Spatial regulation of proneural gene activity: auto- and cross-activation of *achaete* is antagonized by *extramacrochaetae*

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The spatially restricted activities of *achaete* (*ac*) and *scute* (*sc*) are thought to define proneural clusters of potential sensory organ precursor cells in the imaginal discs of *Drosophila*. These genes encode transcriptional regulators of the basic helix–loop–helix (bHLH) class. We have found that direct, positive transcriptional autoregulation by the *ac* protein and cross-regulation by *sc* are essential for high-level expression of the *ac* promoter in the proneural cluster pattern and that autoactivation is important for the bristle-promoting function of the *ac* gene. These auto- and cross-regulatory activities are antagonized in a dose-dependent manner by the inhibitory HLH protein encoded by the *extramacrochaetae* (*emc*) gene. We have found that *emc* is expressed in the wing imaginal disc in a pattern strongly complementary to that of the proneural clusters. Our results indicate that *emc* plays an essential early role in defining territories of bristle-forming potential.

[**Key Words:** *Drosophila*; neurogenesis; sensory organ development; transcriptional autoregulation; helix–loop–helix proteins; protein–protein interaction; pattern formation]

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The various types of sensory organs, or sensilla, that constitute the adult peripheral nervous system (PNS) of *Drosophila* are distributed on the body surface in a largely invariant pattern, the control of which offers a complex problem in the mechanisms of postembryonic pattern formation. Adult sensory organs develop during the late larval and early pupal stages within the imaginal discs and histoblast nests, epithelial sheets that give rise to the cuticular structures of the fly. Each sensillum is a multicellular sensory unit. For example, the mechanosensory bristles, typified by the macrochaetes and microchaetes of the head and thorax, are composed of a single bipolar neuron and three non-neuronal accessory cells. These four cells are the progeny, via a fixed lineage, of a single sensory organ precursor (SOP) cell (Hartenstein and Posakony 1989).

Genetic and molecular studies have identified two principal steps in the development of most adult sensory organs. In the first step, the localized activity of two genes, *achaete* (*ac*) and *scute* (*sc*), appears to confer on "proneural clusters" of cells in the imaginal disc the potential to become SOPs (Cubas et al. 1991; Skeath and Carroll 1991). Then, inhibitory cell–cell interactions mediated by the neurogenic class of genes restrict the expression of the SOP cell fate in each proneural cluster to a single cell. This cell comes to accumulate the highest amount of *ac* and *sc* protein and soon divides to give rise to an individual sensory organ; the remaining members

of the proneural cluster become epidermal cells (Dietrich and Campos-Ortega 1984; Hartenstein and Posakony 1990; Simpson 1990; Cubas et al. 1991; Skeath and Carroll 1991). Thus, the mechanisms responsible for the spatially restricted activity of *ac* and *sc* in proneural clusters in the imaginal disc play an essential role in establishing the spatial pattern of sensory organs in the adult fly.

Both the *ac* and *sc* genes encode proteins of the basic helix–loop–helix (bHLH) class (Villares and Cabrera 1987; Alonso and Cabrera 1988; Murre et al. 1989) that are capable of sequence-specific DNA binding in vitro (Cabrera and Alonso 1991; Van Doren et al. 1991). The SOP cell fate also requires the activity of another, ubiquitously expressed bHLH protein that is the product of the *daughterless* (*da*) gene (Caudy et al. 1988a,b; Cronmiller et al. 1988; Cline 1989; Ghysen and O'Kane 1989). In vitro, the intact *ac* and *sc* proteins exhibit DNA-binding activity only when they are part of hetero-oligomeric complexes with *da* (Cabrera and Alonso 1991; Van Doren et al. 1991). The sensillum-promoting activities of the *ac*, *sc*, and *da* genes are antagonized by the negative regulators *extramacrochaetae* (*emc*) and *hairy* (*h*). In *emc* and *h* mutants, the *ac* and *sc* proteins are expressed ectopically in the wing imaginal disc (Cubas et al. 1991; Skeath and Carroll 1991), and sensory organs appear at ectopic sites in the adult (Moscoso del Prado and Garcia-Bellido 1984; Lindsley and Zimm 1992). The *emc* and *h* genes also encode HLH proteins (Rushlow et al. 1989;

Ellis et al. 1990; Garrell and Modolell 199). In particular, the *emc* protein contains the HLH dimerization domain characteristic of the HLH family but lacks the conserved basic region that constitutes the DNA-binding domain of the bHLH proteins, suggesting that its activity as a negative regulator is likely to depend on protein–protein, and not protein–DNA, interactions (Ellis et al. 1990; Garrell and Modolell 1990).

Recent evidence suggests that the *ac* and *sc* promoters are initially activated in the wing imaginal disc at distinct sites and that cross-activation between the two genes causes them to be expressed in the same final pattern (Martínez and Modolell 1991; Skeath and Carroll 1991). In addition, it has been shown that expression of the *ac* and *sc* promoters in the proneural clusters requires the activity of the respective endogenous genes (Martínez and Modolell 1991). That the *ac* and *sc* proteins could act directly as transcriptional regulators of the *ac* gene was strongly suggested by the finding that the *ac* promoter contains three E-box (CANNTG) sites that are bound specifically in vitro by *da/ac* and *da/sc* hetero-oligomeric protein complexes (Van Doren et al. 1991). It was demonstrated further that the *emc* protein is capable of specifically antagonizing this DNA-binding activity in a dose-dependent manner, suggesting that it acts at least in part as an inhibitor of *ac* and *sc* auto- and cross-regulation (Van Doren et al. 1991).

In this study we have investigated the specific functions of the *ac*, *sc*, and *emc* proteins that underlie their

control of adult sensory organ patterning. We have found that *ac* and *sc*, when combined with *da*, can directly activate the *ac* promoter. This direct transcriptional auto- and cross-regulation is essential for the correct expression of *ac* in proneural clusters and for its bristle-promoting function. We have also found that the expression pattern of *emc* in the wing imaginal disc is largely complementary to that of *ac* and that *emc* can interfere with *ac*- and *sc*-dependent activation of the *ac* promoter. We conclude that the *emc* protein plays an important early role in bristle patterning in part by post-translationally inhibiting the auto- and cross-regulatory activities of *ac* and *sc* in specific regions of the wing imaginal disc.

Results

Transcriptional activation of the ac promoter

We tested the ability of the *da*, *ac*, and *sc* proteins to activate transcription of the *ac* promoter in a cotransfection assay in *Drosophila* tissue culture cells. The reporter gene consisted of a 0.9-kb *ac* promoter fragment (Fig. 1A), containing the three previously identified *da/ac-sc* DNA-binding sites (T5E1–T5E3; Van Doren et al. 1991), fused to the coding sequence of the bacterial chloramphenicol acetyltransferase (CAT) gene. The level of expression of this construct was measured either alone or in the presence of expression vectors containing *da*,

Figure 1. *ac* and *sc*, in combination with *da*, are potent activators of the *ac* promoter. (A) Diagram showing the upstream region of the *ac* gene used to construct a promoter–CAT reporter gene. Previously identified E-box binding sites for *da/ac-sc* protein complexes (T5E1–T5E3) are indicated (Van Doren et al. 1991). In the mutant reporter construct, the E box (CANNTG) sequence at all three sites was mutated (to AANNTT). (B,C) Pooled data from five (B) and at least four (C) independent tissue culture cotransfection experiments testing the ability of the *ac*, *sc*, and *da* expression vectors, individually and in combination, to elevate the expression of the *ac*–CAT reporter gene over the level given by the reporter alone (control). Solid bars represent data for the wild-type *ac* promoter; shaded bars show data for the mutant promoter. Standard errors are indicated.

ac, or sc protein-coding sequences under the control of the constitutive actin 5C promoter. The results are shown in Figure 1, B and C. Very little activation of the ac-CAT reporter gene above basal levels (two- to four-fold) is observed when the cells are cotransfected with the ac or sc expression vectors. Cotransfection of a da expression vector reproducibly gives an 8- to 10-fold activation of ac promoter expression. However, the highest levels of reporter gene activation, >100-fold, are observed when a combination of the ac and da, or the sc and da, expression vectors are present in the transfection mixture. In contrast, a second ac-CAT reporter construct, identical to the first except for the introduction of two base pair mutations in each of the three da/ac-sc binding sites, fails to show significant activation under any of these conditions (Fig. 1B,C). This result strongly suggests that the ac and sc proteins act directly through the E-box binding sites to regulate the ac promoter.

The relative activities of the da, ac, and sc proteins, individually and in combination, as activators of ac transcription (Fig. 1) closely parallel their relative in vitro DNA-binding activities on the T5E1–T5E3 sites (Van Doren et al. 1991). The ac and sc proteins alone fail to bind to these sites, a weak binding activity of da is observed on the T5E1 site only, and the highest binding activity on all three sites is exhibited by hetero-oligomeric complexes of da and ac or da and sc. Thus, the relative potency of the da + ac and da + sc combinations in activating the expression of the ac reporter is very likely to reflect the formation and function of da/ac and da/sc protein complexes in the transfected cells.

These experiments indicate that the ac and sc proteins, in combination with the da protein, are capable of high-level, direct transcriptional auto- and cross-activation of the ac gene.

Proper expression of an ac promoter-lacZ fusion gene in transgenic flies requires da/ac-sc DNA-binding sites

The same 0.9-kb ac promoter fragment used for the cotransfection studies [see Fig. 1A] directs expression of an *Escherichia coli lacZ* reporter gene in the wing imaginal disc in a pattern that resembles the normal pattern of ac protein expression (Fig. 2A; Cubas et al. 1991; Martínez and Modolell 1991; Skeath and Carroll 1991). Moreover, we have verified that the spatial pattern of lacZ transcript accumulation from this ac-lacZ reporter in the wing disc is qualitatively similar to the distribution of the endogenous ac transcript (P.A. Powell, unpubl.). High-level expression of this reporter gene in the proneural cluster pattern is abolished in the *sc*¹⁰⁻¹ genetic background (Fig. 2B; Martínez and Modolell 1991). The *sc*¹⁰⁻¹ mutation severely reduces or inactivates the function of both the ac and sc genes (García-Bellido 1979; Campuzano et al. 1985; Villares and Cabrera 1987), and adult male flies of the genotype *sc*¹⁰⁻¹/Y completely lack sensory bristles on the thorax and head. Thus, the normal activity of the ac promoter in the wing disc is dependent on the function of the

endogenous ac and sc genes (Martínez and Modolell 1991).

Because the cotransfection experiments indicated that da/ac- and da/sc-dependent activation of the ac promoter in cultured cells requires the integrity of the three E-box binding sites within the 0.9-kb fragment (Fig. 1B,C), we investigated whether these sites are also required for the normal activity of the ac promoter in vivo. As shown in Figure 2, C and D, the high-level proneural cluster expression pattern of the wild-type promoter-lacZ fusion gene in the wing imaginal disc is abolished by mutation of the three da/ac-sc binding sites [this is the same mutant promoter used for the cotransfection experiments (Fig. 1B,C); the E box (CANNTG) sequence at all three sites was mutated to AANNTT]. The diffuse residual expression observed in the wing pouch and notum regions of the disc with the mutant promoter (Fig. 2D) is very similar to the residual expression exhibited by the wild-type promoter in an ac-sc mutant background (Fig. 2B) and can also be seen underlying the proneural cluster expression pattern of the wild-type promoter in a wild-type background (Fig. 2A,C). It is possible that some or all of this expression is reflective of the initial, ac/sc-independent activation of the ac promoter in the wing disc that is revealed by the reporter gene owing to the stability of the β-galactosidase protein.

These experiments demonstrate that mutation of the endogenous ac and sc genes (Fig. 2B), and mutation of the specific E-box binding sites for da/ac and da/sc protein complexes in the ac promoter (Fig. 2D), have very similar effects on ac promoter activity in the wing imaginal disc. Both specifically eliminate the characteristic high-level proneural cluster pattern of ac expression. Taken together with the in vitro DNA binding (Van Doren et al. 1991) and transcriptional activation (Fig. 1B,C) data, these results lead us to conclude that direct transcriptional autoregulation by the ac protein, and cross-regulation by sc, play an essential role in achieving the normal level of ac expression in the wing imaginal disc.

Ectopic sc expression results in ectopic ac promoter activity

If the ac and sc proteins are direct transcriptional regulators of the ac promoter, then ectopic expression and function of these proteins in imaginal discs should result in ectopic activation of the promoter. We tested this prediction by examining the spatial pattern of expression of the ac-lacZ promoter-reporter fusion gene in transgenic flies that also carried a sc cDNA under the control of a heat shock promoter. When third-instar larvae of this type are subjected to heat shock regimens that give large numbers of ectopic macrochaete bristles on the notum of adult flies (Rodríguez et al. 1990; A. Singson, unpubl.), additional territories of lacZ expression are observed in the notum and wing primordia of the wing imaginal disc (Fig. 2E). Moreover, these new zones of ac promoter activity are located in the regions in which the precursors to the ectopic bristles are expected to appear (Bryant 1978; Huang et al. 1991).

Figure 2. *da/ac-sc* binding sites are required for correct expression of the *ac* promoter in vivo. The wild-type *ac* promoter fragment diagrammed in Fig. 1A was placed upstream of the *E. coli lacZ* gene, and transgenic fly lines carrying this reporter construct (*ac-lacZ*) were obtained by P element-mediated germ-line transformation. Transgenic lines carrying the same *ac-lacZ* reporter construct with the *da/ac-sc* binding sites mutated [*X1,2,3 ac-lacZ*; the E box (CANNTG) sequence at all three sites was mutated to AANNTT] were also established. All panels show late third-instar wing imaginal discs stained for β -galactosidase activity.

(A) β -Galactosidase staining pattern for a representative *ac-lacZ* insertion. This pattern has been observed in all eight lines tested, with varying levels of intensity and of diffuse background staining. Strong staining is present at the locations of *ac*- and *sc*-expressing proneural clusters (Cubas et al. 1991; Skeath and Carroll 1991). (B) Disc of a sibling animal from the same experiment as in A, which is mutant for both *ac* and *sc* (*sc*¹⁰⁻¹/Y). The high-level expression of the *ac-lacZ* reporter in the proneural cluster pattern is abolished. (C) Disc from a different *ac-lacZ* transformant line from that shown in A. This disc is somewhat older; the full expression pattern of the reporter gene is evident. (D) β -Galactosidase staining pattern for a representative *X1,2,3 ac-lacZ* (mutant promoter) insertion line. The strong proneural cluster staining observed in all *ac-lacZ* lines was observed in none of the *X1,2,3 ac-lacZ* lines tested (six lines). The diffuse residual staining was observed to varying degrees in different lines and is strikingly similar to the residual *ac*- and *sc*-independent staining seen in B. (E) *ac-lacZ* expression in a wing disc in which *sc* is overexpressed under the control of a heat shock promoter. Late third-instar larvae carrying two copies of the *Hs-sc* construct were subjected to heat shock immediately before dissection and β -galactosidase staining of discs. Areas of ectopic expression of the *ac* reporter are clearly evident by comparison to C. Regions that fail to activate reporter expression under these conditions are indicated by arrows (cf. to Fig. 6).

Rescue of bristle development by the *ac* gene in transgenic flies

The results presented above demonstrate the functional importance of the three *da/ac-sc* binding sites in the *ac* upstream region for the normal level of expression of the *ac* gene in the imaginal disc. To investigate the requirement for these sequences in promoting bristle development in the fly, we first tested the ability of the wild-type *ac* gene to rescue bristle development in transgenic flies carrying the *sc*¹⁰⁻¹ mutation. As shown in Table 1 and Figure 3A, two copies of a 2.2-kb *ac* genomic DNA fragment (including the complete *ac* transcription unit and the same 0.9 kb of 5'-flanking sequence used in the experiments described above) restore an average of nearly 40% of the wild-type number of microchaete bristles to *sc*¹⁰⁻¹ mutant flies. These bristles are organized in a well-spaced array that very strongly resembles the microchaete pattern of wild-type flies. If the three *da/ac-sc* DNA-binding sites in the 5'-flanking region of *ac* are mutated, this rescue activity is greatly compromised (Table 1; Fig. 3B). This result indicates that these binding sites are functionally important in vivo in promoting the development of thoracic bristles. Because the rescue assay is carried out in a genetic background lacking *sc*⁺ function, we suggest that the difference in rescue activity between the wild-type and mutant constructs is a measure of positive transcriptional autoregulation by the *ac* transgene.

emc inhibition of *ac* promoter activation

In an earlier study, we showed that the inhibitory HLH protein encoded by the *emc* gene specifically antagonizes the in vitro binding of *da/da*, *da/ac*, and *da/sc* protein

complexes to the E-box sites upstream of the *ac* gene (Van Doren et al. 1991). It was suggested from these results that *emc* might act in vivo as a negative regulator of auto- and cross-regulation of the *ac* and *sc* genes. We tested the effect of *emc* on the activation of the *ac* promoter by *da* and *ac*, or *da* and *sc*, in the tissue culture cotransfection assay. Figure 4 shows that transfection of increasing amounts of *emc*-encoding DNA progressively diminishes the level of *da/ac*- or *da/sc*-dependent expression of the *ac* reporter gene. The higher amounts of *emc* DNA tested reduce activation of the *ac* promoter to levels very close to those observed in the absence of activator DNA. Thus, in the cotransfection model of *ac* auto- and cross-regulation, *emc* behaves as a dose-dependent inhibitor capable of abolishing the transcriptional activating function of the *da/ac* and *da/sc* combinations.

Ectopic activity of the *ac* promoter in *emc* mutant imaginal discs

The *emc* gene is named for the recessive ectopic bristle phenotype conferred by partial loss-of-function alleles (Botas et al. 1982; Moscoso del Prado and Garc-Bellido 1984; Garca-Alonso and Garca-Bellido 1988). Figure 5, A and B, illustrates the mutant phenotype of a strong hypomorphic allele, *emc*²⁰, isolated in our laboratory (Lindsley and Zimm 1992). Ectopic macrochaetes appear at many positions on each heminotum, particularly between and anterior to the dorsocentral bristles, medial to the presutural bristle, between the postalar and supraalar bristles, and lateral to the dorsocentrals. We used this mutant to examine the effect of loss of *emc* function on the activity of the 0.9-kb *ac* promoter fragment. In wing imaginal discs from third-instar larvae homozygous for *emc*²⁰, the *ac-lacZ* reporter gene is expressed at high levels in many ectopic positions (Fig. 5C, D). These show a striking correspondence to the positions at which the precursors to the ectopic bristles shown in Figure 5B would be expected to arise (Bryant 1978; Huang et al. 1991). Thus, reduction of *emc* function in the wing imaginal disc permits the high-level activity of the *ac* promoter in territories from which it is normally excluded. This is very likely to be the basis of the ectopic appearance of cells expressing *ac* protein in *emc* mutant wing discs (Skeath and Carroll 1991).

Inhibition of *ac* promoter activity in areas of high *emc* expression in the wing disc

To investigate the spatial relationship between the pattern of *ac* promoter activity and the pattern of *emc* expression, we subjected wing imaginal discs from late third-instar larvae carrying the *ac-lacZ* reporter to both β -galactosidase activity staining and in situ hybridization using an *emc* probe. Figure 6A shows that there is a remarkable degree of complementarity between the distribution of *emc* transcripts (dark purple) and the pattern of *lacZ* expression driven by the *ac* promoter (blue). In both the notum and wing pouch regions of the disc, *ac* promoter activity is excluded from areas of highest *emc*

Table 1. *da/ac-sc* binding sites in the *ac* promoter contribute to bristle development

Binding sites intact		Binding sites mutated	
line (no.)	mean $\pm$ S.E.	line (no.)	mean $\pm$ S.E.
C3-2 (5)	46.4 $\pm$ 3.8	D2-1 (5)	6.4 $\pm$ 2.2
C6-2 (3)	51.3 $\pm$ 10.4	D4-1 (5)	11.4 $\pm$ 1.9
C4-1 (5)	75.0 $\pm$ 5.3	D9-1 (5)	17.8 $\pm$ 4.2
C1-1 (6)	85.5 $\pm$ 6.2	D6-2 (7)	24.1 $\pm$ 2.4
C7-4 (5)	136.2 $\pm$ 5.7	D7-1 (8)	59.5 $\pm$ 2.0
Mean	79.2		23.8

Male flies mutant for both *ac* and *sc* (genotype *sc*¹⁰⁻¹/Y) were made homozygous for a single transgene insert containing a 2.2-kb fragment of the *ac* gene. The three E-box binding sites in the promoter of this construct were either intact or mutated (CANNTG vs. AANNTT). The ability of the two constructs to promote bristle development was assayed by counting the number of microchaetes on the notum of the indicated number of flies. A wild-type male fly has ~210 notum microchaetes (Hartenstein and Posakony 1989), whereas *sc*¹⁰⁻¹/Y flies have none (Garca-Bellido 1979). Five independent inserts for each construct were tested. The means of the two groups are significantly different by the Mann-Whitney U Test ($P = 0.028$).

Figure 3. *da/ac-sc* binding sites in the *ac* promoter contribute to bristle development. Representative results from the bristle rescue experiment are described in Table 1. Shown are dissected nota of transgenic flies carrying a 2.2-kb fragment of the *ac* gene in a genetic background that is mutant for both *ac* and *sc* (genotype *sc*¹⁰⁻¹/*Y*). *sc*¹⁰⁻¹ flies are completely devoid of bristles on the notum [for details, see the legend to Table 1]. (A) *ac* gene with wild-type *da/ac-sc* binding sites (line C4-1). (B) *ac* gene with mutant *da/ac-sc* binding sites (line D6-2).

transcript accumulation and appears most intense in “holes” in the *emc* expression pattern. The borders of the *lacZ*-expressing territories often appear to abut directly on territories of high *emc* expression. To demonstrate more clearly the relationship between areas of low *emc* expression and the sites of appearance of SOPs, we carried out similar double-staining experiments with wing discs from late third-instar larvae carrying the enhancer trap transposon insertion A101, which is expressed in all SOPs and their progeny (Huang et al. 1991).

Figure 4. *emc* interferes with *da/ac*- and *da/sc*-dependent activation of the *ac* promoter. Shown are the results of representative tissue culture cotransfection experiments using the wild-type *ac*-CAT reporter construct (see legend to Fig. 1). Increasing amounts of *emc* expression vector (0–3 µg) were included with a combination of either *ac* and *da*, or *sc* and *da*, expression vectors (0.5 µg each) in the cotransfection. The total amount of expression vector DNA was kept constant by including appropriate amounts of the parental expression vector with no insert. *emc* inhibits in a dose-dependent manner the normally strong activation of the *ac* promoter by either *ac* + *da* (○) or *sc* + *da* (▲).

A typical result is shown in Figure 6B. In the notum region of the wing disc, it is clear that macrochaete SOPs arise in territories that are relatively devoid of *emc* transcript. In most instances, the SOP is surrounded by a “halo” of cells with low or no *emc* expression; these are presumably the remaining cells of the proneural cluster that express *ac* (and *sc*) but do not become SOPs. Figure 6B also demonstrates that the apparent exclusion of the in situ hybridization signal from areas of β-galactosidase positivity in Figure 6A is not an artifact of the staining procedure. A higher magnification view of the wing pouch region of a wing disc from the same A101/*emc* double-staining experiment is shown in Figure 6C. The two rows of A101-positive nuclei on the left side of the disc correspond to the precursors of the chemosensory bristles of the anterior wing margin (Hartenstein and Posakony 1989). These rows of precursors are clearly separated by a thin strip of high *emc* transcript accumulation. Figure 6C also shows a “stripe” of strong *emc* expression in the wing pouch region that lies approximately along the anterior/posterior compartment boundary, in the area between the L3 and L4 wing vein primordia (see also Fig. 6B).

We further investigated the relationship between *emc* expression and *ac/sc* activity in experiments illustrated in Figure 6, D and E. Wing discs taken from early to mid-third-instar larvae carrying the *ac-lacZ* reporter exhibit a pattern of *emc* transcript distribution that shows similarities to the pattern of later discs (Fig. 6D). *ac-lacZ* reporter expression is not yet detectable at the anterior wing margin in discs at this earlier stage, yet *emc* transcripts are already evident there, suggesting that *emc* expression in some areas precedes the establishment of high-level *ac* promoter activity. We also tested the possibility that the low level of *emc* transcript in *ac*-expressing proneural clusters is the result of a repressive action of *ac* and/or *sc* on *emc* expression in these areas. We examined the *emc* transcript distribution in wing discs from late third-instar male larvae carrying the *sc*¹⁰⁻¹ mutation, which inactivates both *ac* and *sc* (see above). As shown in Figure 6E, the *emc* expression pattern is evidently unchanged from that observed in wild-type discs of comparable age (see Fig. 6A,B). In particular, the ter-

Figure 5. The *ac* promoter is ectopically activated in an *emc* mutant background. (A,B) Scanning electron micrographs of wild-type (w^{1118}) and *emc*²⁰ flies, respectively. Note the pattern of ectopic macrochaetes on the head and notum of the *emc*²⁰ fly (B); examples are marked by the white arrow and arrowhead. (C,D) β -Galactosidase staining shows patterns of *lacZ* expression from the *ac-lacZ* reporter in wing discs of the same genetic backgrounds [wild type (C), *emc*²⁰ (D)]. Positions of ectopic *ac* promoter activity are clearly apparent in the *emc*²⁰ disc (D). The arrow and the arrowhead indicate ectopic *lacZ*-expressing clusters in positions where the corresponding ectopic macrochaetes marked in B would develop, according to the fate map of Bryant (1978). (E) Cuticle preparation of the notum of an adult fly containing four copies of the *Hs-emc* construct (see text), which was subjected to heat shock beginning at 6 hr after puparium formation, during the time of microchaete SOP determination. Anterior is at the top. Overexpression of *emc* results in clear regions of microchaete loss, particularly anterior and lateral to the dorsocentral macrochaetes (cf. E and A).

ritories of high and low expression in the notum region of the disc are readily apparent. This experiment demonstrates explicitly that the spatial modulations in *emc* transcript distribution in the late third-instar wing disc do not result from *ac*- or *sc*-dependent regulation.

We showed above that overexpression of *sc* driven by a heat shock promoter leads to high-level expression of the *ac* promoter in most, but not all, regions of the wing imaginal disc (Fig. 2E). A comparison of Figure 2E with Figure 6 reveals a close correspondence between areas that fail to activate the *ac-lacZ* reporter in response to *sc* overexpression (arrows in Fig. 2E) and areas of highest

emc transcript accumulation (e.g., Fig. 6B). This observation offers strong evidence that *ac* promoter activity is antagonized in territories of high *emc* expression in the wing disc.

Phenotypic consequences of overexpression of emc

Our interpretation of the results presented here is that spatial variations in the level of *emc* expression play an important role in determining where in the wild-type imaginal disc auto- and cross-regulation of the *ac* gene can occur (see Discussion). If this view is correct, we

Figure 6. Distribution of *emc* transcripts in wing imaginal discs of third-instar larvae. Whole imaginal discs were hybridized in situ using a digoxigenin-labeled *emc* RNA probe (purple). Discs were also stained for β -galactosidase activity (blue) to reveal the pattern of expression of either the *ac-lacZ* reporter construct (A,D) or the A101 enhancer trap transposon (B,C,E). A101 is a marker for SOP cells and their progeny (Huang et al. 1991). Anterior is to the left. (A) Wing disc from a late third-instar larva carrying the *ac-lacZ* reporter. (B) Wing disc from a late third-instar larva carrying the A101 marker. (C) Higher magnification view of the wing pouch region of a wing disc from a late third-instar larva carrying the A101 marker. The white arrow indicates the accumulation of *emc* transcripts (purple) between the double row of chemosensory bristle SOPs at the anterior wing margin (blue). The black arrow indicates the intense stripe of *emc* expression approximately along the anterior-posterior compartment boundary in the wing pouch region. (D) Wing disc from a mid-third-instar larva carrying the *ac-lacZ* reporter. (E) Wing disc from a late third-instar larva mutant for both *ac* and *sc* (genotype *sc*¹⁰⁻¹/Y) and carrying the A101 marker. The absence of β -galactosidase staining verifies the *sc*¹⁰⁻¹ background.

may expect that broad overexpression of *emc* (resulting in abnormally high levels of *emc* activity in normal proneural clusters) would cause the failure of normal bristles to develop. We tested this hypothesis by establishing lines of transgenic flies in which a wild-type *emc* cDNA is expressed under the control of the *Drosophila Hsp70* promoter. When animals carrying four copies of this fusion gene are subjected to a heat shock regimen as early pupae, before the divisions of the microchaete precursor cells (Hartenstein and Posakony 1989), the resulting adult flies lack a large number of microchaete bristles on the head and notum (Fig. 5E). In contrast, the macrochaete bristles, which had already commenced differentiation at the time the heat shock was applied (Hartenstein and Posakony 1989), are unaffected. Earlier heat shocks, applied in the third larval instar before the division of the macrochaete SOPs, result in a reproducible pattern of macrochaete loss (data not shown). The anterior dorso-centrals, sternopleurals, presuturals, ocellars, and post-verticals are especially sensitive to this treatment. Thus,

broad overexpression of the wild-type *emc* protein is capable of interfering with normal sensory organ development, suggesting that the areas of low *emc* expression that we have observed in the wing disc (Fig. 6) are essential for the establishment of the normal adult bristle pattern.

Discussion

Direct transcriptional activation of the ac promoter by the ac and sc proteins

Several pieces of evidence lead us to the conclusion that the *ac* promoter is subject to direct transcriptional activation in vivo by protein complexes that include the *ac* and *sc* proteins. First, as we have described previously, hetero-oligomeric complexes of *da* and *ac*, or *da* and *sc*, bind in a sequence-specific manner in vitro to three E-box sites upstream of the *ac* transcription start (Van Doren et al. 1991). Second, in a cotransfection assay in

Drosophila tissue culture cells, these same protein combinations are capable of activating by >100-fold the expression of a CAT reporter gene driven by a 0.9-kb fragment of the *ac* promoter; mutation of the three *da/ac-sc* binding sites abolishes this activation (Fig. 1). Third, the same 0.9-kb promoter fragment drives high-level expression of a *lacZ* reporter gene in the wing imaginal discs of third-instar larvae in a proneural cluster pattern that is similar to that of the endogenous *ac* gene (Fig. 2; Martínez and Modolell 1991). High-level expression of the reporter in the cluster pattern is abolished in animals carrying loss-of-function mutations in the endogenous *ac* and *sc* genes (Fig. 2; Martínez and Modolell 1991). Finally, mutation of the three E-box binding sites greatly reduces or abolishes reporter gene expression in the proneural cluster pattern in vivo (Fig. 2).

These data do not strictly rule out the possibility that, in vivo, a protein or protein complex not involving *ac* or *sc* is responsible for the E box-dependent activation of *ac* transcription in proneural clusters, and that *ac* and *sc* act indirectly. Such a hypothetical activator, however, must (1) be dependent for its expression or activity on *ac* and *sc* gene function, and (2) require the integrity of the core nucleotides of the E-box sites to which *da/ac* and *da/sc* complexes bind in vitro. Given these constraints, we believe the simplest interpretation of the evidence is that the *ac* and *sc* proteins participate directly in transcriptional activation of the *ac* gene in the proneural clusters of the wing imaginal disc.

The genetically defined requirement for both *da* and *ac/sc* function in bristle development can be satisfactorily accounted for if these bHLH proteins function in vivo as *da/ac* or *da/sc* hetero-oligomeric complexes (Van Doren et al. 1991). However, although *da* is essential for the specification or execution of the SOP cell fate [at least in the larval PNS (Ghysen and O'Kane 1989)], it is not known whether high-level expression of *ac* or *sc* in imaginal disc proneural clusters or SOPs requires *da*⁺ function. It is possible that, in vivo, post-translational modifications, for example, allow *ac* and *sc* to function in auto- and cross-regulation independently of *da*. Similarly, *da* may have important functions in adult PNS development that do not require the presence of *ac* or *sc*. Nevertheless, the results described here (Fig. 1) and those of earlier studies (Cabrera and Alonso 1991; Van Doren et al. 1991) indicate that the intact *ac* and *sc* proteins are highly active in sequence-specific DNA binding and transcriptional activation only in the presence of the *da* protein.

Role of auto- and cross-regulation of ac transcription in the development of proneural clusters

Given our conclusion that *ac* transcription in the wing disc is in part controlled by direct autoactivation, as well as cross-regulation by *sc*, it is useful to consider what function these activities serve in the process of sensory organ development. Their principal effect is to rapidly bring about a high level of *ac* and *sc* expression in the cells of the proneural clusters. This, in turn, may help to

ensure that the competence to adopt the SOP cell fate is conferred in a robust manner on these cells, particularly the ultimate single SOP. Given the relatively transient nature of *ac/sc* expression in both the clusters and in the SOP (Cubas et al. 1991; Skeath and Carroll 1991), high-level expression of these genes may be necessary to achieve the required levels of target gene activity in a timely fashion.

An issue of obvious importance is whether *ac* autoactivation is important in vivo in promoting bristle development. We have addressed this question by an experiment that measures the ability of an *ac* transgene to rescue microchaete development on the notum in a genetic background lacking endogenous *ac* and *sc* function. In these flies, the only source of functional *ac* protein is the transgene itself, cross-activation of the transgene by *sc* is eliminated, and there is no direct *sc* contribution to SOP determination. Thus, this appears to be a very stringent test of *ac* autoregulation. Our finding that mutation of the three *da/ac-sc* DNA-binding sites in the *ac* promoter severely compromises the microchaete-promoting activity of the *ac* gene (Fig. 3; Table 1) provides direct evidence that *ac* autoactivation makes an important contribution to bristle patterning.

Mutual cross-activation of *ac* and *sc* appears to be responsible for the expression of both of these genes in the same final pattern following their initial activation in complementary patterns (Martínez and Modolell 1991; Skeath and Carroll 1991). It is not yet clear how important *ac-sc* cross-regulation is for most bristle development; however, because *sc*-dependent bristles do not generally require *ac*, and vice versa, only a single macrochaete, the posterior supra-alar, is genetically dependent on both *ac* and *sc* activity (Ghysen and Dambly-Chaudière 1988). It is possible that in many proneural clusters, mutual cross-activation between *ac* and *sc* is a simple consequence of their autoregulatory activities, because the two proteins bind to the same sites in vitro (Cabrera and Alonso 1991; Van Doren et al. 1991).

The emc protein interferes post-translationally with ac auto- and cross-regulation

We have shown in this paper (Fig. 5) that an *ac-lacZ* reporter gene is expressed in ectopic proneural clusters in the wing imaginal discs of larvae carrying a strong partial loss-of-function mutation of *emc*. This indicates that the wild-type function of *emc* includes acting as a negative regulator of *ac* promoter activity. Formally, the *emc* protein could accomplish this by direct transcriptional repression of *ac*, mediated by DNA binding. However, we believe that the evidence presented here and in our previous reports (Ellis et al. 1990; Van Doren et al. 1991) is most consistent with the interpretation that *emc* negatively regulates *ac* transcription by interfering post-translationally with direct transcriptional autoactivation by *ac* and cross-activation by *sc*. First, the predicted *emc* protein shares the HLH dimerization domain of the bHLH proteins but lacks the conserved set of basic residues that constitute their DNA-binding domain, sug-

gesting that it acts as a negative regulator by a mechanism involving protein–protein, rather than protein–DNA, interactions (Ellis et al. 1990; Garrell and Modolell 1990). Second, in vitro, *emc* antagonizes in a dose-dependent manner the sequence-specific binding of *da/ac* and *da/sc* protein complexes to the E-box sites in the *ac* promoter that we have shown are required for high-level expression of the promoter in proneural clusters (Fig. 2; Van Doren et al. 1991). Recently, we have found that the HLH domain of *emc* is both necessary and sufficient for this in vitro activity (P.A. Powell and M. Van Doren, unpubl.). Third, in tissue culture cotransfection experiments, *emc* behaves as a dose-dependent inhibitor of *da/ac*- and *da/sc*-dependent transcriptional activation of the *ac* promoter (Fig. 4).

We propose from these findings that post-translational inhibition of *ac* and *sc* auto- and cross-activation is one of the principal mechanisms by which *emc* negatively regulates *ac/sc* activity and, hence, sensory organ determination in the imaginal disc. (Transcriptional regulation of downstream genes by *ac* and *sc* will also be inhibited by this mechanism.) Our conclusion that transcription of the *ac* gene is controlled in part by regulating its autoactivation function offers a precedent that may possibly be extended to other eukaryotic genes encoding transcription factors. It seems clear that any transcriptional regulator whose normal expression is dependent on an autoregulatory activity is potentially subject to spatial and temporal control at this level.

emc and the spatial regulation of proneural gene activity in the wing imaginal disc

Three observations reported here indicate that *emc* plays an important role in defining the spatial pattern of proneural clusters in the wing imaginal disc. First, *emc* transcripts are distributed nonuniformly in the disc (Fig. 6). Both the wing pouch and notum regions exhibit territories of high and low *emc* RNA accumulation. Second, the spatial pattern of *ac/sc* activity is, to a large degree, complementary to the pattern of *emc* expression. Thus, the *ac lacZ* reporter is apparently not activated in territories of high *emc* expression; conversely, the highest levels of activation occur within zones of minimal *emc* transcript accumulation (Figs. 2E and 6A,D). We have ruled out the possibility that this reciprocity is the result of a repressive action of *ac* and *sc* on *emc* by showing that the spatial pattern of *emc* transcript accumulation is established normally in the absence of *ac* and *sc* function (Fig. 6E). Finally, the *ac* promoter is strongly active in ectopic clusters of cells in the wing discs of an *emc* hypomorphic mutant (Fig. 5D), indicating that *emc* function is required in at least some parts of the disc to inhibit high-level *ac* transcription and thus SOP potential.

Our view of the role of *emc* in controlling the spatial pattern of *ac* and *sc* transcription, and thus the pattern of functional proneural clusters, is summarized in Figure 7. As described earlier (Van Doren et al. 1991), the normal development of the imaginal disc must include the activation of the *ac* and *sc* promoters not only in the areas in

which proneural clusters appear, but also in territories where *emc* acts to suppress bristle development (Fig. 7A). We propose that the relatively high level of *emc* protein in these latter regions engages much or all of the available *da*, *ac*, or *sc* protein in hetero-oligomeric complexes that do not bind E-box sites with high affinity. These inactive *emc*-containing complexes are formed at the expense of complexes that are competent for sequence-specific, high-affinity DNA-binding and transcriptional activation (e.g., *da/ac*, and *da/sc*), thus preventing the establishment of stable auto- and cross-regulatory loops that would otherwise maintain *ac*- and *sc* transcription at the levels characteristic of proneural clusters (Fig. 7B, right). In contrast, in territories of low or no *emc* expression (Fig. 7A,B, left), the *ac* and *sc* protein resulting from the initial activation of these genes is able to form functional dimeric complexes with *da*, establish stable auto- and cross-activation of both *ac* and *sc*, and thus confer on the cells in these territories (the proneural clusters) the potential to adopt the SOP fate (by regulation of downstream target genes). In this model ectopic bristles develop in *emc* hypomorphic mutants (Fig. 7A, bottom) in part because the general reduction in *emc* activity in the disc causes the local *emc* activity in certain areas to fall below the threshold level necessary to inhibit *ac* and *sc* auto- and cross-activation, and this, in turn, results in the appearance of new proneural clusters and ultimately ectopic SOPs. Similarly, overexpression of *sc* allows it to overcome *emc* inhibition in certain areas and thus establish its auto- and cross-regulatory activities, resulting in ectopic proneural clusters (Fig. 2E) and ectopic bristles (Rodriguez et al. 1990; A. Singson, unpubl.). The bristle loss phenotype of the *Hs-emc* flies (Fig. 5E) can be explained in opposite terms. Broad overexpression of *emc* presumably results in elevating *emc* activity within areas that normally would have given rise to proneural clusters. This higher level of *emc* is capable of antagonizing *ac* and *sc* auto- and cross-activation, thus preventing SOP determination at these sites.

The spatial variation in *emc* transcript levels in the third-instar wing imaginal disc offers positional information of only moderate resolution (Fig. 6). Thus, our results indicate that although *emc* can help to define territories in which proneural clusters will arise and can limit the size of these clusters, it is insufficient to directly specify single SOPs. This is consistent with genetic observations that lateral inhibition, mediated by the neurogenic genes, is required to restrict the expression of the SOP fate to a single cell in each proneural cluster.

Although we believe that *emc* plays a critical early role in defining territories of bristle-forming potential in the wing imaginal disc, it is clear that other factors also function in this process. For example, the initial activation of the *ac* and *sc* genes is clearly patterned, because the two genes are first expressed in largely nonoverlapping spatial domains (Martinez and Modolell 1991; Skeath and Carroll 1991). In addition, we observe a number of territories in the disc that appear to lack both *ac* promoter activity and *emc* expression (Fig. 6), further

Figure 7. Schematic diagrams illustrating models of how spatial variations in *emc* expression control the pattern of *ac* auto- and cross-regulation in the imaginal disc (A), and the establishment of positive autoactivation by the *ac* gene and its inhibition by *emc* (B) (for details, see text). (A) The scale at left indicates levels of expression (and/or activity) of *ac* and *emc*. The broken lines represent the initial level of expression of *ac*. Hatched areas represent proneural clusters, where auto- and cross-activation increase *ac* transcription to higher levels. (Top) Wild-type fly; (bottom) *emc* hypomorphic mutant, with ectopic proneural cluster (central hatched region). (B) (Left) Establishment of positive autoregulation of the *ac* gene in areas of low or no *emc* expression. (Right) Inhibition of *ac* autoactivation in areas of high *emc* expression.

indicating that *ac* expression is controlled by mechanisms not involving *emc*.

The nonuniform accumulation of *emc* transcripts in the wing disc immediately raises the question of what controls the spatial pattern of *emc* expression. For example, the strong stripe of *emc* RNA that we have observed approximately along the anterior/posterior compartment boundary in the wing pouch region may well be under the control of genes associated with this boundary, such as *engrailed* (Kornberg et al. 1985; Brower 1986), *cubitus interruptus* (Eaton and Kornberg 1990), and *decapentaplegic* (Masucci et al. 1990; Posakony et al. 1991). Thus, investigating the transcriptional regulation of *emc* expression in the wing disc may be a profitable approach to defining a connection between bristle patterning and the genes that provide positional information in the disc.

Materials and methods

Drosophila stocks

Flies were raised on standard yeast/cornmeal/molasses/agar medium. The *Hs-emc* stock was homozygous for two different P[*Hs-emc*] insertions (see below). *Hs-sc* stocks were homozygous for one P[*Hs-sc*] insertion and were also homozygous for one P[*ac-lacZ*] insertion. All other stocks are described in Lindley and Zimm (1992).

Tissue culture

Drosophila Schneider S2 cells were maintained in Schneider's *Drosophila* medium (GIBCO), supplemented with 12% fetal bo-

vine serum, and were grown at room temperature. Cells were split 1 : 5 twice weekly.

Mutagenesis of the *ac* upstream region

Unless otherwise indicated, all molecular biology procedures are as described (Ausubel et al. 1987; Sambrook et al. 1989). A 2.2-kb *EcoRI* fragment containing the *ac* transcription unit (Villares and Cabrera 1987) was isolated from a *Drosophila* genomic DNA library. A 0.27-kb *DraI*-*Bam*HI fragment of this clone was ligated into *SmaI*/*Bam*HI-digested pUC19. The resulting plasmid (pT5D-B) contains from -321 to -51 of the *ac* upstream region, which includes all three previously identified *da/ac-sc* binding sites: T5E1, T5E2, and T5E3 (Van Doren et al. 1991). Site-specific mutagenesis was performed using this plasmid, mutant oligonucleotides, convenient restriction sites, and the polymerase chain reaction (PCR) to generate a plasmid with mutations in these three sites (pT5D-BX1,2,3). The core E-box nucleotide sequence in each of the sites was changed from CANN TG to AANN TT, and this, as well as all PCR-synthesized DNA, was verified by double-stranded DNA sequencing. Owing to an apparent polymorphic difference between the published sequence of the *ac* upstream region [used to design the oligonucleotides (Villares and Cabrera 1987)] and the sequence of our *ac* clone, the wild-type and mutated *ac* upstream regions also differ at position -248 (G vs. A). Because this polymorphism was found in *ac* genomic DNA clones from two wild-type stocks, we do not believe that this contributes significantly to the observed results.

Plasmid construction

The 2.2-kb *EcoRI* fragment of *ac* genomic DNA was subcloned into the *EcoRI* site of a derivative of pUC19 in which the *Bam*HI

site had previously been eliminated. This plasmid (pT5-2.2) contains 875 bp of *ac* upstream sequence, the entire *ac* transcription unit, and ~0.35 kb of downstream sequence (based on the 3' end of our *ac* cDNA). A 0.9-kb *EcoRI*-*Eco47III* fragment from this clone was blunt-end-ligated into the *HincII* site of the same pUC19 derivative. This plasmid (pT5-0.9) contains from -876 to +14 of the *ac* transcription unit. In addition, the wild-type *NaeI*-*BamHI* fragment in each of these plasmids was exchanged for the mutated *NaeI*-*BamHI* fragment from pT5D-BX1,2,3 (see above). This created the plasmids pT5-2.2X1,2,3 and pT5-0.9X1,2,3, which are identical to pT5-2.2 and pT5-0.9, respectively, except that the T5E1-T5E3 sites are specifically mutated. To create reporter constructs for cotransfection analysis, 0.9-kb *PstI*-*XbaI* fragments from both pT5-0.9 and pT5-0.9X1,2,3 were cloned into *PstI*/*XbaI*-digested pC4CAT (Thummel et al. 1988), generating pT5-0.9CAT and pT5-0.9X1,2,3CAT, respectively. These constructs have 876 bp of *ac* upstream sequence (including the *ac* transcription start site), with the *da/ac-sc* binding sites either intact or mutated, fused to the CAT-coding sequences. To create expression constructs for cotransfection analysis, cDNAs including the entire coding regions of *ac*, *sc*, *da* (A. Singson, unpubl.), and *emc* (Ellis et al. 1990) were placed under the control of the *Drosophila* actin 5C promoter in the vector pAct5CPPA (Han et al. 1989). For *ac*, an *Eco47III*-*MscI* fragment was first blunt-end-ligated into the *SmaI* site of pKST1 + to create pKST5. A *BamHI*-*KpnI* fragment of pKST5 was then ligated into *BamHI*/*KpnI*-digested pAct5CPPA, generating pAct5. For *sc*, an exonuclease-trimmed cDNA fragment [beginning at position 584 and ending at position 1806 of the published genomic DNA sequence (Villares and Cabrera 1987)] was cloned into the *XbaI* site of pKST1 + using *XbaI* linkers to create pKST4. A *SacI*-*BamHI* fragment of pKST4 was then ligated into *SacI*/*BglIII*-digested pAct5CPPA, generating pAct4. For *da*, a *SacI*-*BamHI* fragment of pKSDa (Van Doren et al. 1991) was ligated into *SacI*/*BglIII*-digested pAct5CPPA, generating pActda. For *emc*, a *RsaI*-*RsaI* fragment was first blunt-end-ligated into the *XbaI* site of pGEM4 to make pGEM4emc (H. Ellis, unpubl.). pGEM4emc was digested with *SalI* and then subjected to a 2-bp fill-in using dCTP, dTTP, and Klenow DNA polymerase. A fragment containing the *emc* coding region was then liberated with a second digestion using *BamHI*. pAct5CPPA was digested with *BamHI* and also subjected to a 2-bp fill-in using dATP and dGTP, thereby generating a terminus compatible with ligation to the modified *SalI* terminus described above. pAct5CPPA was then digested with *BglIII*, and the two DNAs were ligated. Plasmid constructs for P element-mediated transformation made use of the CaSpeR vector (Pirrotta et al. 1988). For the bristle rescue experiments, the 2.2-kb *EcoRI* fragments from pT5-2.2 and pT5-2.2X1,2,3 were cloned into the *EcoRI* site of CaSpeR in the same orientation (pCSPT52.2 and pCSPT52.2X1,2,3). The *ac-lacZ* reporter gene was constructed using the vector CaSpeR*lacZ* (J. S. Margolis and J. W. Posakony, in prep.). This vector contains the *E. coli lacZ* coding region and the SV40 small t polyadenylation site cloned into the multiple cloning site of CaSpeR. The identical *PstI*-*XbaI* fragments from pT5-0.9 and pT5-0.9X1,2,3 used to generate the CAT constructs were cloned into *PstI*/*XbaI*-digested CaSpeR*lacZ*, generating pCLZT5-0.9 and pCLZT5-0.9X1,2,3. These plasmids contain the identical 876 bp of *ac* upstream sequence as in the CAT constructs, with the *da/ac-sc* binding sites either intact or mutated, controlling expression of the *lacZ* gene. The CaSpeR-*Hsp70* transformation vector for heat shock experiments is as described (Bang and Posakony 1992). For P[*Hs-sc*], the *XbaI*-linked fragment of *sc* cDNA described above was directly cloned into CaSpeR-*Hsp70*. To make the P[*Hs-emc*] construct, an *XbaI* fragment containing the *emc*-

coding region was isolated from pGEM4emc (above) and cloned into CaSpeR-*Hsp70*.

Cotransfection analysis

Cotransfection of plasmid DNAs into *Drosophila* Schneider cells was carried out using calcium phosphate precipitation (Sambrook et al. 1989). Approximately 2×10^6 to 4×10^6 cells were plated in each 60-mm culture dish 1 day before transfection. A total of 10 μ g of DNA was used for each duplicate transfection and included 2 μ g of a plasmid containing a *copia* long terminal repeat (LTR)-*lacZ* fusion gene (Han et al. 1989), 2 μ g of either the pT5-0.9CAT or pT5-0.9X1,2,3CAT reporter constructs, 4 μ g total of expression vector DNAs (with or without cDNA insert; see above), and 2 μ g of pGEM4 (the DNA for a set of duplicates was precipitated together and then divided between the samples). The cells were then incubated for 2 days with the precipitate. Cells were harvested by resuspension and were washed twice in PBS before being resuspended in 100 μ l of 0.25 M Tris-HCl (pH 8.0). Cells were lysed by four cycles of freeze/thaw in dry ice/ethanol cycle, and lysates were clarified by centrifuging 5 min in a microcentrifuge. β -Galactosidase and CAT assays were performed as described (Gorman 1985). For all assays, CAT activity was normalized to β -galactosidase activity to control for transfection efficiency.

P element-mediated germ-line transformation

Transgenic flies were generated by standard P element-mediated germ-line transformation techniques (Rubin and Spradling 1982) using *w¹¹¹⁸* as the recipient strain.

β -Galactosidase staining of imaginal discs

Imaginal discs of third-instar larvae were dissected in PBS and fixed for 2 min on ice in 1% glutaraldehyde in PBS. They were then stained for β -galactosidase activity as described (Romani et al. 1989).

Double labeling by β -galactosidase activity staining and in situ hybridization

Double staining of wing discs to detect β -galactosidase activity and *emc* transcript distribution was performed sequentially. Discs were first stained histochemically for β -galactosidase activity as described above. Following several washes in PBS + 0.1% Tween 20, discs were fixed as described (Schweisguth and Posakony 1992), and subsequent proteinase K and postfixation steps were performed according to Jiang et al. (1991). Discs were then subjected to in situ hybridization (Tautz and Pfeifle 1989) with a digoxigenin-labeled RNA probe synthesized from a full-length *emc* cDNA clone (pNB5b; Ellis et al. 1990).

Heat shock experiments

Heat shock treatments of *Hs-sc* (late third-instar larvae) and *Hs-emc* (staged pupae) animals were carried out as described (Rodriguez et al. 1990; Bang and Posakony 1992). Immediately following the final heat shock, animals were dissected immediately for β -galactosidase staining (larvae in the *Hs-sc* experiments) or were allowed to continue development to adulthood (*Hs-emc* pupae and some *Hs-sc* larvae).

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Note added in proof

Recently, Cubas and Modolell [EMBO J. 11: 3385–3393 (1992)] reported results consistent with those described here concerning the spatial pattern of *emc* transcript accumulation in the wing imaginal disc. We note that these investigators have used the term "proneural fields" to describe the groups of cells referred to herein as "proneural clusters."

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