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Neurogenic genes control gene expression at the transcriptional level in early neurogenesis and in mesectoderm specification

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SUMMARY

The development of the central nervous system in the *Drosophila* embryo is initiated by the acquisition of neural potential by clusters of ectodermal cells, promoted by the activity of proneural genes. Proneural gene function is antagonized by neurogenic genes, resulting in the realization of the neural potential in a single cell per cluster. To analyse the relationship between proneural and neurogenic genes, we have studied, in specific proneural clusters and neuroblasts of wild-type and neurogenic mutants embryos, the expression at the RNA and protein levels of *lethal of scute*, the most important known proneural gene in central neurogenesis. We find that the restriction of *lethal of scute* expression that accompanies the restriction of the neural potential to the delaminating neuroblast is regulated at the transcriptional level by neurogenic genes. These genes, however, do not control the size of proneural clusters. Morover, available antibodies do not provide evidence for an hypothetical posttranscriptional regulation of proneural proteins by neurogenic genes. We also find that neurogenic genes are required for the specification of the mesectoderm. This has been shown for *neuralized* and *Notch*, and could also be the case for *Delta* and for the *Enhancer of split* gene complex. Neurogenic genes would control at the transcriptional level the repression of proneural genes and the activation of *single-minded* in the anlage of the mesectoderm.

Key words: *Drosophila*, neurogenic genes, *lethal of scute*, neurogenesis, *single-minded*, mesectoderm specification

INTRODUCTION

Neuroblasts, the progenitor cells of the central nervous system (CNS) in insects, delaminate from the embryonic neuroectoderm of *Drosophila* in a stereotyped manner that involves several waves of segregation (Hartenstein and Campos-Ortega, 1984; Doe, 1992). It has been shown, at least for thirteen neuroblasts that segregate in each hemisegment during the first two waves (S1 and S2), that their formation is initiated by the acquisition of neural potential by small groups of ectodermal cells, called proneural clusters (see Campos-Ortega, 1993, for a recent review). Proneural clusters in the embryonic neuroectoderm are defined by the expression of *achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*), genes of the *achaete-scute* complex (*AS-C*) (Cabrera et al., 1987; Romani et al., 1987; Martín-Bermudo et al., 1991; Skeath and Carroll, 1992; Ruiz-Gómez and Ghysen, 1993). During segregation, each neuroblast accumulates the highest levels of AS-C proteins, whereas in the remaining cells of the proneural cluster these proteins gradually dissapear. This pattern of expression and the analysis of mutant phenotypes indicates that the function of the *AS-C* genes is to confer upon cells of the clusters the capacity to become neuroblasts (reviewed by Campuzano and Modolell, 1992; Campos-Ortega, 1993). The restriction of the neural fate to a single cell of a proneural cluster is mediated by neurogenic genes, through an inhibitory process that involves intercellular communication (reviewed by Ghysen et al., 1993). According to Cabrera (1990, 1992), the molecular outcome of the inhibitory process would be the accumulation of an active, unphosphorylated form of the AS-C proteins exclusively in the presumptive neuroblasts. This contrasts with another report which suggests that the control of the AS-C proteins is largely transcriptional (Skeath and Carroll, 1992).

It has been proposed that neurogenic genes constitute a functional cassette that mediates cell interactions in many developmental processes (Ruohola et al., 1991). One such process could be the specification of the mesectoderm in the early embryo, for the analysis of mesectoderm-specific gene expression in different mutants has lead to the suggestion that mesectoderm specification requires an inductive signal from the mesoderm (see Nambu et al., 1993, and references therein). Additional evidence for the induction derives from the ectopic transplantation of mesodermal cells, which results in the expression in the surrounding ectoderm of *single-minded* (*sim*), a master regulatory gene for mesectodermal development (Leptin and Roth, 1994; Nambu et al., 1991).

In this paper, we first attempt to clarify the discrepancies existing in the control of proneural gene expression by neuro-

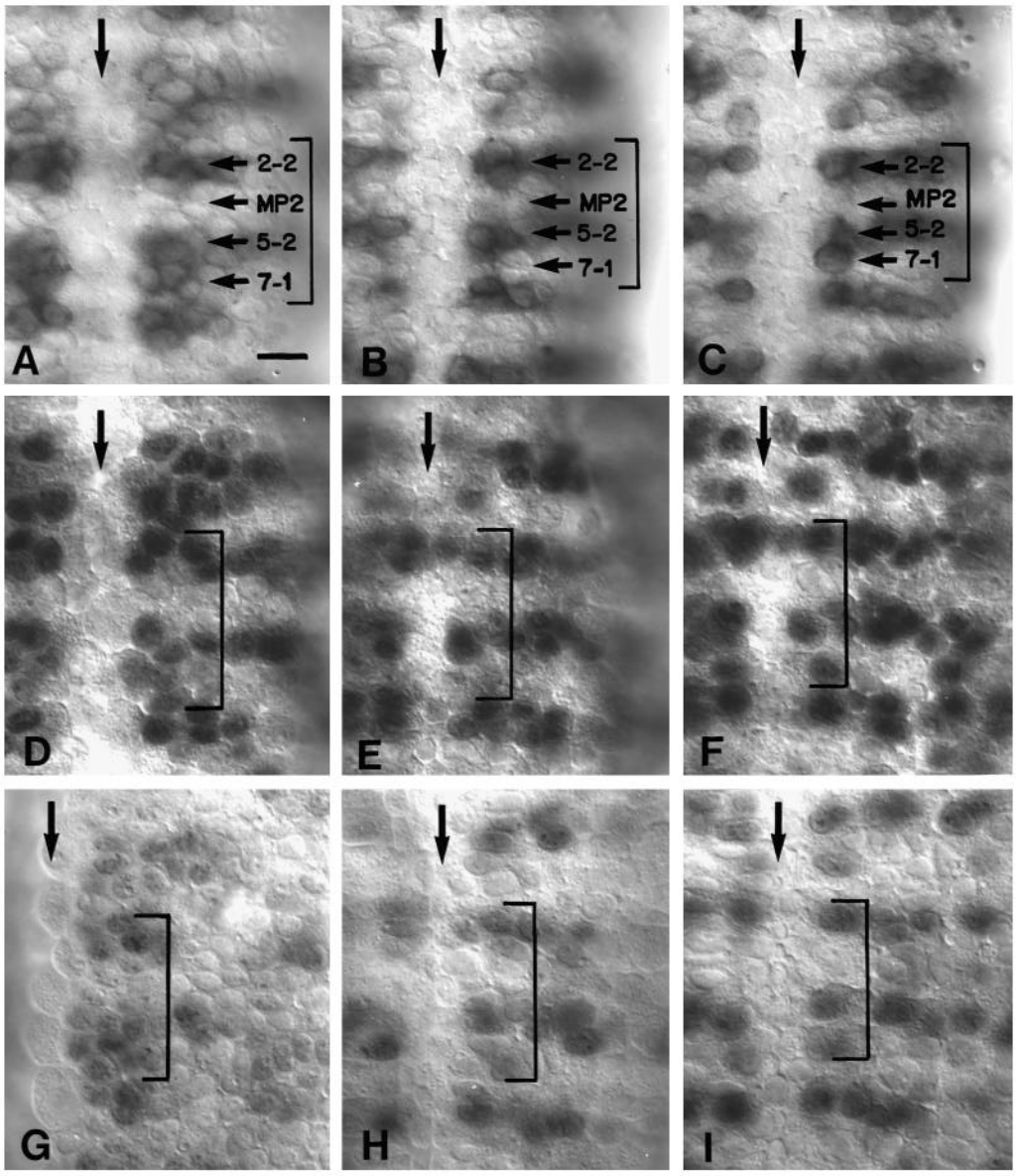

Fig. 1. Expression of *l'sc* in a wild-type embryo, during segregation of S1 neuroblasts, at the RNA (A-C) and protein (D-I) levels. (A) Detail of an early stage 8 embryo. Parallel and adjacent to the midline (marked by a vertical arrow in all pictures), in the region of the neuroectoderm that will give rise to the first four neuroblasts per hemisegment of the medial row (neuroblasts 2-2, MP2, 5-2 and 7-1), *l'sc* RNA is detected in consecutive groups of about fifteen ectodermal cells, separated by smaller groups of about five non-expressing cells. A group of *l'sc*-expressing cells is the composite of three individual, but contiguous, proneural clusters of 4-6 cells each (two cells along the anteroposterior axis and two to three cells along the dorsoventral axis). These are the clusters of neuroblasts 5-2 and 7-1 of an hemisegment and of neuroblast 2-2 of the following hemisegment, respectively. The non-expressing cells are the proneural cluster of neuroblast MP2. The extent of a segment is marked by a large bracket. (B) Shortly after segregation of S1 neuroblasts, *l'sc* RNA is still found in only 2-3 cells of clusters 2-2 and 5-2 and barely detected in cluster 7-1. (C) At a deeper plane of focus, RNA is detected in neuroblasts 2-2, 5-2 and 7-1. L'sc protein detection, with either an antibody against the entire protein (D-F), or with an antibody against a C-terminal peptide (provided by the late C. Cabrera) (G-I), yields essentially identical results, although the latter antibody stains more faintly. In both cases, the patterns of protein accumulation in the neuroectoderm, before (D,G), and after (E,H) neuroblast segregation, as well as in the neuroblast layer (F,I), closely correspond to the pattern of transcription. The embryo in E,F, slightly older than those in B,C and H,I, is beginning to express *l'sc* in the proneural cluster of the median neuroblast at the midline. Anterior is to the top. Scale bar, 20 μm.

genic genes. Our results indicate that neurogenic genes principally regulate *l'sc* expression at the transcriptional level, without affecting the domains of proneural gene expression. Secondly, we analyse whether neurogenic genes intervene in the transmission of the inductive signal that leads to mesectoderm specification. The results indicate that *N* and *neuralized* (*neu*) are required for the establishment of the mesectodermal fate and suggest also that *Dl* and members of the *Enhancer of split* gene complex (*E(spl)-C*) may be involved in the same process.

MATERIALS AND METHODS

Fly strains

The following *Drosophila* strains were used: wild-type Oregon-R, N^{55e11}, neu^{IF65}, $Df(3R)Dl^{FX3}$, $Df(3R)E(spl)^{RB25.1}$ (the latter three mutations balanced over a TM3 *lacZ* chromosome), ovo^{D1} (Lindsley and Zimm, 1992), P[w^+, *sim-lacZ*] (the *sim* promoter fused to *lacZ*, a gift from S. Crews) (Nambu et al., 1991), FRT^{101} and $hsFLP^{38}$ (Chou and Perrimon, 1992).

Germ-line clones

Female germ-line clones were induced in N^{55e11}/ovo^{D1} larvae by X-irradiation (Jiménez and Campos-Ortega, 1982), or using the FRT/FLP technique (Chou and Perrimon, 1992), as described by Menne and Klämbt (1994). Virgins of the appropriate genotype were crossed either to wild-type males or to P[w^+, *sim-lacZ*] males, to study *sim* expression. Heterozygous $N^{55e11}/+$ female embryos were recognized with an anti-Sex lethal antibody.

Immunohistochemistry

Two classes of anti-L'sc polyclonal antibodies were used. One class is a rat antibody raised against a fusion protein that contains the entire

L'sc sequence, except the first fifteen amino acids (Martín-Bermudo et al., 1991). A second class are different antibodies raised against the synthetic peptide DDEELLDYISSWQE, corresponding to the C terminus of the translated sequence of *l'sc* (Alonso and Cabrera, 1988; Martín-Bermudo et al., 1993). Of this latter class, a rabbit serum was a gift from the late C. Cabrera. In addition, we raised and affinity purified similar antibodies in rats and in one rabbit, following essentially the protocol described by Cabrera (1990). Other antibodies used were: anti-Ac (from J. Skeath and S. Carroll), anti-β-gal (Cappel), anti-Sex lethal (from L. García-Alonso), anti-Hunchback (from P. Macdonald) and anti-Snail (from A. Alberga). Embryos were fixed and stained essentially as described previously (Martín-Bermudo et al., 1991, 1993). Staged embryos were used in most instances, and were collected at 20 minutes (early stage 8), or 35-40 minutes (late stage 8) after the onset of gastrulation at 25ºC. In some cases, embryos embedded in Epon were sectioned at 5 μm.

Other procedures

In situ hybridization essentially followed the protocol of Tautz and Pfeifle (1989). After staining, embryos were dehydrated first through an ethanol series, then two times in acetone for a total of 3-5 minutes, transferred to a mixture of acetone:Epon (1:1) and inmediately spread over a microscope slide. Once the acetone had evaporated, the embryos were dissected and mounted in Epon.

RESULTS

Wild-type expression of *lethal of scute* during early neurogenesis

The detection of AS-C proteins during early neurogenesis with different antibodies led to different hypothesis about the control of *AS-C* expression (Cabrera, 1990, 1992; Martín-Bermudo et al., 1991; Skeath and Carroll, 1992). To clarify this issue, we first analysed in detail *l'sc* RNA and protein expression patterns during wild-type segregation of neuroblasts in stage 8. For simplicity, we restrict our description to the ventral-most region of the neuroectoderm, which gives rise to four S1 neuroblasts (2-2, MP2, 5-2 and 7-1) per hemisegment (Doe, 1992).

l'sc RNA is found at early stage 8 in the proneural clusters of neuroblasts 2-2, 5-2 and 7-1 (Fig. 1A). Neuroblast MP2 arises from a cluster expressing *ac* and *sc* but not *l'sc* (Martín-Bermudo et al., 1991; Skeath and Carroll, 1992; Ruiz-Gómez and Ghysen, 1993). At late stage 8, *l'sc* transcription has decayed in the neuroectoderm (Fig. 1B) but it is still active in neuroblasts 2-2, 5-2 and 7-1, which have just segregated (Fig. 1C). The pattern of L'sc protein accumulation closely follows that of transcription, as detected either with an antibody raised against the entire protein (Fig. 1D-F; see also Martín-Bermudo et al., 1991), or with any of those raised against a C-terminal peptide (Fig. 1G-I). These results strongly suggest that the control of the spatial distribution of L'sc is largely transcriptional.

lethal of scute expression in neurogenic mutants

To analyse, at the same level of resolution as above, how the expression of proneural genes is modified in neurogenic mutants, we studied the RNA and protein patterns of *l'sc* in mutants for *neu*, *Dl*, *N*, and the *E(spl)-C*. The analysis was restricted to the zygotic lack-of-function situation, except for *N*. In this case, N^- embryos were derived from homozygous N^- female germ line clones, for we found it necessary to eliminate the maternal contribution in order to detect mutant phenotypes during stage 8.

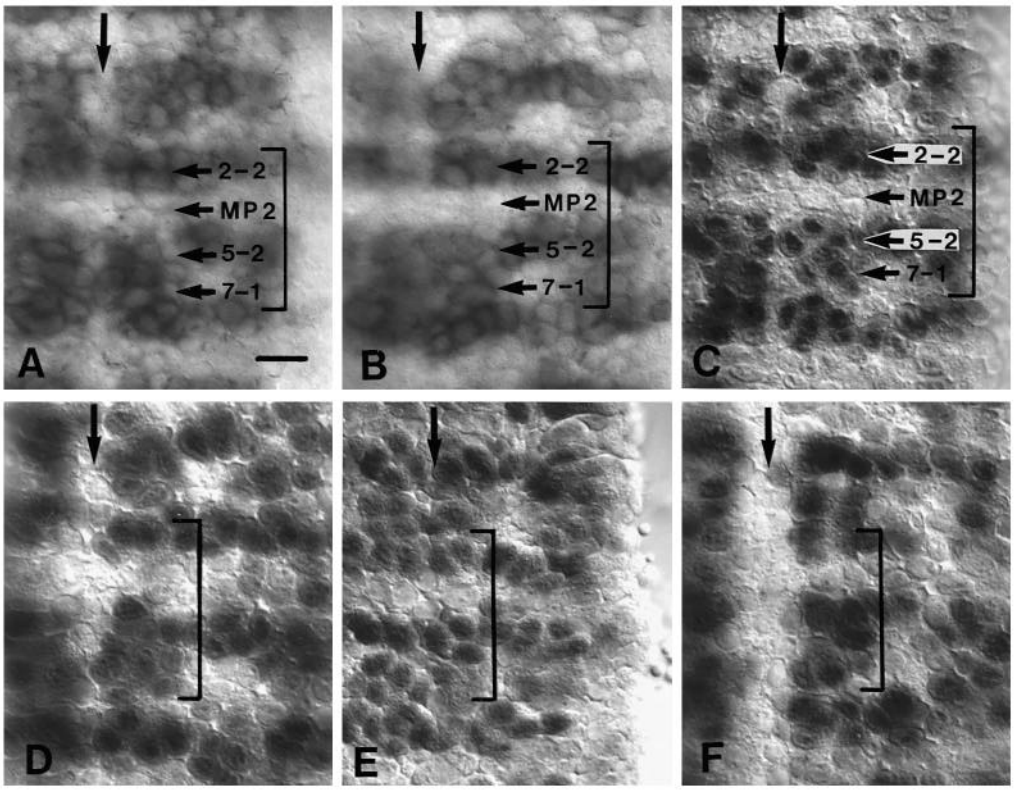

Fig. 2. Expression of *l'sc* in neurogenic mutants during segregation of S1 neuroblasts. (A) At early stage 8, the pattern of transcription in proneural clusters of a neu^- embryo is essentially like that of the wild type (Fig. 1A), except for the presence of transcripts in the territory normally occupied by the mesectoderm at the midline (vertical arrow). (B) Unlike the wild type (Fig. 1B), transcription in neu^- embryos is fully maintained in the clusters when S1 neuroblasts delaminate at late stage 8. (C-F) At that stage, the L'sc protein is also detected in all cells of the three *l'sc*-expressing clusters. This is seen in pictures focused at an intermediate plane between the neuroectoderm and the neuroblast layer of embryos mutant for *neu* (C), *Dl* (D), *N* (E) and *E(spl)-C* (F). Note how proneural clusters clearly fuse at the midline in neu^- and N^- embryos. Note also that the size of the clusters remains constant throughout stage 8. This can be seen in neu^- embryos by comparing the RNA pattern (A) at the proneural cluster stage with the RNA (B) or protein (C) patterns when neuroblasts segregate. Likewise, in all late stage 8 mutants (B-F), the MP2 cluster remains devoid of *l'sc* expression, indicating that no enlargement of neighbouring *l'sc*-expressing clusters has taken place. Anterior is to the top. Scale bar, 20 μm.

Proneural clusters in the four mutants, revealed by *l'sc* transcription, are basically normal (Fig. 2A). The only difference from the wild type is that the bilaterally symmetric clusters in *neu*$^-$ (Fig. 2A) and *N*$^-$ (not shown) embryos tend to fuse at the midline (see below). At late stage 8, *l'sc* transcription does not become restricted to the neuroblast and persists in entire clusters, which otherwise maintain their initial size (Fig. 2B).

As in the wild type, the pattern of L'sc protein accumulation closely follows that of transcription in neurogenic mutants. Mutant proneural clusters look essentially normal, as shown by double staining for L'sc and Ac. In the wild-type control (Fig. 3A), S1 clusters occupy the entire ventral-most region of the neuroectoderm, expressing either *ac*, *l'sc*, or both. The precise cell-by-cell apposition of those clusters that express either *ac* or *l'sc* suggests that in this region a cell never belongs simultaneously to more than one cluster. Proneural clusters in *N*$^-$ embryos are also not intermingled (Fig. 3B), indicating that they are not enlarged, at least along the anteroposterior axis. Unlike the wild type, most proneural cells in late stage 8 mutants still bear high levels of L'sc (Figs 2C-F and 3C) and already show the characteristic morphology and patterns of gene expression of neuroblasts (not shown; Jiménez and Campos-Ortega, 1990; Campos-Ortega and Haenlin, 1992). As already noticed for the RNA, the domain of L'sc accumulation is the same as in earlier stages.

Neurogenic gene function is required for the specification of the mesectoderm

The mesectoderm forms during gastrulation, when two single-cell wide rows join at the ventral midline. In a stage 8 wild-type embryo these two rows are still devoid of proneural gene expression (Fig. 1), but already express *sim* (Fig. 4A; Thomas et al., 1988). As shown in Fig. 2, the presumptive mesectoderm appears to be almost absent in *N*$^-$ and *neu*$^-$ embryos. In addition, defective *sim* expression suggests that most presumptive mesectodermal cells have not adopted their normal fate (Fig. 4B,C). Slight defects in *sim* expression are also detected in *Dl*$^-$ and in *E(spl)-C*$^-$ embryos (Fig. 4D,E). In cross sections, mesectodermal cells are readily observable in the wild type (Fig. 5A), but seem to have adopted a neuroectodermal fate in *N*$^-$ (Fig. 5B) and *neu*$^-$ embryos (not shown). Comparison of Figs 1A and 2A, or of Fig. 3A and 3B, suggests that the medial proneural clusters of the two mutants might be ventrally enlarged. Alternatively, the position of the entire neuroectoderm may be ventrally shifted by one cell diameter, similar to the more extensive shift that it undergoes when the mesoderm is removed (Rao et al., 1991).

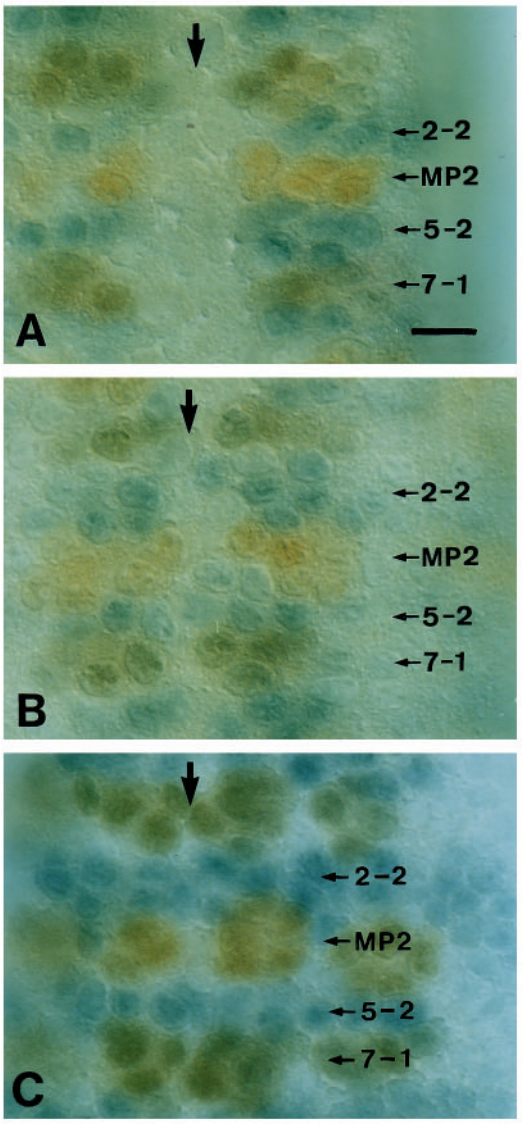

Fig. 3. Double staining of embryos with anti-L'sc and anti-Ac antibodies. (A) An early stage 8 wild-type embryo. (B) An early stage 8 *N*$^-$ embryo. (C) A late stage 8 *neu*$^-$ embryo. In all cases, clusters 2-2 and 5-2 express only *l'sc* (blue), cluster MP2 expresses only *ac* (light brown), and cluster 7-1 co-expresses both genes (dark brown). No enlargement of the clusters along the anterior-posterior axis occurs in the mutants. This is best shown by the lack of cells coexpressing *l'sc* and *ac* around the border between clusters 2-2 and MP2 and between clusters MP2 and 5-2. Whereas in the wild type the two rows of mesectodermal cells (vertical arrow) are devoid of proneural gene expression, many cells occupying equivalent positions express *l'sc* and/or *ac* in the mutants. Due to their irregular size, it is not possible to discern whether the mutant clusters are expanded, or just slightly shifted, by one cell diameter towards the midline. Anterior is to the top. Scale bar, 20 μm.

DISCUSSION

Control of *l'sc* expression by neurogenic genes

In the wild-type CNS, the proneural proteins become restricted from a cluster of cells to a single delaminating neuroblast. As for *ac* (Skeath and Carroll, 1992), we have shown that the restriction of *l'sc* is mainly accomplished at the transcriptional level. In neurogenic mutants, the transcriptional repression of *l'sc* fails to occur (see also Brand and Campos-Ortega, 1988) and all proneural cells accumulate high levels of L'sc protein, as is the case for *ac* (Skeath and Carroll, 1992; Ruiz-Gómez and Ghysen, 1993). A different mode of control of *l'sc* activity was proposed by Cabrera (1992), using an antibody against a C-terminal peptide of L'sc that contains a putative tyrosine phosphorylation site (Cabrera, 1990). With that antibody, L'sc was apparently detected only in neuroblasts, leading to the postulation that two forms of the protein exist. One form, accumulating in neuroblasts, would be unphosphorylated and func-

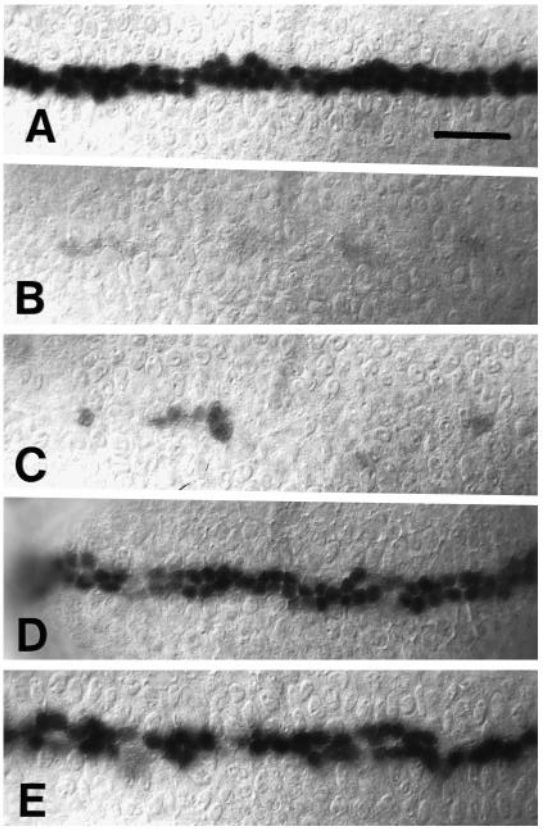

Fig. 4. The mesectoderm in neurogenic mutants, as determined by *lacZ* expression under the control of the *sim* promoter. (A) Detail of the mesectoderm of a stage 8 wild-type embryo, expressing high levels of β-galactosidase. In N^- (B) and neu^- (C) backgrounds, β-galactosidase is detected at low levels in only a few cells, suggesting a defective specification of the mesectoderm. Similar results have been obtained by in situ hybridization with a *sim* probe (not shown). In Dl^- (D) and *E(spl)*-C^- (E) backgrounds, a few mesectodermal cells fail to express *lacZ*. Anterior is to the left. Scale bar, 50 μm.

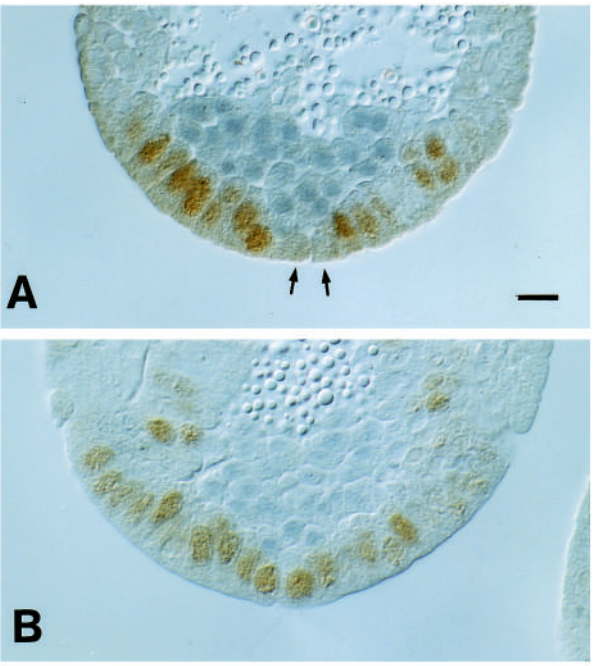

Fig. 5. Cross sections of early stage 8 embryos double stained with anti-L'sc and anti-Snail antibodies. (A) A wild-type embryo expressing *l'sc* in nuclei of proneural cells in the ectoderm (brown) and *snail* in the nuclei of the mesoderm (blue). By this stage, mesodermal expression of *snail* is decaying, thus the lower and variable intensity of the staining. The two mesectodermal cells at the ventral midline (arrows) are devoid of both *l'sc* and *snail* expression. In the N^- embryo shown in B, cells at the ventral midline are now expressing *l'sc*, suggesting that the presumptive mesectoderm has adopted a neuroectodermal fate.

tionally active. The other form, present in every cell that transcribes *l'sc*, would be phosphorylated and inactive. Accordingly, the anti-peptide antibody was found to stain a larger number of cells in Dl^- and N^- embryos, suggesting that neurogenic genes control *l'sc* at the posttranscriptional level (Cabrera, 1990, 1992). Our observations that proneural cells are similarly stained with the two available classes of anti-L'sc antibodies would suggest that both states of phosphorylation of L'sc, if present, would coexist in proneural cells. However, our observations are equally compatible with the possibility that antibodies against the C-terminal peptide cannot discriminate between different protein forms. In this context, it has been shown that neither the deletion of the C-terminal region, nor the substitution of the tyrosine in this domain by a residue that cannot be phosphorylated, have pronounced influence on the proneural activity of L'sc assayed in imaginal discs (Hinz et al., 1994).

Our analysis of embryos double stained for Ac and L'sc demonstrate, as already noted (Brand and Campos-Ortega, 1988; Ruiz-Gómez and Ghysen, 1993), that neurogenic genes do not play a role in the emergence of proneural clusters in the embryo, except for a possible slight ventral enlargement of the medial clusters in N^- and neu^- mutants. However, the possibility that the domains of proneural gene expression later become enlarged in neurogenic mutants has been a controversial issue (Brand and Campos-Ortega, 1988; Campos-Ortega, 1993; Ruiz-Gómez and Ghysen, 1993). In this regard, our results also support the view that, in neurogenic mutants, the domains of proneural gene expression in the CNS do not become enlarged at the stage when S1 neuroblasts normally delaminate.

Neurogenic genes and mesectoderm specification

As indicated in the Introduction, several lines of evidence suggest that mesectodermal specification partially requires an inductive signal from the mesoderm. Our observation that *N* is required for the activation of *sim* (see also Menne and Klämbt, 1994) and for the early repression of proneural genes in the presumptive mesectoderm suggests that mesectoderm induction involves cell communication. We have also found that *neu* participates in the same process, raising the possibility that the family of neurogenic genes participate in the specification of the mesectoderm, in a way analogous to their role during neuroblast formation (de la Concha et al., 1988). Thus, expression of *neu* and *Dl* in the mesoderm (Boulianne et al., 1991; Price et al., 1993; Kopczynski and Muskavitch, 1989; Haenlin et al., 1990) would correlate with their participation in the emission of the inductive signal. Similarly, specific expression of four *E(spl)-C* genes in the mesectoderm (Knust et al., 1992) suggests that they function to implement the reception of the signal. Maternal expression of *Dl* and of *m3*,

a fifth *E(spl)-C* gene (Kopczynski and Muskavitch, 1989; Haenlin et al., 1990; Knust et al., 1987), could explain the lack of a strong mesectodermal phenotype in zygotic Dl^{-} and *E(spl)-C*$^{-}$ mutants.

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