The EMBO Journal vol.12 no.3 pp.1121–1130, 1993

The expression and role of a proneural gene, *achaete*, in the development of the larval nervous system of *Drosophila*

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Communicated by A.Ghysen

The proneural genes of *Drosophila* are required for the segregation of neural precursors from the ectoderm. One of the proneural genes, *achaete* (*ac*), is responsible for the formation of a subset of larval and adult sense organs. We have examined the pattern of expression of *ac* and its regulation during embryogenesis. *ac* is expressed at particular locations of the ectoderm according to a highly dynamic and complex pattern. At each location the expression of *ac* occurs in two stages. First, *ac* is expressed in a cluster of cells ('competent' stage). This pattern of expression depends on an array of *cis*-regulatory sites which promote the expression of *ac* in clusters of cells at particular locations. Second, one cell of each cluster accumulates *ac* transcripts at a high level ('determined' stage) and becomes the neural progenitor cell. The restriction of the determined stage to one cell of the cluster requires the gene *Notch*. The *trans*-activation of *ac* by *sc* (*scute*) or by other genes of the *achaete–scute* complex (AS-C) plays no role in defining the initial pattern of expression of *ac*, but might play a role in the shift from the competent to the determined level. We propose that the requirements of particular sense organs for different AS-C genes reflect the organization of this gene complex, rather than functional differences between the genes.

Key words: *achaete*/*cis*-regulatory sequences/*Drosophila*/neurogenesis/proneural function

Introduction

In insects, the different cells that form a sense organ (SO) are derived from a single precursor cell called a 'sensory mother cell' (SMC, Bate, 1978; Bodmer *et al.*, 1989; Hartenstein and Posakony, 1989). SMCs are ectodermal in origin and are determined very close to the position where the final SOs are located. Thus, the pattern of SOs reflects the pattern of the SMCs. The genetic programme that underlies the emergence of this pattern in *Drosophila* has attracted interest for two reasons. First, pattern formation is a crucial aspect of morphogenesis, and the reproducible pattern of SOs is an appealing system for a genetic analysis of how patterns are formed. Second, the PNS is much simpler and more accessible than the CNS, yet what we learn about the emergence of the PNS may give us clues about the more complex processes that are involved in the development of the CNS.

Several genes have been identified, whose inactivation causes a total or partial absence of SOs in the adult fly (García-Bellido and Santamaría, 1978) or in the late embryo (Dambly-Chaudière and Ghysen, 1987). These genes have been called proneural genes (Ghysen and Dambly-Chaudière, 1989; Romani *et al.*, 1989) because their expression confers to epidermal cells the ability to become SMCs. The best known of the proneural genes are the four genes of the *achaete–scute* complex (AS-C): *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*) (Muller and Prokofyeva, 1935; García-Bellido, 1979; Campuzano *et al.*, 1985; Dambly-Chaudière and Ghysen, 1987; González *et al.*, 1989). The products of the four AS-C genes share a conserved motif, the basic region–helix–loop–helix (b-HLH) sequence, also found in several vertebrate transcriptional regulators (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Caudy *et al.*, 1988; González *et al.*, 1989; Murre *et al.*, 1989a). This motif can mediate the formation of homo- or heterodimers and the binding to specific DNA sequences (Murre *et al.*, 1989b; Cabrera and Alonso, 1991; Garrell and Campuzano, 1991).

Of the four AS-C genes, *ac* and *sc* are essential for the establishment of two complementary subsets of external SOs in the adult (García-Bellido and Santamaría, 1978). The expression of *ac* and *sc* in the wing imaginal disc has been examined in detail, in relation to the formation of the large bristles that form at reproducible sites of the adult thorax. Both genes are expressed according to a reproducible spatial and temporal pattern, in clusters of cells from which the SMCs of the adult SOs will originate (Romani *et al.*, 1989; Cubas *et al.*, 1991; Skeath and Carroll, 1991; Campuzano and Modolell, 1992). The two genes are initially activated in different regions, but their final distribution overlaps completely as a result of the *trans*-activation of *ac* by *sc* and of *sc* by *ac* (Martinez and Modolell, 1991; Skeath and Carroll, 1991). At later times the expression of both *ac* and *sc* becomes restricted to one or two cells of the cluster. This restriction requires the activity of another family of genes involved in defining the pattern of SOs, the 'neurogenic' gene family. The inactivation of any of the neurogenic genes results in the formation of large numbers of neural precursors, both in the CNS (Lehmann *et al.*, 1983) and in the PNS (Hartenstein and Campos-Ortega, 1986; de Celis *et al.*, 1991; Goriely *et al.*, 1991; Heitzler and Simpson, 1991).

One difficulty in the analysis of the adult pattern of SOs is that the complete pattern is quite complicated, so that most of the work done so far has been limited to one type of sense organs (the mechanosensory bristles) in one tissue (the dorsal mesothorax, or notum). Another limitation is that most mutations that alter important features of neurogenesis are lethal and have to be studied either in clones or as weak alleles that allow homozygous individuals to reach adulthood. These limitations do not apply to the larval PNS. Each body segment of the larva bears only ~15 external SOs on each side of the midline. The SOs appear at constant positions

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on the body segments, and their arrangements in the thoracic segments T2 and T3 (T pattern) and in the first seven abdominal segments (A pattern) have been completely described (Campos-Ortega and Hartenstein, 1985; Dambly-Chaudière and Ghysen, 1986; Bodmer and Jan, 1987). Since the entire complement of larval SOs is already present in the late embryo, the effect of mutations that are lethal to the larva can nevertheless be assessed.

In the larva, as in the adult, the complete removal of the AS-C eliminates all external sense organs. The role of the different AS-C genes is different in the two systems, however: while in the adult *ac* and *sc* promote the formation of two complementary subsets of SOs and *ase* has at most a minimal role, in the larva *ac* and *sc* affect the same subset of SOs, whereas *ase* is responsible for the generation of the complementary subset (Dambly-Chaudière and Ghysen, 1987). In addition to their role in the formation of the larval and adult PNS, the AS-C genes are also involved in the formation of the CNS (García-Bellido and Santamaría, 1978; Jiménez and Campos-Ortega, 1979). Here again the different AS-C genes appear to play different roles. The inactivation of *l'sc* causes a reduction in the number of neuroblasts (NBs). The inactivation of *ac*, *sc* or *ase* has by itself no detectable effect, but enhances the defect due to the inactivation of *l'sc* (Jiménez and Campos-Ortega, 1987). Thus different AS-C genes, or combinations of them, seem to be required for the emergence of different precursors in the CNS, in the larval PNS, and in the adult PNS. These results might suggest that the combination of AS-C genes expressed in a particular precursor contributes to specify the fate of that precursor (Cabrera *et al.*, 1987).

Here we examine the wild type pattern of *ac* expression, its *cis*-regulation, its *trans*-regulation by other AS-C members and by the neurogenic *Notch* (*N*) gene, and the relationship between the expression of *ac* and the generation of sense organs. We do not find any evidence that the specific combination of AS-C genes expressed at one site plays any role in defining the fate of the progenitor cell that is formed at that site. We propose that individual sense organs depend mostly or exclusively on one of the AS-C genes because the

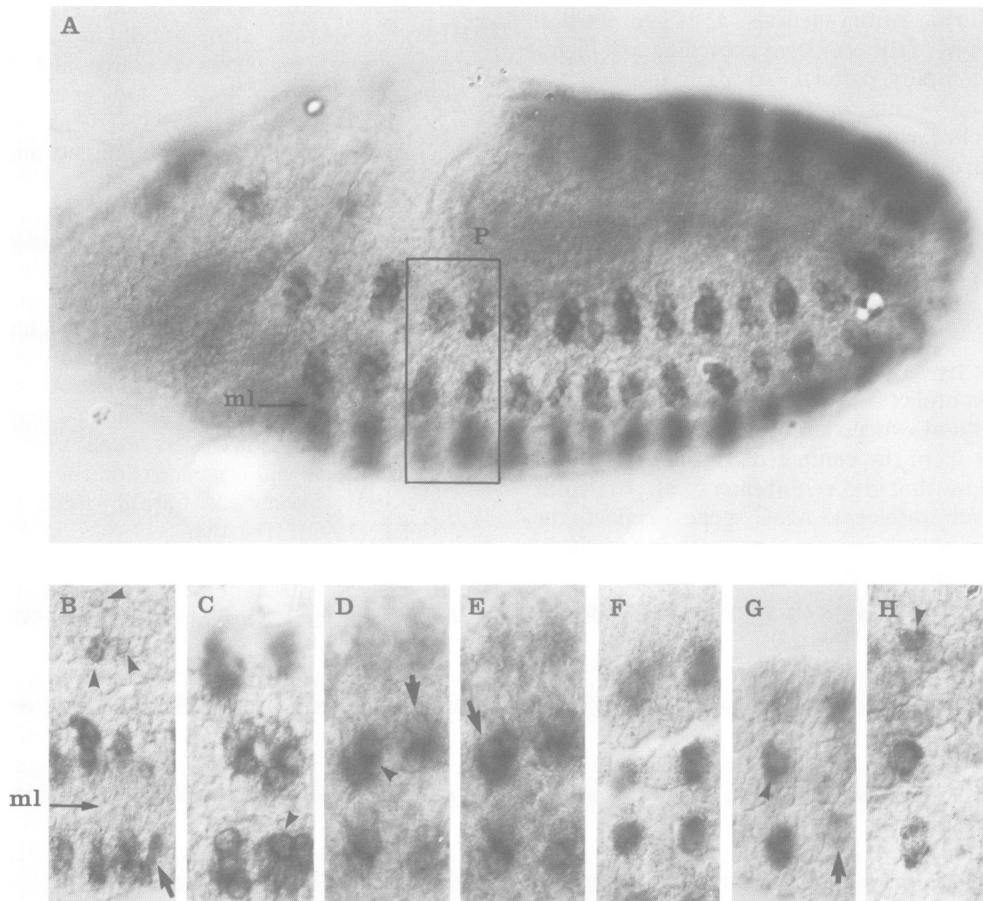

Fig. 1. Early patterns of *ac* expression. Embryonic stages are according to Campos-Ortega and Hartenstein (1985). (**A**) Stage 8 embryo showing *ac* expression in clusters of epidermal cells. The framed portion corresponds to part of the ventral neurogenic region of one segment, including the midline (ml), and is represented in (B)−(H) at different developmental stages. P indicates the position of the posterior compartment. (**B**) Stage 7 embryo, *ac* is expressed by groups of ectodermal cells close to the ventral midline, and by a few cells located more laterally (arrowheads). In these early embryos the number of proneural clusters along the A−P axis varies from 12 to 16, suggesting that the final bisegmental pattern is accomplished progressively (arrow). (**C**) Stage 8 embryo; the pattern of *ac* clusters has become bisegmental. The arrowhead points to one cell in the cluster that accumulates higher amounts of *ac* RNA. (**D** and **E**) Different focal planes of the same early stage 9 embryo. Arrowhead in (D) points to the epidermal foot of the segregating NB indicated by an arrow in (E). Note the slight staining of the epidermal cells in the cluster arrowed in (D). (**F**) Stage 9 embryo showing the four *ac*-expressing NBs per hemisegment. (**G**) Late stage 9 embryo; only the anterior medial NB continues to express high levels of *ac* (arrowhead), the arrow points to the posterior medial NB where *ac* expression has become barely detectable. (**H**) Stage 10 embryo, *ac* expression is maintained in the anterior medial NB by the time *ac* expression in the PNS neurogenic region becomes detectable (arrowhead). (C)−(H) are reproduced to the same scale, whereas (B) has been slightly reduced.

cis-regulatory sites active at the corresponding location act mostly or exclusively on that particular gene.

Results

ac expression in the CNS

The first wave of *ac* expression occurs in the early gastrula (stage 7) in 12–16 clusters of cells aligned on each side of the ventral midline. During stage 8 the early segmental pattern evolves into a bisegmental one, probably by the splitting of the previous clusters (arrow in Figure 1B and C). At the same time, *ac* becomes expressed in a second row of clusters, lateral to the first row (arrowhead in Figure 1B). At the end of stage 8 the pattern comprises four clusters in each hemisegment (Figure 1A). By this time the pattern has become quite regular: each cluster contains four to six ectodermal cells, and the different clusters are clearly separated by cells that do not express *ac* (Figure 1A). Then one cell, usually close to the centre of the cluster, accumulates higher levels of *ac* RNA, and begins to delaminate from the epidermis. Intermediate figures can be observed in which the delaminating cell has almost completely segregated from the epidermis, except for a 'foot' that remains inserted in the epidermal layer (Figure 1D and E). Once the cell has completely segregated, it continues to express high levels of *ac* RNA, while the remaining cells of the cluster turn off *ac* expression (Figure 1F).

The segregation of NBs in the germ band occurs in three waves which give rise to three different NB populations (Campos-Ortega and Hartenstein, 1985). The first wave generates two rows of NBs, a medial and a lateral one, on each side of the midline. Each row contains four NBs per segment. Our results show that two of the four medial and two of the four lateral NBs in each hemisegment are derived from *ac*-expressing cells. Double labelling using anti-ac and anti-en antibodies identifies one NB in each row as belonging to the posterior compartment (Figure 2), corroborating previous results (Cabrera *et al.*, 1987; Skeath and Carroll, 1992; Skeath *et al.*, 1992).

The different clusters differ in the dynamics of *ac* expression. Neuroblast segregation occurs earlier in the medial than in the lateral row (see Figures 1E–F and 2A), but the two lateral NBs and the posterior medial NB turn off *ac* expression at the same time. The expression of *ac* in the anterior medial NB stays longer and overlaps the beginning of *ac* expression in the dorsal ectoderm which will give rise to the dorsal and lateral PNS (Figures 1H and 2B). In all cases, NBs turn off *ac* expression before they undergo

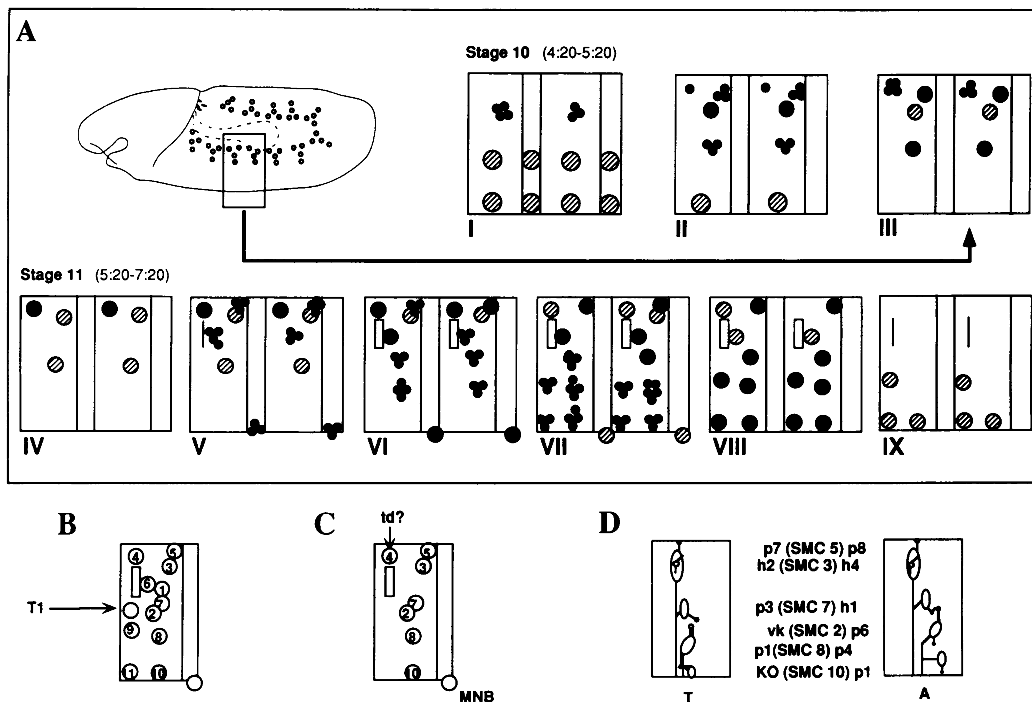

Fig. 3. *ac* expression in the PNS. **(A)** From stage 10 to 11, *ac* expression occurs mostly in the PNS neurogenic region. Each panel represents two consecutive hemisegments in the embryo, anterior is to the left and ventral to the bottom. Each hemisegment is subdivided into a larger anterior, and a smaller posterior compartment. Small black circles indicate epidermal cells and large circles subepidermal cells. The very large circles in panels I and II are neuroblasts. Striped circles indicate cells that contain low levels of *ac* RNA. The different stages of evolution of the *ac* expression pattern are numbered from I to IX. The embryo pictured in the upper left corner is at stage III. **(B)** Schematic representation of all *ac*-expressing subepidermal cells (SMC) in the PNS, and of the median neuroblast (MNB). The number in each SMC indicates their temporal sequence of appearance. The unlabelled circle shows the position of an additional *ac*-expressing SMC that is found only in segment T1. **(C)** The subset of SMCs that is formed when only *ac* and the surrounding regulatory region is present, in *Df(1)260-1/Dp(1)sc⁸* embryos. **(D)** The subset of external sense organs that is formed in the segments T2 and T3 (T pattern) and A1–A7 (A pattern) of *Df(1)260-1/Dp(1)sc⁸* embryos. The names of the different sense organs, and their tentative assignment to each *ac*-dependent SMC, is shown in the middle panel. The assignment of SMC 3 to h2 (h4 in the abdominal pattern) and of SMC 5 to p7 (p8 in the abdominal pattern) is confirmed by the tight correspondence found in *In(1)ac³* embryos between missing *ac* clusters and absence of SOs. Double labelling with anti-ac and anti-βgal antibodies (Figure 4A) identifies SMCs 1 and 2 with cells A and V in the A37 sequence (Ghysen and O'Kane, 1989). These two cells give rise to the two poly-innervated sense organs in each hemisegment: the lateral (lk) and ventral kölbchen (vk) in the thoracic segments, the hair h3 and the papilla p6 in the abdominal segments (Dambly-Chaudière *et al.*, 1992). The assignment of SMCs 7, 8 and 10 to p3 (h1 in the abdomen), p1 (p4 in the abdomen) and KO (p1 in the abdomen) is based on their position and consistent with the occasional absence of both the SMC and the assigned sense organ in the *ac* deficiency. The other *ac*-expressing SMCs (6, 9 and 11) probably give rise to *ase*-dependent sense organs.

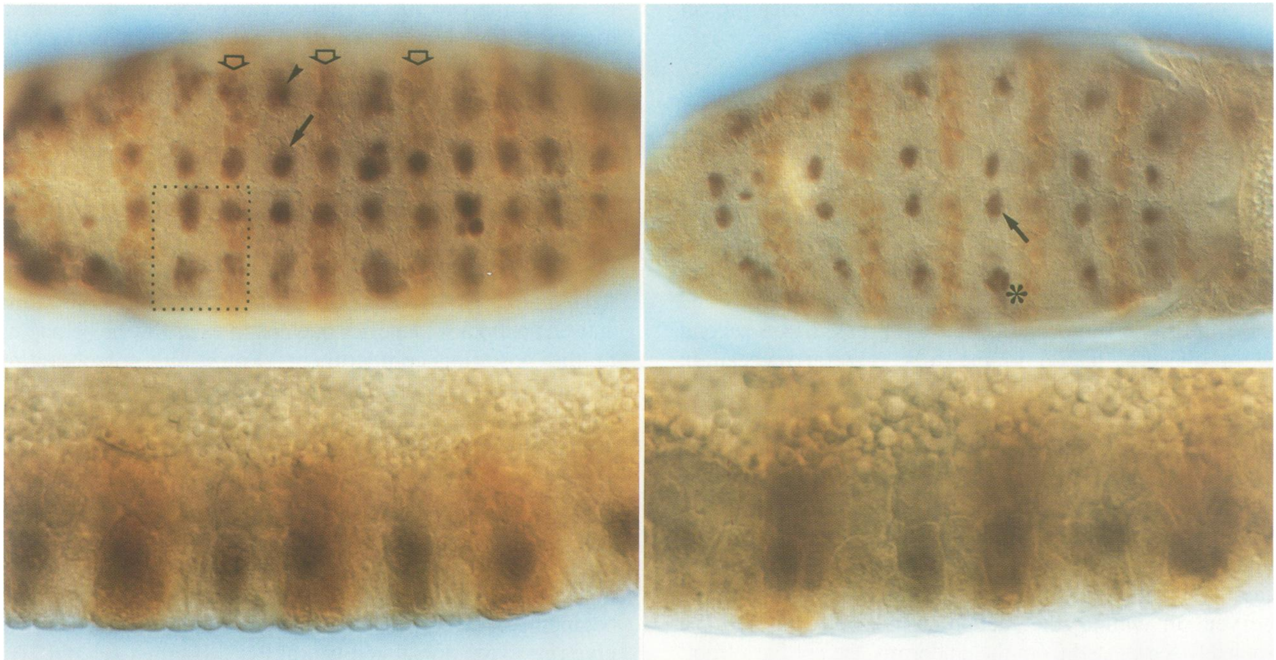

Fig. 2. Distribution of the *ac*, *sc* and *en* gene products in the ventral neurogenic region. Double labelling with anti-ac (dark brown) and anti-en (light brown) antibodies in wild type embryos. **(Upper left)** Four clusters express *ac* per hemisegment (dotted box). Two of the four clusters arise in the posterior compartment (stripe of *en*-expressing cells, large arrows). In most segments an NB has already segregated from each medial cluster (arrow), in a few cases an NB has also segregated from a lateral cluster (arrowhead). **(Upper right)** *ac* expression persists until stage 10 in the anterior medial NB. At this stage *ac* becomes expressed in more dorsal proneural clusters; these will produce the first SMCs of the PNS (asterisk). **(Bottom left)** and **(Bottom right)** Lateral view of the ventral region of a stage 9 embryo showing the similarity in the distribution of *ac* and *sc* gene products in the two medial NBs, in three consecutive segments. The posterior medial NB can be identified by its location at the level of the stripe of *en*-expressing cells.

their first division, as also happens in the precursors of the adult and larval PNS (Cubas *et al.*, 1991; Skeath and Carroll, 1991; this work).

At early stage 11, coinciding with the third wave of neuroblast segregation, there is a second round of *ac* expression in the CNS that includes several clusters per segment located in the midline. Double labelling with anti-ac and anti-en antibodies identifies one of these clusters as the one from which the median neuroblast (MNB) will segregate (not shown).

ac expression in the embryonic PNS

From the beginning of stage 10 until mid-stage 11, for a period of 2 h, *ac* is turned on in the lateral region of the embryonic ectoderm. During this time, the pattern of *ac* expression evolves rapidly. Eleven ectodermal clusters appear in a stereotyped sequence in the anterior compartment of each hemisegment, as is schematically represented in Figure 3A. At each location the expression follows the same sequence that was described above in the CNS, from a round cluster of ectodermal cells to the delaminated neural precursor. Here again, the time spent in each stage varies for the different positions. Double labelling with anti-ac and anti-βgal antibodies in the transformant line A37, where the SMCs express the *lacZ* gene, unequivocally identifies the *ac*-expressing delaminated cells as SMCs (Figure 4A).

The SMCs that derive from the first two clusters of *ac* expression, SMCs 1 and 2, are the precursors of the multiply innervated SOs (Dambly-Chaudière *et al.*, 1992). Not only are they the first to appear, but their progeny will also be the first to differentiate (Hartenstein, 1988; Bodmer *et al.*, 1989). The other *ac*-expressing SMCs appear in a dorsal to ventral sequence. Figure 3B shows a summary of all SMCs that express *ac* at one time or another during stages 10 and 11.

Although the final distribution of SOs differs in the thoracic and abdominal segments, we observed no difference in the patterns of *ac* expression of these segments, with the exception of one additional cluster in T1 (see Figure 3B). This cluster most probably corresponds to the SMC of papilla 4 (p4) of T1, which is *ac* dependent (Dambly-Chaudière and Ghysen, 1987), and has no homologue in the following segments.

Cis-regulation of embryonic ac expression

Most of the control sequences responsible for the adult pattern of *ac* expression are included in a 0.8 kb sequence upstream of the coding region (Ruiz-Gómez and Modolell, 1987; Martínez and Modolell, 1991). We have investigated whether the embryonic expression of *ac* depends on the same regulatory region, or whether it depends on several discrete sites extending over a larger region, as is the case for the adult *sc* pattern (Campuzano *et al.*, 1985; Leyns *et al.*, 1991). To answer this question, we have studied the modifications of the pattern of expression of *ac* in AS-C mutations whose alterations have been molecularly mapped. This is represented in Figure 5. The different phenotypes suggest that the complex pattern of *ac* expression results from the superimposition of several simpler patterns, each of which depends on a discrete control site.

The three most dorsal SMCs (SMCs 3, 4 and 5, open circles in Figure 5) are absent in sc^{3B} embryos where the distal-most part of AS-C is disconnected from the coding region of *ac* by the insertion of a gypsy transposable element.

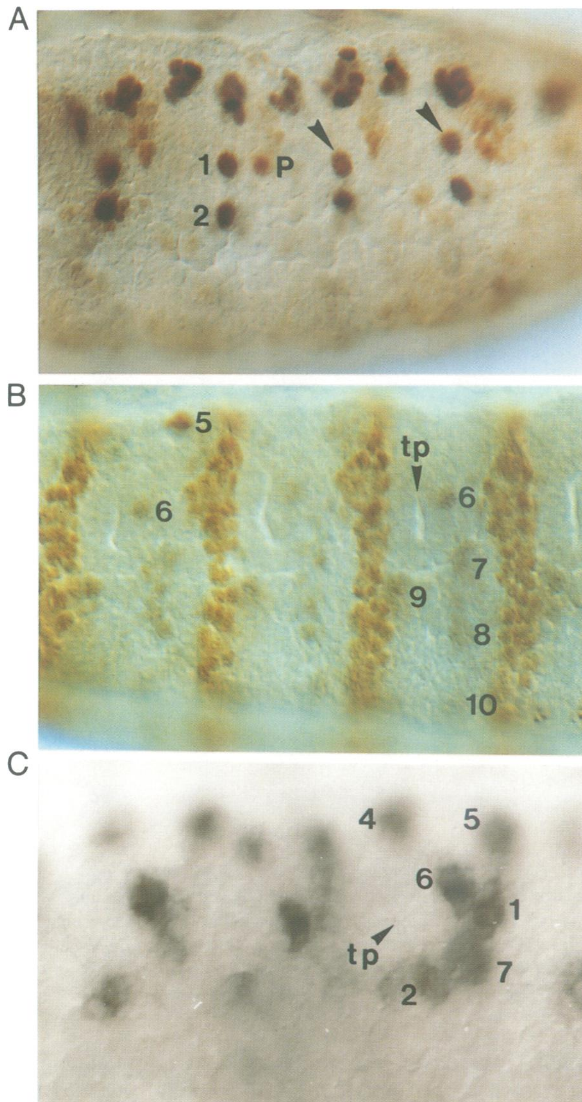

Fig. 4. (**A**) The cell where *ac* expression reaches a high level of expression will become a SMC, as monitored by *lacZ* expression in the A37 line. A37 embryos were double labelled with anti-ac and anti-βgal antibodies; black cells correspond to *ac*-expressing cells while brown cells express *lacZ*. Double labelling of individual cells is difficult to assess with the peroxidase method. It can be done, however, at a very early stage in the formation of the PNS (early stage II in Figure 3A), when one posterior cell expresses *lacZ* in each segment in the A37 line. At this stage the P cells, which form chordotonal organs, express *lacZ* but not *ac*. In some segments a second cell located just anterior to the P cell, begins to express *lacZ*. This cell, which was called the A cell, is identical to SMC 1. The next SMC to form, SMC 2, never expresses *lacZ* before the P cell has divided (Ghysen and O'Kane, 1989), but is already expressing *ac*. By comparing with the P cell (pure *lacZ* expression) and SMC 2 (pure *ac* expression) as internal controls, it is then possible to decide whether the SMC 1 is doubly labelled or not. In some cases there is no doubt that this cell expresses both *lacZ* and *ac* (arrows). (**B**) Pattern of expression of *sc* corresponding to stage VII of the expression of *ac* shown in Figure 3A. The anti-en labelling (brown stripes) helps define the position of the *sc*-expressing cells in the anterior compartment of each segment. The general distribution of *sc*-expressing clusters and SMCs is undistinguishable from that of *ac*-expressing cells, although we cannot say whether the two patterns are identical at the single cell level. (**C**) Pattern of expression of *ac* in the PNS of N^{55e11} neurogenic mutants. A stage V–VI embryo showing *ac* expression in the dorsal neurogenic region. Note that at each site several cells exhibit a high level of expression of *ac*. In panels B and C, cells are numbered as in Figure 3B. tp, tracheal pit.

This suggests that the expression of *ac* in the corresponding clusters depends on a site that is distal to the sc^{3B} breakpoint (site 1). These dorsal SMCs are present in Hw^1 embryos, suggesting that site 1 is located between the distal-most Hw^1 and the sc^{3B} insertions. It is possible, however, that site 1 is located to the left of the distal Hw^1 insertion, since it is unclear whether this insertion has any effect on the phenotype.

The formation of the two dorso-lateral SMCs (SMCs 1 and 6, stippled circles in Figure 5) is prevented when the continuity of the distal region is broken by the breakpoint ac^3, but not in sc^{3B} embryos, suggesting that a site necessary for the expression of *ac* in the corresponding region is located distal to the breakpoint ac^3 and proximal to the breakpoint sc^{3B} (site 2). Since the same dorsolateral SMCs are also absent when the more proximal part of AS-C is disconnected from the *ac* coding region by the sc^8 breakpoint, it seems likely that site 2 is a composite site comprising one element located between sc^{3B} and ac^3 and one element that is proximal to sc^8. The absence of SMCs 1 and 6 in Hw^1 embryos is probably due to the presence of the gypsy insertion between the proximal element of site 2 and the transcription unit.

SMC 7 (cross-hatched) is absent in Hw^1 embryos. This is not due to the distal insertion of Hw^1, since breakpoints closer to the coding region (sc^{3B} and ac^3) do not affect this cell, and therefore the effect of Hw^1 is probably due to the gypsy insertion. Since this SMC is present in sc^8 embryos, the corresponding site (site 4) should be located between the gypsy insertion of Hw^1 and the sc^8 breakpoint.

The anterior ventral SMCs (SMCs 9 and 11, black circles) do not form in sc^8 embryos, indicating that the corresponding control site (site 5) is proximal to this breakpoint. The location of this site has been further defined by the observation that the anterior ventral SMCs are absent in sc^{S1} but not in sc^4 embryos. This indicates that site 5 must be located between the breakpoints of sc^{S1} and sc^4 (proximal to the former but distal to the latter), that is, at a distance of >30 kb from the *ac* coding region. The existence of site 5 has been directly confirmed by observing the expression of *lacZ* in the two anterior–ventral SMCs in a transformant line where 3.7 kb of DNA from the region between the breakpoints of sc^{S1} and sc^4 have been fused to the hsp promoter and the *lacZ* gene (J.L.Gómez-Skarmeta, D.Beamonte, I.Rodriguez and J.Modolell, unpublished result).

Finally, the posterior ventral SMCs (SMCs 2, 8 and 10, striped circles) are present in the Hw^{1R1} mutant, where sites 2 and 5 are disconnected from the coding region by the gypsy insertion of Hw^1, and where sites 1 and 2 are disconnected by the breakpoint associated with the second mutation of Hw^{1R1}. They are also present in ac^3 embryos, suggesting that these SMCs depend on a site (site 3) located between the ac^3 breakpoint and the gypsy insertion of Hw^1, that is, closely associated to the transcribed region.

The results show that the complete pattern of expression of *ac* during embryogenesis depends on a complex regulatory region spanning ~35 kb and extending upstream and downstream of the *ac* transcription unit. This region contains discrete sites, each of which regulates the expression of ac at one position of the ectoderm. The positions of the clusters that depend on each site give us some clues about the nature of the regulatory signals. For example, the three clusters

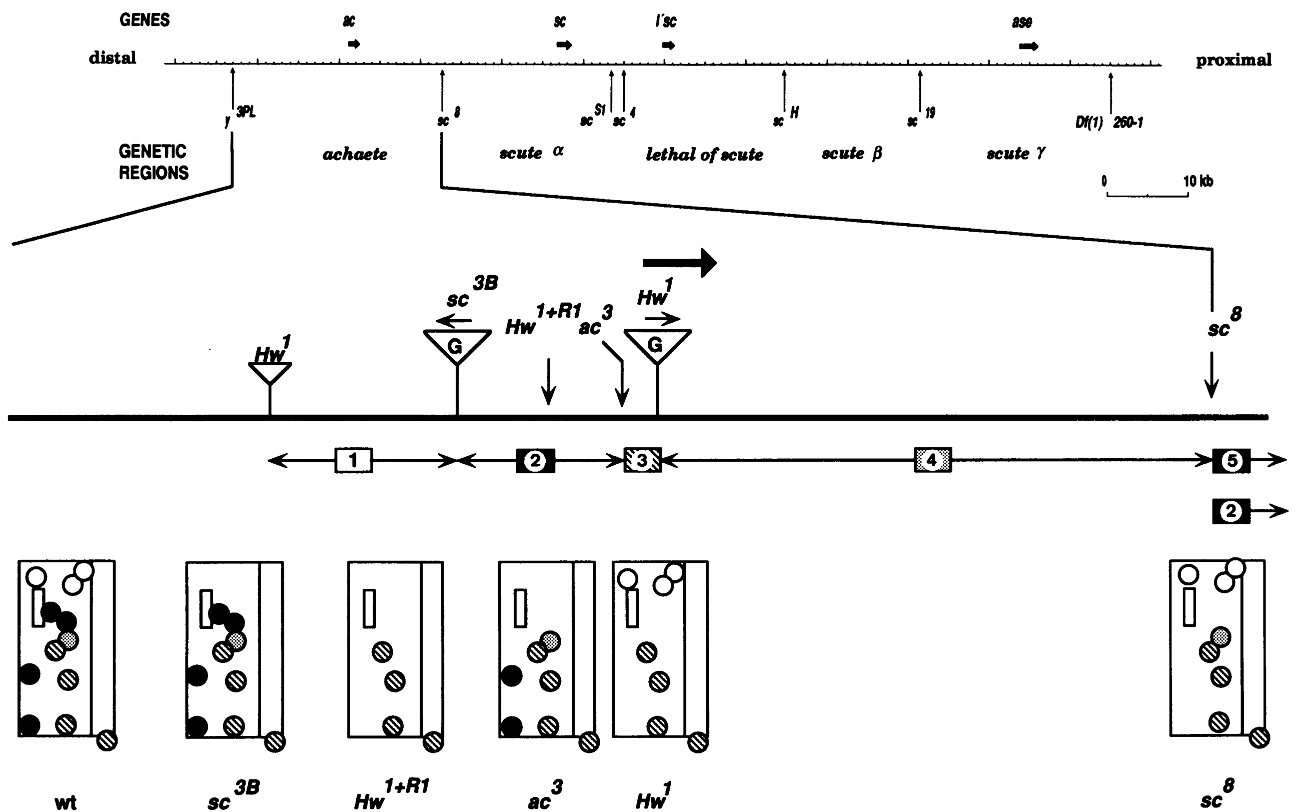

Fig. 5. The effect of different *ac* mutations on the embryonic pattern of expression of *ac* reveals the existence of discrete control sites in the regulatory region. The upper part represents the AS-C DNA. The different breakpoints of AS-C mutations, used to subdivide the complex into five genetic regions (*ac*, *scα*, *l'sc*, *scβ* and *scγ*), are indicated. In the middle the *ac* region has been amplified. The *ac* transcription unit is represented by the thick horizontal arrow, breakpoints by vertical arrows and insertions by triangles. The G marks the *gypsy* transposable element. Below the AS-C DNA, the numbered rectangles represent the different regulatory sites. In the lower part the subepidermal cells that express *ac* in the wild type and in different mutations are indicated. The different phenotypes reveal the existence of five discrete control sites (see text for details).

that depend on site 1 form in the most dorsal region of the hemisegment, and the three clusters that remain in Hw^{1+R1} are all at the same antero-posterior position, suggesting that the expression of *ac* in the embryo could be regulated by the genes that define the embryonic A−P and D−V axes. Direct confirmation of this hypothesis has been recently obtained in the case of the expression of *ac* in the anteriormost neuroblasts (Skeath *et al.*, 1992).

Co-expression of ac, sc and l'sc in the CNS

It has been reported that in the embryonic neurogenic region the *ac*, *sc* and *l'sc* genes are expressed in partially overlapping patterns (Cabrera *et al.*, 1987; Romani *et al.*, 1987). This led to the proposal that the different combinations of AS-C gene products present in each NB contribute to establish its identity (Cabrera *et al.*, 1987). We have analysed the expression of *sc* during embryogenesis and compared it with that of *ac*. The two genes have indistinguishable patterns of expression in the neurogenic region prior to the segregation of the NBs. The only difference is that the level of expression of *sc* is significantly lower than that of *ac*. Double labelling using anti-sc and anti-en antibodies corroborates the RNA data (Figure 2C and D). We also compared the expression of the two genes in the dorsal and lateral ectoderm at later stages, when the SMCs are formed. Here again, the expression of *sc* is weaker than that of *ac*. Besides this quantitative difference we did not detect any variation between the spatial and temporal pattern of expression of *sc* and of *ac*, either at the level of the transcripts (not shown) or at the level of the proteins (Figure 4B, compare with panel VII in Figure 3). The observation that *ac* and *sc* are expressed in the same cells suggests that *sc* may contribute to some extent to the formation of the *ac*-dependent SOs. This would explain why the SOs that are formed when *ac* alone is present have a higher frequency of appearance when *sc* (and/or *l'sc*) are also present (Dambly-Chaudière and Ghysen, 1987).

As a further test of the idea that the combination of AS-C genes expressed in a neural precursor might specify its fate, we examined in more detail the median neuroblast (MNB). The MNB expresses *ac* and *sc* (data not shown) and *l'sc* (F.Jiménez, personal communication), and is absent in the $Df(1)sc^{19}$ which eliminates the *ac*, *sc* and *l'sc* genes (Cabrera *et al.*, 1987). We observed that the MNB is present and correctly expresses *en* in $Df(1)260\text{-}1/Dp(1)sc^{8}$ embryos, where *ac* is the only AS-C gene present.

Trans-regulation of ac expression by other AS-C genes

Trans-activation of *ac* by *sc*, and of *sc* by *ac*, has been demonstrated in the wing disc, and is responsible for the final identity in the patterns of expression of the two genes (Martínez and Modolell, 1991). We investigated whether similar *trans*-activations take place during embryogenesis,

and if so whether they are responsible for the identity of the patterns of expression of *ac* and *sc* reported above.

We have used the HSSC 2-3 transformant line, in which *sc* expression is directed by the inducible hsp-70 promoter in all the cells (Rodríguez *et al.*, 1990). *sc* expression was induced in the early embryo by heat shock pulses at 37°C for 30 or 60 min. After the heat shock treatments, *ac* expression, as revealed by an anti-ac antibody, was present in all embryonic cells, indicating *trans*-activation of *ac* by the *sc* gene product (data not shown).

We then investigated the possibility that the final pattern of *ac* expression results, at least in part, from cross-activation by other AS-C gene products. We examined the expression of *ac* in the absence of the *sc*, *l'sc* or *ase* genes. No evident effects were seen when either *l'sc* or *ase* were deleted (in $In(1)sc^{4L}sc^{9R}$ and sc^2 mutants respectively), or in the rearrangements $In(1)sc^4$ and $In(1)sc^9$ used to construct the *l'sc* deficiency (small effects on the global amounts of *ac* RNA cannot be ruled out, due to the limitations of the detection method). The sc^- ($In(1)sc^{8L}sc^{4R}$) condition promoted an altered pattern of *ac* expression. This, however, is due to an effect of the sc^8 breakpoint on the *cis*-regulation of *ac* and not to the removal of *sc*, since the alteration observed in $In(1)sc^{8L}sc^{4R}$ is the same as that described for $In(1)sc^8$, where *sc* is present (Figure 5). Final proof that the product of the *sc* gene is not required to set up the normal *ac* pattern was obtained by using the Hw^{49c+R5} mutant, which carries an 8 bp deletion in the *sc* sequence. This mutation creates a frameshift that results in an inactive sc truncated product (Balcells *et al.*, 1988). Since the *ac* expression in Hw^{49c+R5} embryos is not altered, this result together with the previous ones indicates that the pattern of *ac* expression is not controlled by other members of the AS-C.

Regulation of ac PNS expression by the neurogenic gene Notch

Lack of function mutations at the neurogenic loci affect the development of the embryonic PNS, giving rise to a hyperplasic PNS containing three to four more cells than in the wild type (Hartenstein and Campos-Ortega, 1986). The effect of neurogenic mutations on the generation of embryonic SMCs has been reported recently (Goriely *et al.*, 1991). In *Notch* (*N*) neurogenic mutants more SMCs develop in each position, but neither their location nor their temporal sequence were affected. It has been reported, however, that the *ac* domains of expression are enlarged in *N* mutant embryos (Brand and Campos-Ortega, 1989). In order to study the role of neurogenic genes on the formation and evolution of the *ac* clusters we have looked at the *ac* expression pattern in embryos hemizygous for N^{55e11}, an *N* null allele.

We found that the position and size of *ac* proneural clusters in the dorsal PNS is not modified. However, *ac* expression is maintained in most or all cells of the clusters for a longer period of time than in the wild type, and consequently more clusters coexist in one embryo, giving rise to images very similar to the one presented as a summary in Figure 3 (see Figure 4C). Within the resolution of our analysis, the results show that *N* plays no detectable role in the emergence of the proneural clusters, but is involved in limiting the number of these cells that differentiate as SMCs. The same conclusion has been reached in the case of the expression of *ac* in the ventral region of neurogenic mutants (Cabrera, 1990; Skeath and Carroll, 1992).

Correspondence between ac proneural clusters and larval sense organs

In order to facilitate the assignment of each proneural cluster to a particular sense organ of the larva, we concentrated on the analysis of the region of AS-C distal to the breakpoint sc^8 (Figure 5). The presence of this region in *Df(1)260-1*/$Dpsc^8$ embryos is sufficient to promote the development of the subset of larval sense organs that are *ac*-dependent (Dambly-Chaudière and Ghysen, 1987). The pattern of expression of *ac* in *Df(1)260-1*/$Dpsc^8$ embryos is shown in Figure 3C. In this genetic combination, the number of *ac* clusters which appear is smaller than in wild type embryos (compare Figure 3C and B) and corresponds approximately to the number of *ac*-dependent SOs. We have taken advantage of this simplified pattern, and have also relied on data obtained from the analysis of *ac* expression and final pattern of SOs in *ac* mutants, to establish the correlation between *ac*-expressing clusters and ac-dependent SOs. The resulting assignment is presented in Figure 3D.

Discussion

The wild type pattern

We have examined at the cellular level the pattern of expression of *ac* during embryogenesis. Our results reveal that the expression of *ac* is highly dynamic and follows a precise spatial and temporal pattern.

The initial expression of *ac* occurs in clusters of ectodermal cells that occupy very reproducible positions in each segment. The sequence of appearance of the different clusters is completely reproducible, thus providing a direct cause for the observation that the emergence of the sensory mother cells follows a highly reproducible sequence (Ghysen and O'Kane, 1989). There are variations between clusters: some of them appear to keep on expressing *ac* for longer periods than others. Likewise, the time that *ac* remains expressed in the progenitor cell depends on the position. Whether these reproducible differences play any role in defining the subsequent fate of the progenitor cells is not known.

The spatial and temporal sequence of *ac* expression within a cluster is also reproducible: first the expression is turned on in several adjacent cells, then one cell, usually at the centre of the cluster, expresses *ac* at a higher level and begins to delaminate. The expression in the delaminated mother cell is maintained for a determined period of time. These two major stages of *ac* expression are common to embryos and discs: an early stage characterized by the expression in clusters of cells according to a well defined spatial and temporal pattern, followed in a later stage by a transient overexpression in one cell of the cluster, the progenitor cell. For reasons that will become apparent in the next sections, we will call these two stages, and the corresponding levels of AS-C expression, the 'competent' and the 'determined' stages and levels.

Cis-regulation of ac expression at the 'competent' stage

Our results suggest that the expression of *ac* during embryogenesis depends on an array of *cis*-regulatory sites that would activate *ac* in clusters of cells at particular

positions in the embryonic ectoderm. Since relatively few *ac* rearrangements are available, we cannot pinpoint the different sites with the same accuracy as in the case of the *sc* regulatory sites (Ruiz-Gómez and Modolell, 1987). The available data demonstrate, however, that different breakpoints affect specifically different clusters and that the putative regulatory sites are dispersed upstream and downstream of the coding region.

Trans-regulation by other AS-C genes

We observed that the ectopic expression of *sc* induces an ectopic expression of *ac*, demonstrating that *sc* is capable of *trans*-activating *ac* in the embryo as it does in the wing imaginal discs (Martínez and Modolell, 1991; Skeath and Carroll, 1991). On the other hand, the pattern of expression of *ac* is not detectably modified in embryos that are deficient for either *sc*, *l'sc* or *ase*. We conclude that, in the embryo, *trans*-activation by AS-C products plays no role in setting up the pattern of expression of *ac*. In the wing disc, the clusters where *ac* is expressed due to *trans*-activation by *sc* correspond to sense organs that develop normally in the absence of *ac*, suggesting that *ac* has no role in the corresponding mother cells. Thus, we think that, in most cases, *trans*-activation of AS-C genes by each other is not necessary for the formation of the SMCs. Given the high homology between the different AS-C genes, it is possible that *trans*-activation simply reflects the self-activation of each gene by its own product (Martínez and Modolell, 1991). Self-activation, on the other hand, might be instrumental in shifting the level of expression of AS-C from the lower, 'competent' level to the higher, 'determined' level.

Trans-regulation by Notch and the 'determined' stage

The inactivation of *N*, or of any of several genes collectively called 'neurogenic', results in a hypertrophy of the neural tissue at the expense of the epidermis (Lehmann *et al.*, 1983). In particular, the *ac*-dependent progenitor cells for the embryonic PNS are formed in large excess in all neurogenic mutants (Goriely *et al.*, 1991). The present data demonstrate that the initial expression of *ac* in clusters is completely normal in N^- embryos, both for the clusters that will produce NBs and for those that will generate SMCs. The late expression is abnormal in that most or all cells of the clusters shift to, and stay at, the high level of expression that is normally observed only in the progenitor cell.

We conclude that the function of *N*, in this particular process, is to restrict to one cell of the cluster the access to a high level of AS-C expression. This restriction might be achieved by a competitive system that would amplify minor differences in *ac* levels between neighbouring cells (de Celis *et al.*, 1991; Goriely *et al.*, 1991; Heitzler and Simpson, 1991), until one cell reaches the high level of *ac* expression that makes the transition from the 'competent' to the 'determined' level.

Functional redundancy of AS-C genes

The study of the CNS and PNS phenotypes of partial deficiences of the AS-C has revealed that AS-C genes can to a large extent substitute for each other in the formation of neural progenitor cells. Thus, there are many examples of mutual substitutions between *ac*, *sc* and *l'sc* both in the CNS (Jiménez and Campos-Ortega, 1979), in the embryonic PNS (Dambly-Chaudière and Ghysen, 1987), and in the adult PNS (Balcells *et al.*, 1988; Held, 1990; Rodríguez *et al.*, 1990). The observation that the absence of one of the AS-C genes can often be partially rescued by the others suggests that these genes are functionally equivalent.

It might be that the different AS-C genes are equivalent only to the extent that they can all promote the formation of a progenitor cell, but that they differ in the specificity that they confer to that cell. However, our results make this hypothesis somewhat unlikely in the case of the *ac*-dependent progenitor cells. First, we observed that most or all SMCs that express *ac* also express *sc*, as was also observed in the adult PNS (Romani *et al.*, 1989; Cubas *et al.*, 1991; Martínez and Modolell, 1991; Skeath and Carroll, 1991). Second, we observed that in wild type condition the first mother cell to be formed during embryogenesis, SMC 1, expresses *ac* and *sc*. Yet we know that this SMC is correctly specified (to the extent that it will give rise to the correct poly-innervated SO, Dambly-Chaudière and Ghysen, 1987) by the expression of *ase*, irrespective of the presence or absence of the other AS-C genes. Third, we detected the formation of an *en*-expressing median neuroblast when only *ac* is expressed, though in wild type the proneural cluster from which this NB arises also expresses *sc* and *l'sc* (Martin-Bermudo *et al.*, 1991). It appears, therefore, that the fate of a particular progenitor cell depends on the position, and possibly the time at which it is formed, but not on the particular combination of AS-C genes that it expresses.

The two aspects of gene function

If the different AS-C products are functionally equivalent, yet the formation of different sense organs depends on the activity of different AS-C genes, then one might consider that the real difference between the AS-C genes lies in the battery of *cis*-regulatory elements in which they are embedded. In the case of complex loci like the AS-C, the interspersion of coding sequences and of control sites allows not only different control sites to act on the same coding sequence, but also one control site to act on different coding sequences (Skeath *et al.*, 1992). This may explain many of the puzzling aspects of AS-C expression and requirements.

For example, we have shown that *ac* is expressed in more clusters than those where it is required (Figure 3B and C). This 'dispensable' expression is abolished if the continuity of AS-C is broken by the breakpoint sc^8, indicating that it depends on control sites that are proximal to the breakpoint and probably act mostly on proximal AS-C genes. This suggests that the expression of *ac* in these clusters is driven by control regions that are located far away and reflects long-range effects of the *cis*-regulation of other AS-C genes. On the other hand, all the sense organs that were genetically characterized as *ac*-dependent correspond to clusters that are formed under the control of sites that are distal to sc^8, and therefore close to the *ac* coding region (see Figure 5).

It is also likely that control sites close to *ac* act on *sc* and possibly *l'sc*. This would explain the observation that *sc* is expressed in the same cells that express *ac*, though at a lower level. In some *ac*-dependent clusters the simultaneous expression of *sc* (and *l'sc*) may have an additive effect, explaining why the frequency with which the corresponding SOs are formed when only *ac* is present (in *Df(1)260-1/Dpsc*^{8} embryos) is <1, and increases when *sc* or/and *l'sc* are also present (Dambly-Chaudière and Ghysen, 1987).

Another revealing case is that of the sense organs that

depend on the $sc\beta$ region. This region does not contain any of the AS-C structural genes, yet a group of sense organs is formed whenever $sc\beta$ is present, i.e. both in sc^{19L} and in sc^{HR}, but not in sc^{HL} or in sc^{19R} (see Dambly-Chaudière and Ghysen, 1987 and Figure 5). It appears, therefore, that these sense organs depend on regulatory sites present in the $sc\beta$ region and which may act indifferently on *ase* (in sc^{HR}) or on *l'sc*, *sc* and/or *ac* (in sc^{19L}).

A third interesting case is that of the four NBs that express *ac* in the first wave of segregation. All four NBs also express *sc* and three of them *l'sc*. The available data suggest that the same control regions of AS-C direct the expression of the three genes. The control region that leads to AS-C expression in the anterior NBs lies distal to the sc^8 breakpoint, close to the *ac* coding region, while the control region for the posterior NBs is proximal to the sc^8 breakpoint (data not shown and M.D.Martín-Bermudo, personal communication). These assignments are entirely consistent with results based on the analysis of AS-C−*lacZ* constructs and showing that one AS-C region distal to the sc^8 breakpoint drives the expression of both *ac* and *sc* in the anterior NBs, while another region proximal to the sc^8 breakpoint controls the expression of the two genes in the posterior NBs (Skeath *et al.*, 1992). The 'anterior NB' regulatory region, possibly due to its location far apart from the *l'sc* gene, promotes the expression of *l'sc* at detectable levels only in the lateral NB.

Conclusion

The analysis of the data reported here, and a comparison with other data on the expression and function of the AS-C genes, leads us to propose that the formation of individual progenitor cells in the embryonic PNS depends on specific regulatory sites, each of which acts through the nearest AS-C gene(s). In this view, to say that a particular AS-C gene is required for the formation of a particular progenitor cell means that no other AS-C gene is properly expressed in the same position in the absence of this particular gene, and inversely, to say that a gene is not required means that at least one other gene is sufficiently expressed in this region.

Why this peculiar arrangement evolved is a matter of conjecture. One possibility is that the trend towards the production of a completely defined and reproducible pattern favoured the independent positioning of each sense organ, which itself necessitated a substantial if progressive increase in the size of the regulatory region. The distance at which a regulatory sequence can act is presumably not without limits, and therefore the increase in size of the regulatory region would itself be limited unless it was accompanied by an increase in the number of coding sequences.

Materials and methods

Drosophila stocks

Most of the *Drosophila* strains used in this work are from A.García-Bellido's collection. Several experiments were performed using *lacZ* labelled balancer chromosomes (kindly provided by Y.Hiromi) to reliably detect the mutant embryos.

Antibodies

Antibodies against ac and sc products were kindly provided by J.Skeath and S.Carroll (Skeath and Carroll, 1991). The anti-en mAb has been described previously (DiNardo *et al.*, 1985).

Immunocytochemistry

Embryos were dechorionated, fixed and their vitelline envelopes removed as described by Goriely *et al.* (1991). After removing the vitelline envelopes embryos were rinsed three times with fresh methanol and used immediately. The embryos were rehydrated in PBS:methanol (1:1), rinsed three times in PBT (0.3% Triton X-100 in PBS), blocked in PBT containing 10 mg/ml of bovine serum albumin (PBTB) for 30 min at room temperature and incubated overnight at 4°C in the first primary antibody (anti-ac antibody diluted 1:100 in PBTB). After several washes in PBT, the embryos were incubated with the appropriate biotinylated secondary antibody (Amersham) for 2 h, washed and stained using the Vectastain Elite ABC kit (Vectorlabs) and diaminobenzidine. In the case of double labelling with anti-en or anti-βgal antibodies, nickel and cobalt ions were added to the staining reaction as described in Adams (1981). The embryos were then washed and successively incubated with the second primary antibody and the second biotinylated secondary antibody as described above. No ions were added to the second staining reaction. Finally, embryos were dehydrated and mounted in Canada balsam or embedded in Epon.

Other methods

Whole mount *in situ* hybridizations with digoxigenin-labelled probes (Boehringer Mannheim Biochemicals) were performed as described by Tautz and Pfeifle (1989), with minor modifications. Larvae were prepared for microscopic examination of the external sense organs as described in Dambly-Chaudière and Ghysen (1986). Photographs were taken with a Zeiss Axiophot microscope.

Acknowledgements

We are most grateful to J.Modolell, in whose laboratory part of this work have been done, to members of his laboratory and to J.F.de Celis for their encouragement and stimulating discussions. We thank J.Skeath and S.Carroll for providing antibodies, C.Dambly-Chaudière, J.L.Gómez-Skarmeta, A.Goriely, F.Jiménez and M.D.Martín-Bermudo for communicating unpublished results; J.A.Campos-Ortega, S.Campuzano, C.Dambly-Chaudière, J.F.de Celis, Y.N.Jan and J.Modolell for comments on the manuscript and P.Aganzo for help in the computer drawing of figures. M.Ruiz-Gómez was a postdoctoral fellow of the Consejo Superior de Investigaciones Científicas. A.Ghysen is maître de recherches of the Fonds National de la Recherche Scientifique, Belgium. This work was supported by grants from the Communauté française de Belgique and the Human Frontier Science Programme Organization to A.G., from Dirección General de Investigación Científica y Técnica (PB87-0433-C02-01 and PB90-0085) and Comunidad Autónoma de Madrid to J.Modolell and from Fundación Ramón Areces to the Centro de Biología Molecular Severo Ochoa.

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Received on November 9, 1992; revised on December 8, 1992