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# Regulation of proneural gene expression and cell fate during neuroblast segregation in the *Drosophila* embryo

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## Summary

**The *Drosophila* embryonic central nervous system develops from sets of progenitor neuroblasts which segregate from the neuroectoderm during early embryogenesis. Cells within this region can follow either the neural or epidermal developmental pathway, a decision guided by two opposing classes of genes. The proneural genes, including the members of the *achaete-scute* complex (AS-C), promote neurogenesis, while the neurogenic genes prevent neurogenesis and facilitate epidermal development. To understand the role that proneural gene expression and regulation play in the choice between neurogenesis and epidermogenesis, we examined the temporal and spatial expression pattern of the *achaete* (*ac*) regulatory protein in normal and neurogenic mutant embryos. The *ac* protein is first expressed in a repeating pattern of four ectodermal cell clusters per hemisegment. Even though 5-7 cells initially express *ac* in each cluster, only one, the neuroblast, continues to express *ac*. The repression of *ac* in the remaining cells of the cluster requires zygotic neurogenic gene function. In embryos lacking any one of five genes, the restriction of *ac* expression to single cells does not occur; instead, all cells of each cluster continue to express *ac*, enlarge, delaminate and become neuroblasts. It appears that one key function of the neurogenic genes is to silence proneural gene expression within the nonsegregating cells of the initial ectodermal clusters, thereby permitting epidermal development.**

Key words: proneural genes, neurogenic genes, neuroblast.

## Introduction

The neuroectoderm of insect embryos consists of a uniform sheet of cells, all of which possess the potential to become neuroblasts (NBs) (Bate, 1976; Doe and Goodman, 1985). Cell ablation studies performed on grasshopper embryos have shown that the fate of individual cells is guided by cell-cell interactions between neighboring cells (Taghert et al., 1984; Doe and Goodman, 1985). In the neurogenic region, one cell from a cluster of 5-6 cells normally enlarges, delaminates and becomes a neuroblast (NB). If all of the cells of the cluster are ablated, no NB forms. However, if different portions of the cluster are ablated, leaving a number of neuroectodermal cells intact, a NB is always formed. Further, if one waits and ablates only the cell that is enlarging to become the NB, one of the remaining cells forms the NB. Finally, if the NB is ablated just before its first cell division, no new NB is formed. These experiments suggest that (1) the NB arises from these neuroectodermal cells; (2) every cell of the cluster shares a common NB-forming potential (i.e. an equivalence group); (3) local inhibition of the remaining cells by the enlarging NB ensures that only one NB arises from the equivalent group of cells; (4) all cells of the cluster retain their NB-forming potential at least while the NB is enlarging but lose this potential by the time the NB is about to divide. The molecular basis of these cellular events is not well understood. The central questions are: (1) which genes give the neuroectodermal cells their NB-forming potential?; (2) which genes are required to suppress this potential in the remaining cells of the cluster after one cell is chosen to become a NB? and (3) how do these genes interact at the molecular level to specify both the neural fate of the one cell chosen to become a NB and the non-neural/epidermal fate of the other cells?

Genetic analysis of *Drosophila* neurogenesis and epidermogenesis has identified a modest number of genes that function to allow a single neural precursor to arise from an initially equivalent group of cells (Stern, 1954; Garcia-Bellido and Santamaria, 1978; Garcia-Bellido, 1979; Lehmann et al., 1983). These genes can largely be grouped into two classes: (1) the proneural genes, which promote neurogenesis, and (2) the neurogenic genes, which suppress neurogenesis and facilitate epidermal development (for reviews see Ghysen and Dambly-Chaudiere, 1989 and Jan and Jan,

1990 and references therein). Genetic experiments performed on both *Drosophila* embryos and adult flies suggest that the proneural genes are required for the initial commitment of cells to the neural fate. In *Drosophila* embryos and adult flies homozygous for null alleles of the proneural genes of the AS-C, *achaete* (*ac*), *scute* (*sc*) and *lethal of scute (l'sc)*, fewer than the normal number of sensory structures and neural precursor cells, NBs or sensory mother cells (the cells that give rise to the sensory structures), arise (Garcia-Bellido and Santamaria, 1978; Garcia-Bellido, 1979; Jimenez and Campos-Ortega, 1979; Dambly-Chaudiere and Ghysen, 1987; Jimenez and Campos-Ortega, 1990). Although the mechanisms by which *ac*, *sc*, and *l'sc* promote neurogenesis appear to be the same, each of these genes directs the specification of largely independent but partially overlapping neural precursor cells (Garcia-Bellido and Santamaria, 1978; Garcia-Bellido, 1979; Jimenez and Campos-Ortega, 1979; Dambly-Chaudiere and Ghysen, 1988; Jimenez and Campos-Ortega, 1990). Conversely, an increased number of sensory structures and neural precursors are found in *Drosophila* embryos and adult flies carrying either duplications of the AS-C or hypermorphic alleles of *ac* or *sc* (Lindsley and Grell, 1968; Garcia-Bellido, 1979; Campuzano et al., 1985; Jimenez and Campos-Ortega, 1990). The transcript patterns of the *ac*, *sc* and *l'sc* genes strongly correlate with NB segregation and sensory mother cell formation (Cabrera et al., 1987; Romani et al., 1989). Molecular studies of these genes have revealed that they each encode proteins that possess a basic helix-loop-helix (bHLH) motif (Villares and Cabrera, 1987), which is found in a number of proteins involved in transcriptional regulation and cell determination. The HLH domain is required for homo- and heterodimer formation between HLH proteins and the basic region just to the amino-terminal side of the HLH domain appears to confer DNA-binding specificity and transcriptional activating properties on dimers of these bHLH proteins (Murre et al., 1989; Davis et al., 1990). In fact, recent results suggest that heterodimers between *l'sc* and *daughterless*, a universally expressed bHLH gene also required for neurogenesis, may transcriptionally activate certain genes within NBs (Cabrera and Alonso, 1991).

Once a cell is chosen to become a NB, it inhibits the neighboring cells of the cluster from following suit via a process termed lateral inhibition (for review see Simpson, 1990). Mutations in any one of the neurogenic genes appear to cripple this process and result in neural hyperplasia at the expense of epidermis (Lehmann et al., 1983). Many lines of evidence strongly suggest that the neurogenic genes function in a cell-communication pathway that ultimately suppresses neurogenesis (for review see Campos-Ortega, 1991 and references therein). However, the molecular basis through which these genes prevent neurogenesis is not well understood. Initially, it was shown that *l'sc* protein accumulated only within NBs while *l'sc* RNA was detected in larger cell clusters (Cabrera, 1990). Further, it was shown that in certain neurogenic mutant backgrounds *l'sc* protein accumulated in all of the cells that expressed *l'sc* RNA (Cabrera, 1990). This suggested that the neurogenic genes opposed neurogenesis within the other cells of the cluster by preventing proneural protein accumulation within them. More recently, however, using a different antibody preparation, *l'sc* protein has been detected in wild-type embryos in essentially the same pattern as *l'sc* RNA (Martin-Bermudo et al., 1991). Thus, the dynamics of proneural protein distribution and the effect the neurogenic genes have on proneural protein expression are a matter of some dispute.

Here, we show the *ac* RNA and protein patterns to be essentially identical and that the dynamics of the *ac* expression pattern reflect at the molecular level the processes of singling out one cell from an initially equivalent cluster of cells to become a NB and of suppressing the NB-forming potential in the remaining cells of the cluster. We find that the *ac* protein is first expressed in a segmentally repeating pattern of clusters of 5-7 ectodermal cells arranged in columns along the ventral neuroectoderm. Even though 5-7 cells initially express *ac* protein only one, the NB, retains *ac* protein expression, while the other cells rapidly lose *ac* protein expression. Further, we show that once a cell is chosen to become a NB the neurogenic genes are required to suppress *ac* protein expression in the remaining cells of the cluster, since in embryos lacking any one of five neurogenic genes *ac* expression is not restricted to a single cell. Instead, all cells of each cluster retain *ac* expression, enlarge, delaminate and become NBs.

## Materials and methods

### *Fly strains*

The following fly stocks were used: *big brain*$^{1DO5}$, *Delta*$^{9Q}$, *Enhancer of split*$^{8D06}$, *neuralised*$^{9L19}$, *Notch*$^{55e11}$ and *In(1)y*$^{3PL}$*sc*$^{8R}$. These stocks were obtained from the laboratories of Mark Muskavitch and Spyros Artavanis-Tsakonas, and the Bowling Green and Tübigen Stock Centers.

### *Antibody generation*

A pet3a expression plasmid containing the 0.45 kb *Sma*I to *Pst*I fragment of the *ac* coding region was generously provided to us by Tadashi Uemera. The *ac* protein fragment was prepared for immunizations and ELISA experiments by making a soluble extract from inclusion bodies yielding roughly 15 milligrams of soluble protein per 500 ml initial culture. By SDS-polyacrylamide gel analysis, the *ac* protein fragment was found to constitute between 80 and 90% of the total protein in these preparations.

Six female BALB/c mice were immunized with the *ac* protein fragment. For the first boost, 50 μg of protein was emulsified 1:1 with complete Freund's adjuvant and injected intraperitoneally into the mice. The mice were subsequently boosted with 50-100 μg of protein in PBS at roughly three to four week intervals. Six days after each boost the mice were tail bled and the sera was tested for reactivity against the *ac* protein by staining 0-8 hour old embryos. After five boosts, serum from one mouse stained embryos in a pattern similar to the *ac* RNA pattern. Spleen lymphocytes from this mouse were fused to NS-1 myeloma cells following established protocols. Roughly 2000 hybridoma supernatants were screened by ELISA for reactivity against the *ac* protein

fragment coated at a concentration of 2 μg/ml. The 158 ELISA-positive supernatants were then screened on 0-8 hour embryos to determine which hybridoma colonies produced antibodies that recognized the *ac* protein fragment immunohistochemically. Of 21 hybridoma colonies that produced antibodies that recognized the *ac* fragment immunohistochemically, 11 were subcloned and kept. Two of these 984A11C1 and 990E5F1, were used in this study.

*Immunohistochemistry and in situ hybridization*

Immunohistochemical detection of *ac* protein was carried out as described in Carroll et al. (1988). mAb 984A11C1 was used at a 1:3 dilution. For double-labelling studies, we essentially followed the protocol of Kania et al. (1990). Embryos were first incubated overnight with mAb 990E5F1 diluted 1:1 in PBT (1×PBS; 0.1% Triton X-100; 1% BSA). After extensive washing for one hour, the embryos were incubated with biotinylated horse antimouse (Vector) for two hours at 4°C and then, after another hour of washes, the embryos were incubated with streptavidin-horseradish peroxidase conjugate (BRL) for one hour at 4°C. After 30 minutes of washes in PBT and another 30 minutes of washes in 100 mM Tris-HCl pH 6.8 the stain was developed in 100 mM Tris-HCl pH. 6.8 with 0.5 mg/ml of diaminobenzidene (DAB) and 0.002% $H_2O_2$. After the reaction was stopped by the addition of 5 μl of sodium azide, the embryos were washed five times in PT (1×PBS; 0.1% Tween-20). The embryos were then stripped of the first set of antibodies by incubating them in 200 mM glycine-HCl pH 2.2 for five minutes. Glycine was removed from the embryos by five washes with PT. The embryos were then reblocked in PBT for three hours. For double labelling embryos with *ac* and *en,* embryos were then incubated overnight in mAb INV4D9 (kindly provided by Nipam Patel) diluted 1:1 in PBT. After extensive washing for one hour, the embryos were incubated for two hours at 4°C with alkaline-phosphatase conjugated to goat anti-mouse (Fisher Biotech). After another hour of washes, the stain was developed as described in Kania et al. (1990). For double labelling embryos with *ac* and *hb,* stripped and reblocked embryos were incubated overnight with a rabbit anti-*hb* antiserum (kindly provided by James Langeland) used at 2 μg/ml in PBT. Detection of *hb* expression was performed as described above for *ac* with the following exception: staining was developed with 0.5 mg/ml of diaminobenzidene (DAB) in the presence of 0.03% (wt/vol) $Co^{2+}$ and $Ni^{2+}$ ions. After staining was completed all embryos were washed five times in PT, transferred to PBS and 10% glycerol and then mounted. In situ hybridization was carried out as described in Tautz and Pfeifle (1989). Embryos were hybridized with 50 μl (1 ng/μl) of a DNA probe from the 2 kb *Eco*RI fragment of an *ac* cDNA, generously provided by Carlos Cabrera.

## Results and discussion

ac *is expressed in clusters of ectodermal cells from which single NBs segregate*

In order to localize the proneural *ac* protein with high resolution in developing embryos, we generated a number of monoclonal antibodies (mAbs) specific for the *ac* protein. Using these mAbs we observe that the *ac* protein first accumulates in a segmentally repeated pattern of clusters of 5-7 ectodermal cells (late stage 8 as defined by Campos-Ortega and Hartenstein, 1985; Fig. 1A,C) and that *ac* protein is localized primarily within the nucleus (Fig. 2B, note the similarity between the localization of the nuclear *engrailed* protein and the *ac* protein). Two medial and two lateral clusters are found per hemisegment (brackets; Fig. 1A). Shortly after the establishment of the cluster pattern, one cell in each cluster, the future NB, comes to express *ac* most intensely and delaminates towards the interior of the embryo while the other cells of the cluster remain in the ectodermal cell layer and continue to express *ac* (data not shown). Next, the cells within the ectodermal cell layer lose *ac* expression (as no cell death occurs in the neuroectoderm during these stages, these cells probably become epidermal cells; Campos-Ortega and Hartenstein, 1985), while the delaminated NB retains it (mid-late stage 9; Fig. 1B,D). Thus, every cluster rapidly resolves to a single enlarged cell, the NB, which still expresses *ac* (mid-late stage 9; Fig. 1B,D). The fate of each cell within a cluster is strongly correlated then, not with the initial presence of the *ac* protein, but with the fate of *ac* expression in that cell: cells that retain *ac* expression become NBs; those that lose *ac* expression lose their NB-forming potential and become epidermal.

Once a NB has delaminated away from the ectoderm and before it undergoes any divisions, *ac* expression in the NB is extinguished. The NBs in the posterior region of each segment are the first to lose *ac* expression (early stage 10; Fig. 1E); shortly thereafter the two anterior NBs also extinguish *ac* expression (data not shown). Interestingly, the dynamics of *ac* protein distribution within the neuroectoderm of the *Drosophila* embryo precisely parallel the dynamics of *ac/sc* protein expression previously observed within the notal regions of the wing imaginal disc (Cubas et al., 1991; Skeath and Carroll, 1991). Thus, as is found in the genesis of the peripheral nervous system of the adult fly, *ac* appears to be involved within the embryo in the initial commitment of an ectodermal cell to become neural but not in the maintenance or final differentiation of that cell type. After stage 10, *ac* is expressed in the peripheral and then again in the central nervous system in rapidly changing and spatially intricate patterns (Skeath, J. and Carroll, S. unpublished observations).

*The ac mRNA and protein patterns coincide*

As previously mentioned the relationship between the domains of proneural gene transcription and protein accumulation has been a matter of dispute. To clarify the relationship between *ac* RNA and protein expression, we compared these patterns by in situ hybridization and immunohistochemical inspection of embryos at the same stage of development. At the ectodermal cluster stage, the *ac* RNA pattern is no broader than the protein pattern (compare Fig. 1C to Fig. 1G), and both patterns quickly resolve to label single NBs from each cluster (compare Fig. 1D to Fig. 1H). Thus, the *ac* RNA and protein patterns appear to be essentially identical as is most likely the case with *l'sc* (Martin-Bermudo et al., 1991). This suggests that the spatial control of these proteins is largely transcriptional, not post-transcriptional (Cabrera, 1990). The original *l'sc* antibody was raised against an epitope

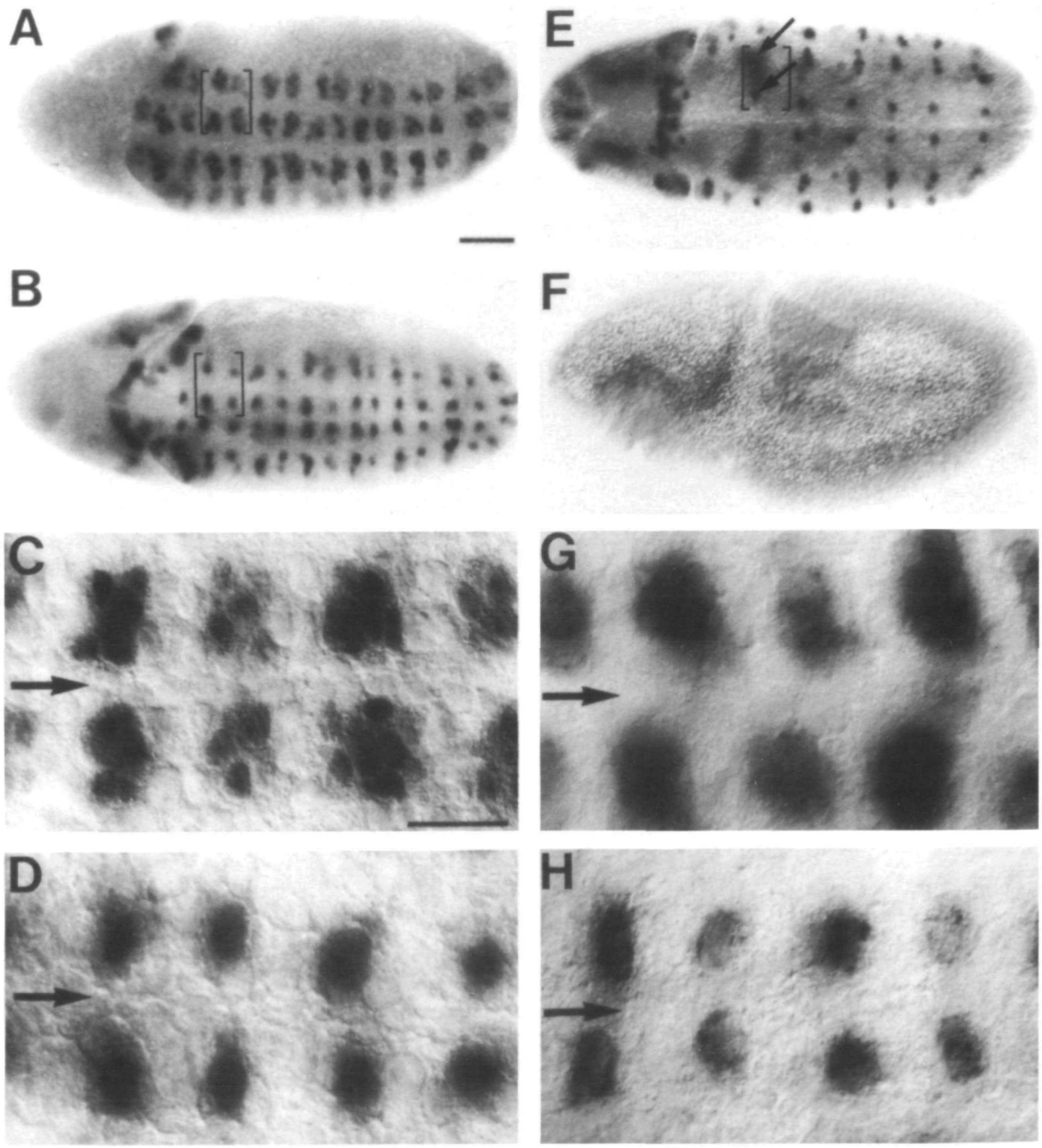


**Fig. 1.** Resolution of the WT *ac* protein and RNA patterns during NB segregation. Ventral views of WT embryos: (A,C,G) late stage 8 (ectodermal cluster stage); (B,D,H) mid-late stage 9; and (E) early stage 10. (F) Lateral view of a late stage 8 $Df(1)y^{3PL}sc^{8R}$ embryo. (A) *ac* protein, as detected by the monoclonal antibody (mAb) 984A11C1, is initially expressed in a repeating pattern of four clusters of 5-7 ectodermal cells per hemisegment (one hemisegment is bracketed in A,B and E). (B) By mid-late stage 9, *ac* protein expression in each cluster has resolved to a single enlarged cell, the NB, yielding 4 NBs per hemisegment (brackets; B). (E) Shortly thereafter, the NBs in the posterior compartment extinguish *ac* expression, while *ac* expression remains in the anterior NBs (arrows; E) until late stage 10 (data not shown). At the ectodermal cluster stage both the *ac* protein (C) and RNA (G) are found in essentially identical patterns of cell clusters. By mid-late stage 9 both the *ac* protein (D) and RNA (H) expression patterns have resolved to just the NBs. (F) The specificity of mAbs 984A11C1 (F) and 990E5F1 (data not shown) for the *ac* protein was verified in that $Df(1)y^{3PL}sc^{8R}$ embryos which carry a deletion of the *ac* gene exhibit no staining. In C,D,G,H arrow points to the ventral midline. Anterior is to the left. In A,B,E,F scale bar=50 $\mu$m. In C,D,G,H scale bar=20 $\mu$m.

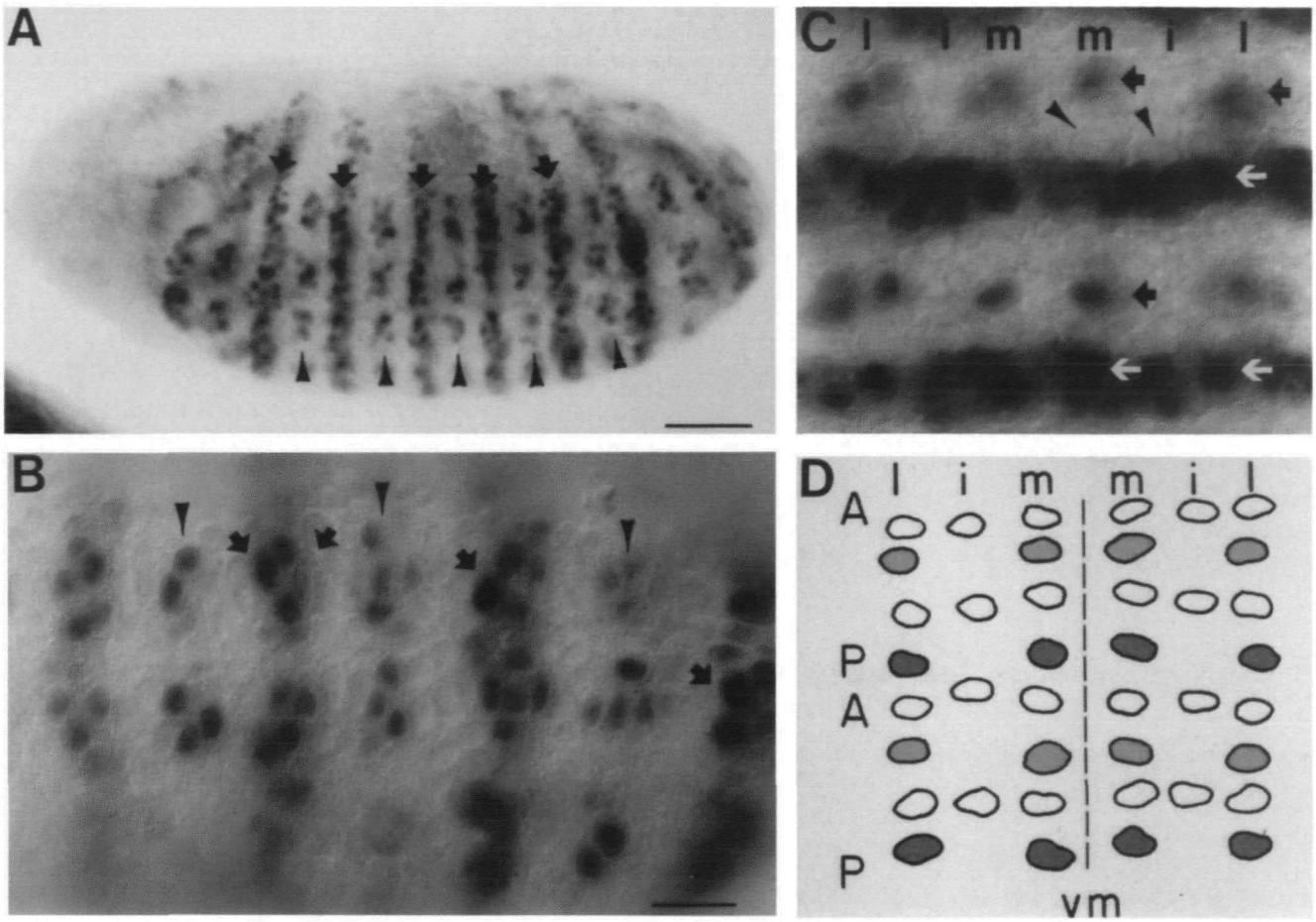


**Fig. 2.** Anteroposterior and dorsoventral registration of *ac*-expressing cell clusters and NBs. WT embryos double labelled for *ac* protein (brown) and *en* protein (blue/black). The domain of *en*-expressing cells marks the posterior compartment in each segment (DiNardo et al., 1985). (A) Ventrolateral view of WT embryo at the ectodermal cluster stage. (B) Ventral view of abdominal segments of an embryo at a similar stage. Note, at this stage every other transverse row of *ac*-positive clusters is contained entirely within an *en* stripe (arrows; A). The more darkly labelled cells in each *en* stripe express *ac* and *en* (arrows; B). The other set of *ac*-positive clusters are found midway between adjacent *en* stripes (arrowheads; A,B). (C) Ventral view of a mid-late stage 9 embryo. By this stage (C,D), the first wave of NB segregation has occurred and NBs are arranged in three longitudinal columns: medial (m), intermediate (i) and lateral (l). The m and l NB columns consist of 4 NBs and the i column consists of 2 NBs per hemisegment at this stage (D; data not shown). *ac* is expressed in every other NB of the m and l columns (white and black arrows; C), but not in the i column. Within each *en* stripe, the more strongly labelled cells, the NBs, coexpress *ac* and *en* (white arrows; C). A number of unlabelled NBs can be distinguished by their cell outlines (arrowheads; C). (D) A diagram of the NB map as deduced from an analysis of embryos doubly labelled for *ac* and *en* and *ac* and *hb* (data not shown). Red circles: *ac*-positive NBs. Blue circles: *ac* and *en*-positive NBs. Dashed line: ventral midline (vm); solid line: segment boundary. A, anterior; P, posterior. In A,B anterior is to the left. In C,D anterior is to the top. A, scale bar=50 μm. B-D, scale bar=20 μm.

containing a putative tyrosine phosphorylation site (Cabrera and Alonso, 1988; Cabrera, 1990), and thus may not recognize a phosphorylated form of *l'sc*. It is possible that dephosphorylation of this site, which is also present in the *ac* and *sc* proteins (Villares and Cabrera, 1987), may occur preferentially in NBs and be involved in the activation of proneural proteins.

### *The anteroposterior and dorsoventral registration of ac-expressing clusters and NBs*

The *ac*-positive NBs represent a subset of the first population of NBs to segregate from the neuroectoderm. The anteroposterior (AP) and dorsoventral (DV) registration of these neuroblasts and the ectodermal cell clusters from which they arise was determined by double labelling embryos with antibodies specific for the *ac* protein and the protein encoded by the segment polarity gene *engrailed* (*en*) (DiNardo et al., 1985; Patel et al., 1989; Fig. 2); and for *ac* and the protein encoded by the segmentation gene *hunchback* (*hb*; data not shown), which is expressed in most if not all NBs (Jimenez and Campos-Ortega, 1990). Two of the four *ac* clusters per hemisegment, one medial (ventral) and one lateral (more dorsal) cluster, are completely contained within each stripe of *en*-expressing cells (arrows Fig. 2A,B); the other two clusters are found midway between adjacent *en* stripes (arrowheads; Fig. 2A,B). It may be important developmentally that the width of the *ac* clusters equals the width of the *en* stripe. This correspondence holds from the onset of *ac* expression until the clusters begin to resolve.

By the time the four *ac*-positive clusters per hemisegment have resolved to four NBs (mid-late stage 9) the NBs are arranged in three longitudinal columns: medial (m), intermediate (i), and lateral (l) (Hartenstein and Campos-Ortega, 1984). The m and l columns consist of four NBs and the i column consists of two NBs each per hemisegment (Fig. 2D, data not shown). In the m and l columns, *ac* is expressed in every other NB (arrows, Fig. 2C,D). Taken together with the observations on embryos double labelled for *ac* and *en*, this demonstrates that in each hemisegment *ac* is expressed in the second most anterior pair of NBs and in the most posterior set of NBs, but not in the intervening rows (Fig. 2D). It has been suggested that the AS-C genes could function to specify NB identity (Cabrera et al., 1987). Although no formal evidence exists to support this idea (see Martin-Bermudo et al., 1991), the fact that only four out of the first ten NBs express *ac* protein (Fig. 2C,D) suggests these genes, alone or in combination with other genes, could perform such a role. With the recent increase in the number of specific markers for NBs and their progeny, it may soon be possible to determine if the genes of the AS-C do, in fact, specify NB identity (Martin-Bermudo et al., 1991). For example, if the loss of *ac*, *sc* or *l'sc* protein expression from a particular NB or the directed misexpression of one of these genes in a NB which normally does not express this gene alters the pattern of gene expression within that NB or its progeny, this would suggest a function for the AS-C genes in specifying the fate of that NB.

### *Neurogenic genes suppress ac expression in the non-segregating cells of the proneural cluster*

The exclusive retention of *ac* expression within one cell, the NB, of an ectodermal cell cluster raised the question of how proneural protein expression is eliminated from the other cells of the cluster, thereby removing their potential to become NBs. The available evidence suggests the neurogenic genes epidermalize these cells via a cell communication pathway that ultimately opposes neurogenesis (Lehmann et al., 1983; de la Concha et al., 1988; Brand and Campos-Ortega, 1989; for reviews see Artavanis-Tsakonas, 1988; Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1990; Simpson, 1990; and Campos-Ortega, 1991). In order to determine the relationship between neurogenic gene function and *ac* protein expression, we assayed the distribution of *ac* protein in embryos mutant for five neurogenic genes. Homozygous *Notch* ($N^{55e11}$; data not shown), *Delta* ($Dl^{9Q}$), *Enhancer of split* ($E(spl)^{8D06}$), *big brain* ($bib^{1D05}$), and *neuralised* ($neu^{9L119}$) mutant embryos exhibit similar effects on *ac* expression: the restriction of *ac* expression from a cluster to a single cell does not occur; instead, most to all cells of the cluster retain *ac* expression at high levels, enlarge, delaminate and apparently become NBs (compare Fig. 3B,C,D to Figs 3A, 1D). It appears, then, that one key function of the neurogenic genes is to silence proneural gene expression within the non-segregating cells of the initial ectodermal clusters, thereby allowing epidermal development.

Even though the resolution of *ac*-positive cell clusters does not occur in neurogenic mutants, the temporal regulation of *ac* is normal. Just after mid-late stage 9, *ac* expression in embryos mutant for any one of the neurogenic genes is lost from the posterior region of each segment leaving two clusters in place of the two anterior NBs found in WT embryos (compare Fig. 3E,F to Fig. 1E). Shortly thereafter, as is observed in WT embryos, *ac* expression is removed from the anterior region of each segment (data not shown). Except in $neu^{9L119}$ embryos (Fig. 3E), the number of *ac*-positive cells per cluster remains relatively constant until these cells cease *ac* expression (Fig. 3F). The number of cells per cluster in $neu^{9L119}$ embryos increases from 5-7 cells (mid-late stage 9; Fig. 3C) to 5-12 cells (early stage 10; Fig. 3E). Division of cells within the cluster, late derepression of *ac* expression, or recruitment of adjacent cells into proneural clusters could account for the increase in *ac*-positive cells in $neu^{9L119}$ mutant embryos.

Our results argue strongly that the neurogenic genes function to silence proneural gene expression in the non-segregating cells of the ectodermal cell cluster. However, a number of questions remain as to the exact mechanism/pathway through which these genes accomplish this end. In what order do the neurogenic genes act within the lateral inhibition pathway and what are the physical interactions that occur between their

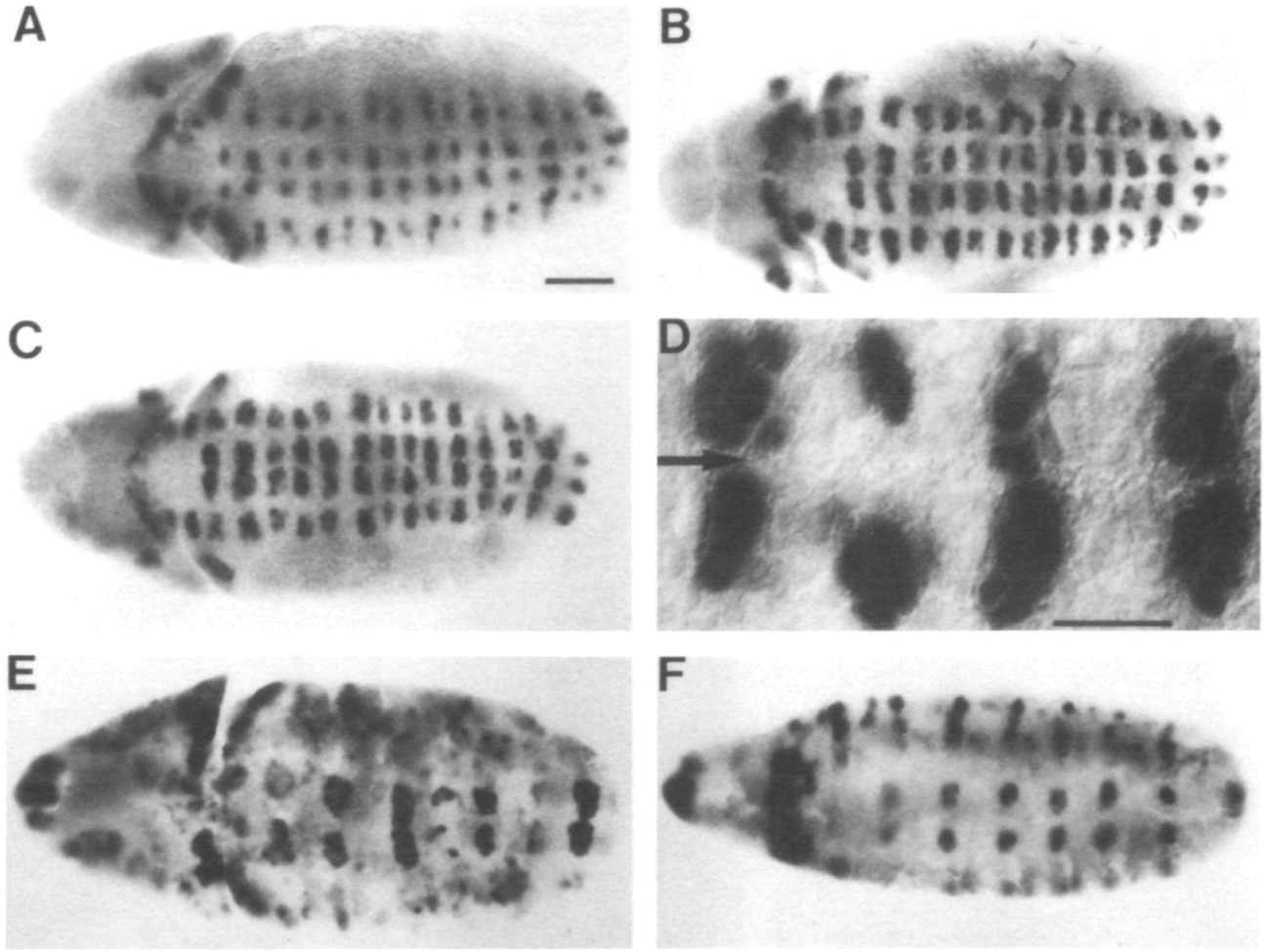


**Fig. 3.** *ac* protein expression fails to resolve in neurogenic mutant embryos. Ventral views of (A) WT embryo and homozygous (B) $bib^{1D05}$, (C) $neu^{9L119}$ and (D) $Dl^{9Q}$ (high magnification) mutant embryos at mid-late stage 9 and of homozygous (E) $neu^{9L119}$ and (F) $E(spl)^{8D06}$ mutant embryos at early stage 10. In comparison to WT embryos at mid-late stage 9 (A) *ac* expression did not resolve to single NBs in neurogenic embryos (compare B,C to A and D to Fig. 1D). Note the similarity in the *ac* expression pattern between neurogenic embryos at mid-late stage 9 and WT embryos at the ectodermal cluster stage (compare B,C to Fig. 1A). This correspondence is especially striking at high magnification (compare D to 1C). By early stage 10, *ac* expression in neurogenic embryos is removed from the posterior compartment leaving two clusters in place of the two anterior NBs found in WT embryos (compare E,F to Fig. 1E). In all neurogenic mutants examined, except the $neu^{9L119}$ embryos, the number of *ac*-positive cells per cluster remained largely constant until they turned off *ac* expression. At early stage 10, the number of cells per cluster in an $E(spl)^{8D06}$ embryo (F) and in $bib^{1D05}$, $Dl^{9Q}$ and $N^{55e11}$ embryos (data not shown) is between 4 and 5, roughly equivalent to the number of cells per cluster in neurogenic embryos at mid-late stage 9 (B,C,D). In $neu^{9L119}$ embryos, the number of cells per cluster increases from between 5 and 7 during mid-late stage 9 (C) to 4-12 cells during early stage 10 (E). Anterior is to the left. In D arrow points to the ventral midline. For A-C,E,F scale bar=50 μm. For D scale bar=20 μm.

gene products to perpetuate the inhibitory signal? A series of experiments have shown that *Dl* appears to act as the signal that passes on the lateral inhibitory signal from one cell to another via its physical interaction with the receptor trans-membrane protein *Notch* (Fehon et al., 1990; Heitzler and Simpson, 1991). It will be important to determine biochemically where the rest of the neurogenic genes fit into the pathway. Further, it will be critical to understand how in molecular terms the neurogenic genes silence proneural gene expression. The *E(spl)* complex appears to act in the last step of lateral inhibition (de la Concha et al., 1988) and encodes several bHLH proteins (Klambt et al., 1989). The gene products of the *E(spl)* complex may then remove proneural gene expression from cells initially competent but not chosen to become NBs. Given their structure, the *E(spl)* complex gene products could perform this function by sequestering transcriptional activators of the proneural genes in 'poisoned' heterodimers (Benezra et al., 1990; Ellis et al., 1990; Garrell and Modolell, 1990) incapable of transcriptional activation or they could block proneural gene transcription directly by binding to the control regions of these genes.

### *Global control of ac gene expression*

The precise and reproducible AP and DV pattern of *ac*-

expressing clusters within each segment suggest that the segmentation genes that establish segment number and polarity and the dorsal-ventral genes which specify the DV pattern of the embryo could directly regulate the initial AP and DV limits of *ac* expression. In fact, preliminary results implicate the pair-rule (but not the segment polarity genes) as being the primary determinants of *l'sc* (Martin-Bermudo et al., 1991) and *ac* expression along the AP axis, while the dorsal-ventral genes appear to repress *ac* expression in the dorsolateral ectoderm (Skeath, J., Panganiban, G, and Carroll, S. unpublished data). In order to determine directly how the early pattern-forming genes regulate the expression of the *ac* gene it will be crucial to define *cis*-acting elements of *ac* which respond to these genes; and then to determine via in vitro DNA-protein binding and in vivo reporter mutagenesis experiments which of the early pattern-regulating genes act directly on *ac* to establish its initial expression pattern.

In conclusion, the dynamics of *ac* protein expression vividly illustrate the early cellular and molecular events of neurogenesis and the roles of the neurogenic genes in facilitating epidermal development. Several key issues remain with regard to the regulation of the spatial pattern of NBs and the specification of NB identity. It will be important to determine which genes establish the repeating pattern of ectodermal cell clusters, what the specific roles are of each neurogenic gene in lateral inhibition of proneural gene expression, and, which genes specify the identity of each NB and its progeny. The elucidation of the mechanisms involved in regulating *ac* should aid us in integrating our knowledge of the roles of the genes involved in the specification of cell types with those involved in guiding the overall organization of the embryo.

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