coincide with the RNA. Notwithstanding these reservations, we have considered the expression of *Notch* in the developing nervous system, where we believe its action to be important in governing cell fate, in an attempt to formulate alternative hypotheses regarding its role and mode of action. In ~5-h-old embryos *Notch* transcript is present through the germ band including epidermal, neural and mesodermal cell layers. The observed pattern of hybridization is strikingly similar to autoradiographs of embryos of the same age depicting [^{3}H]thymidine incorporation in dividing cells (Campos-Ortega, 1982), indicating that these dividing cells express *Notch*. As neural development proceeds, the neuroblasts divide asymmetrically and perpendicular to the embryonic surface (Poulson, 1950; Hartenstein and Campos-Ortega, 1984). Consequently the 'less differentiated', dividing cells are at the periphery of the developing nerve cord whilst the 'more differentiated' ganglion mother cells and neurones are internal. This pattern is

again revealed by [^{3}H]thymidine incorporation in 10-h-old germ band retracted embryos (Campos-Ortega, 1982) and again convincingly mirrors *Notch* expression in these cells at this time (Figure 2c). The implication is that neural *Notch* expression coincides with a 'less differentiated' cellular phenotype. Preliminary analysis of *Notch* transcription in post-embryonic stages conforms to the notion that it is associated with less differentiated and/or dividing cells. For example, in the third instar larva *Notch* transcripts are predominant in the imaginal discs and the proliferation centres of the brain (K.Markopoulou and S.Artavanis-Tsakonas, in preparation).

We have previously proposed that the *Notch* product acts by a cell interaction mechanism to permit segregation of neural from epidermal cell lineages, given the nature of the predicted protein (Wharton *et al.*, 1985b). The discovery in this work that *Notch* is expressed in a larger number of cells, in other tissue types, prompts us to extend this hypothesis to include other developmental events wherever groups of cells need to communicate with each other to allow correct tissue differentiation. For example, in the neurogenic region of the embryo or in the eye disc, groups of cells have been committed by virtue of other, pattern-forming genes, into certain developmental fates. The exact differentiation of individual cells depends on cell interactions. Within the partially committed cell cluster one cell — responding to an unknown, possibly stochastic, developmental cue — differentiates. The consequence of this differentiation is the recruitment of the surrounding cells into specific fates. Within this framework, the involvement of a non-diffusible cell surface molecule with homology to a mammalian growth factor is quite attractive.

The nature of the *Notch* product, deduced from its sequence, makes its expression in both epidermal and neural precursors harder to interpret mechanistically. Since it may act as a signal transducer, one possibility is that it might interact homotypically between adjacent neural and epidermal precursors. Such a model is consistent with the complex genetic interactions between mutations at the *Notch* locus, notably the *Abruptex* mutations. We have sequenced one of the *Abruptex* mutant chromosomes and suggest that it is derived from a single His−Tyr change in the putative extracellular portion of the *Notch* germ product. This modest difference between the *Abruptex*^{E2} mutant product and the wild-type strongly implies that the site of the alteration marks a position on the *Notch* product crucial to its function. The extracellular location which we predict this site enjoys suggests that it may be important in recognition, consistent with our notion that homopolymeric *Notch* polypeptides either on a single cell or between adjacent cells might be crucial to *Notch* function. Studies on the cell autonomy of *N* mutations show that small mutant clones formed by γ-ray-induced mitotic recombination appear to exhibit non-autonomous phenotypes (P.Hoppe and R.Greenspan, personal communication) and in addition N^{55e11} mutant cells transplanted to wild-type hosts may develop epidermal structures (Technau and Campos-Ortega, 1987). Similarly, mixed cell cultures of *Notch* and wild-type cells behave non-autonomously after culture in larvae (Gehring, 1973). Given the fact that large clones in the embryo appear autonomous (Hoppe and Greenspan, 1986), the possibility exists that mutant cells can be rescued only if they are adjacent to wild-type cells. The rescue of N^- cells into their correct fact by an adjacent wild-type cell argues against homotypic interactions between *Notch* proteins on adjacent cells being a necessary prerequisite for differentiating epidermal from neural precursors.

The specific role of *Notch* in regulating neurogenesis may depend on a more limited expression of other neurogenic gene products. Considering the similarity in phenotype of the known zygotic neurogenic mutants and their interactions, it is perhaps not surprising that the context of their expression might determine their specific role in regulating early embryonic neurogenesis. We have chosen to examine the nature of interactions between two zygotic neurogenic genes, *Notch* and *Enhancer of split*, by *in situ* hybridization of *Notch* probes to *E(spl)* mutants. It does not appear to be the case that *E(spl)* regulates *Notch* expression at the level of transcription. *Notch* transcription appears relatively normal in *E(spl)* mutants although there may be ectopic expression in undifferentiated mesoderm of a severe mutant. This latter finding is intriguing in view of the possibility that wild-type *Notch* expression correlates with undifferentiated cells. Our data do not exclude the possibility that subtle differences in transcription rates relate to the mutant phenotype.

The structure of the predicted *Notch* product makes post-transcriptional interactions with other gene products plausible. Given that *E(spl)* does not seem to regulate *Notch* transcription, it is reasonable to assume that the gene products may interact directly. To give some support to this hypothesis we have sequenced the *split* mutant chromosome and found it to be a missense mutation changing an isoleucine to a threonine in the deduced extracellular portion of the *Notch* product. This single amino acid change appears remarkable in view of the fact that it occurs in a single EGF-like repeat, of 36, within a 2703 amino acid protein. The presumptive extracellular location of this amino acid change emphasizes a role the *Notch* product might play outside the cell and points to extracellular interaction with other gene products such as *E(spl)*. In order to characterize further this interaction we are currently examining the structure and expression of the wild-type *E(spl)* gene.

The complex spatial pattern of *Notch* transcription initially seems at odds with a simple role in regulating neurogenesis. In particular, the ubiquitous expression of *Notch* RNA in all cells of the embryo before and during neuroblast segregation is contrary to the expectations of the simplest hypothesis. This result emphasizes the need to understand not just the pattern of *Notch* expression, but also the context of that expression with respect to cellular phenotype. For example, by analogy to the polarized cell surfaces of many vertebrate epithelia (Simons and Fuller, 1985), we might predict that the *Notch* protein be limited to a defined subset of the cell surface in all the cells of an undifferentiated epithelium such as the ventral ectoderm. The movement of cells out of the epithelium, such as is seen with delaminating neuroblasts, would juxtapose *Notch* protein into a new context. A delaminated cell could use *Notch* to recognize its new environment and allow it to adopt a fate suitable to this new position and/or repress such a fate in its neighbours. Certainly this model illustrates one way that cells can generate assymetry with respect to *Notch* expression, even though they both contain *Notch* RNA. One consequence of an asymmetrical *Notch* cell surface distribution would be that the developmental fate of *Notch*-expressing cells when transplanted to a new environment would depend not only on the nature of that environment, but also the orientation of the transplanted cell. The unpredictable fate of wild-type, *Notch*$^+$, cells when faced with such heterotopic transplantation (Technau and Campos-Ortega, 1986) conforms to this notion. In order to address further these mechanistic models we are analysing *Notch* expression in more detail using antibodies derived from fusion proteins containing different regions of the *Notch* coding sequence.

Materials and methods

Drosophila mutants

The *Enhancer of split* stock we have used is $E(spl)^{8D06}/TM6B$. $E(spl)^{8D06}$ is an EMS-induced allele (Jürgens *et al.*, 1984) and exhibits an extreme neurogenic phenotype as judged both from sectioned material and cuticular phenotype. It is deficient for 96F1-97A3/4 (A.Preiss and P.Lewis, personal communication). TM6B is a balancer for the third chromosome (Lindsley and Grell, 1968). *spl* is an X-ray-induced homozygous viable allele which causes small and roughened eyes and duplication and/or deletion of bristles (Lindsley and Grell, 1968). Ax^{E2} is an EMS-induced allele which results in gaps along the posterior portion of the L5 wing vein — it is viable and fertile as a hemi- or homozygote with a slightly more severe phenotype (Foster, 1975).

Flies were reared at 25°C on standard *Drosophila* medium.

In situ hybridization

Probes were synthesized from dual promoter templates using T3 or T7 RNA polymerases as described by Ingham *et al.* (1985) except the *in vitro* transcription reaction volume was 5 μl instead of 20 μl, the smaller reaction volume being achieved by drying down [^{35}S]UTP label in the reaction vessel.

Egg collection, fixation, *in situ* hybridization, washing and autoradiography was as described in Ingham *et al.* (1985).

Isolation of genomic clones

Genomic libraries of partially digested *Bgl*II DNA from *w spl* and Ax^{E2} homozygous adult flies were cloned in the *Bam*HI site of EMBL3 (Frischauf *et al.*, 1983) and screened with ^{32}P-labelled *Notch* probes (Maniatis *et al.*, 1982) to isolate mutant DNA.

DNA sequencing

Seven-kb *Eco*RI or *Cla*I/*Dra*I *Notch* restriction fragments were subcloned into pEMBL18(+) (Dente *et al.*, 1983). Single-stranded DNA from these subclones were primed with synthetic primers derived from wild-type sequence or the M13 universal primer and sequenced by the dideoxynucleotide chain termination procedure (Sanger *et al.*, 1977). In most cases only the sense strand was sequenced.

Acknowledgements

We thank our colleagues in the Art O'Vanis laboratory for helpful suggestions and comments on the manuscript. We are grateful for the continued excellent technical assistance of Ruth Bryant and are indebted to Phil Ingham, Ken Howard, the Levine laboratory (in particular Chris Rushlow) and the Kankel laboratory (in particular Bruce Chase and Greg Fitzgerald) for help and advice on histology and hybridization. D.A.H. was the recipient of an EMBO long-term Post-Doctoral fellowship and T.X. was supported by a Yale University predoctoral fellowship. This research was supported by the National Institutes of Health grant GM29093 awarded to S.A.-T. We gratefully acknowledge the financial help of the Fondation des Treilles (Paris, France) which covered the costs of this publication.

References

Artavanis-Tsakonas,S., Muskavitch,M.A.T. and Yedvobnik,B. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1977–1981.

Artavanis-Tsakonas,S., Grimwade,B.G., Harrison,R.G., Markopoulu,K., Muskavitch,M.A.T., Schlesinger-Bryant,R., Wharton,K. and Yedvobnik,B. (1984) *Dev. Genet.*, **4**, 233–254.

Bock,E. (1942) *Chrysopa perla. Arch. Entwicklungsmech. Organ.*, **141**, 159–247.

Campos-Ortega,J.A. (1982) In Ransom,R. (ed.), *A Handbook of Drosophila Development.* Elsevier, Amsterdam, pp. 153–181.

Campos-Ortega,J.A. and Hartenstein,V. (1985) *The Embryonic Development of Drosophila melanogaster.* Springer, Berlin.

Dente,L., Cesareni,G. and Cortese,R. (1983) *Nucleic Acids Res.*, **11**, 1645–1655.

Dietrich,U. and Campos-Ortega,J.A. (1984) *J. Neurogenet.*, **1**, 315–332.

Doe,C.Q. and Goodman,C.S. (1985) *Dev. Biol.*, **111**, 206–219.

Foster,G.G. (1975) *Genetics*, **81**, 99–120.

Frischauf,A.-M., Lehrach,H., Poustka,A. and Murray,N. (1983) *J. Mol. Biol.*, **170**, 827–842.

Gehring,W.J. (1973) In Ruddle,F. (ed.), *Genetic Mechanisms of Development, Symposia of the Society for Developmental Biology.* Academic Press, New York, pp. 103–128.

Grimwade,B.G., Muskavitch,M.A.T., Welshons,W.J., Yedvobnik,B. and Artavanis-Tsakonas,S. (1984) *Dev. Biol.*, **107**, 503–519.

Haget,A. (1953) *Bull. Biol. France Belg.*, **87**, 123–217.

Hartenstein,V. and Campos-Ortega,J.A. (1984) *Roux's Arch. Dev. Biol.*, **193**, 308–325.

Hoppe,P. and Greenspan,R. (1986) *Cell*, **46**, 773–783.

Ingham,P., Howard,K.R. and Ish-Horowicz,D. (1985) *Nature*, **318**, 439–445.

Jiminez,F. and Campos-Ortega,J.A. (1982) *Roux's Arch. Dev. Biol.*, **191**, 191–120.

Jürgens,G., Wieschaus,E., Nusslein-Volhard,C. and Kluding,H. (1984) *Roux's Arch. Dev. Biol.*, **193**, 283–295.

Kidd,S. and Young,M.W. (1986) *Nature*, **323**, 89–91.

Kidd,S., Lockett,T.J. and Young,M.W. (1983) *Cell*, **34**, 421–433.

Knust,E., Dietrich,U., Tepass,U., Bremer,K.M., Weigel,D., Vässin,H. and Campos-Ortega,J.A. (1987) *EMBO J.*, **6**, 761–766.

Kuner,J., Nakanishi,M., Ali,Z., Drees,B., Gustavson,E., Theis,J., Kavuar,L.M., O'Farrell,P. and Kornberg,T. (1985) *Cell*, **42**, 309–316.

Lehmann,R., Jimenez,F., Dietrich,U. and Campos-Ortega,J.A. (1983) *Roux's Arch. Dev. Biol.*, **192**, 62–74.

Lindsley,D.L. and Grell,E.H. (1968) *Genetic Variations of Drosophila melanogaster*. Carnegie Institute of Washington. Publication no. 627.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

Perrimon,N., Endstrom,L. and Mahowald,A.P. (1984) *Genetics*, **108**, 559–572.

Portin,P. (1975) *Genetics*, **81**, 121–133.

Poulson,D.F. (1937) *Proc. Natl. Acad. Sci. USA*, **23**, 133–137.

Poulson,D.F. (1945) *Am. Naturalist*, **79**, 340–363.

Poulson,D.F. (1950) In Demerec,M. (ed.), *Biology of Drosophila.* Wiley, New York, pp. 168–274.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

Shannon,M.P. (1973) *J. Exp. Zool.*, **183**, 383–400.

Simons,K. and Fuller,S.D. (1985) *Annu. Rev. Cell Biol.*, **1**, 243–288.

Technau,G.M. and Campos-Ortega,J.A. (1986) *Roux's Arch. Dev. Biol.*, **195**, 445–454.

Technau,G.M. and Campos-Ortega,J.A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4500–4504.

Vässin,H., Vielmetter,J. and Campos-Ortega,J.A. (1985) *J. Neurogenet.*, **2**, 291–308.

Welshons,W.J. (1956) *Dros. Inf. Serv.*, **30**, 157–158.

Welshons,W.J. (1965) *Science*, **150**, 1122–1129.

Welshons,W.J. (1971) *Genetics*, **68**, 259–268.

Wharton,K.A., Yedvobnik,B., Finnerty,V.G. and Artavanis-Tsakonas,S. (1985a) *Cell*, **40**, 55–62.

Wharton,K.A., Johansen,K.M., Xu,T. and Artavanis-Tsakonas,S. (1985b) *Cell*, **43**, 567–581.

Wright,T.R.F. (1970) *Adv. Genet.*, **15**, 261–359.

Yedvobnik,M., Muskavitch,M.A.T., Wharton,K.A., Halpern,M.E., Paul,E., Grimwade,B.G. and Artavanis-Tsakonas,S. (1985) *Cold Spring Harbor Symp. Quant. Biol.*, **50**, 841–854.

Received on June 24, 1987; revised on August 6, 1987