Cis-regulation of *achaete* and *scute*: shared enhancer-like elements drive their coexpression in proneural clusters of the imaginal discs

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The pattern of bristles and other sensory organs on the adult cuticle of *Drosophila* is prefigured in the imaginal discs by the pattern of expression of the proneural *achaete* (*ac*) and *scute* (*sc*) genes, two members of the *ac-sc* complex (AS-C). These genes are simultaneously expressed by groups of cells (the proneural clusters) located at constant positions in discs. Their products (transcription factors of the basic–helix–loop–helix family) allow cells to become sensory organ mother cells (SMCs), a fate normally realized by only one or a few cells per cluster. Here we show that the highly complex pattern of proneural clusters is constructed piecemeal, by the action on *ac* and *sc* of site-specific, enhancer-like elements distributed along most of the AS-C (~90 kb). Fragments of AS-C DNA containing these enhancers drive reporter *lacZ* genes in only one or a few proneural clusters. This expression is independent of the *ac* and *sc* endogenous genes, indicating that the enhancers respond to local combinations of factors (prepattern). We show further that the cross-activation between *ac* and *sc*, discovered by means of transgenes containing either *ac* or *sc* promoter fragments linked to *lacZ* and thought to explain the almost identical patterns of *ac* and *sc* expression, does not occur detectably between the endogenous *ac* and *sc* genes in most proneural clusters. Our data indicate that coexpression is accomplished by activation of both *ac* and *sc* by the same set of position-specific enhancers.

[*Key Words*: Achaete-Scute Complex; transcriptional regulation; enhancer elements; proneural genes; *Drosophila*]

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In *Drosophila*, a classical model to study pattern formation is provided by the >1000 bristles and other types of external sensory organs (SOs) that appear on its cuticle in characteristic arrangements. On the head and dorsal mesothorax (notum), large bristles (macrochaetae) occupy remarkably constant positions, so each of them has received an individual name (Lindsley and Zimm 1992). Small bristles (microchaetae) appear in "density" types of arrangements and cover constant areas of the fly's body. In most cases, each SO is derived from a single SO mother cell (SMC) that undergoes two differential divisions (Bodmer et al. 1989; Hartenstein and Posakony 1989). The four progeny cells subsequently differentiate into the components of the SO. SMCs appear during the third-instar larval and early pupal stages, and they do so in precisely defined positions of the imaginal discs, the epithelial sacs that are the precursors of a large part of the adult epidermis (Cubas et al. 1991; Huang et al. 1991). Thus, the accurate positioning of SOs is largely explained by the emergence of SMCs at specific sites.

A group of genes, collectively known as the proneural genes (Ghysen and Dambly-Chaudiere 1989; Romani et al. 1989), confer to cells the ability to become SMCs. All known proneural genes encode transcriptional regulators of the basic–helix–loop–helix (bHLH) family (for review, see Garrell and Campuzano 1991; Jan and Jan 1993). In the case of bristles and other external SOs, the proteins encoded by the proneural genes *achaete* (*ac*) and *scute* (*sc*), two members of the Achaete-Scute Complex (AS-C), are most important for generating the corresponding SMCs (for review, see Campuzano and Modolell 1992). It is thought that Achaete (Ac) and Scute (Sc) proteins commit imaginal disc cells to become SMCs by forming heterodimers with the bHLH protein Daughterless (Da) and activating downstream genes that participate in the neural differentiation program (Caudy et al. 1988; Dambly-Chaudière et al. 1988; Cabrera and Alonso 1991; Van Doren et al. 1991; Vaessin et al. 1994). The expression of

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ac and *sc* is restricted to groups of cells, the proneural clusters that define the positions where SMCs arise (Romani et al. 1989; Cubas et al. 1991; Skeath and Carroll 1991). Both *ac* and *sc* are expressed in all clusters and in apparently the same cells. Over 20 proneural clusters are present in the imaginal wing disc at the late third-instar stage. The position, size, shape, time of emergence, and disappearance of each cluster are very reproducible, implying a complex control of *ac* and *sc* expression (Cubas et al. 1991; Skeath and Carroll 1991). A fixed number of SMCs arise from each cluster, typically one or a few. Moreover, they arise in characteristic positions within a cluster. Cell–cell interactions between the members of a cluster, mediated by the neurogenic genes, prevent additional cells from becoming SMCs (for review, see Simpson 1990; Campos-Ortega 1993). In mutants with strongly reduced or missing proneural clusters of *ac/sc* expression, the corresponding SMCs and SOs disappear, whereas in other mutants with enlarged domains of *ac/sc* expression extra SOs emerge in ectopic positions (Cubas et al. 1991; Skeath and Carroll 1991; Cubas and Modolell 1992). Hence, the spatiotemporal distribution of proneural function within the disc epithelia—the pattern of clusters of *ac/sc* expression—defines the sites where SMCs emerge. [Additional factors controlling SMC positioning are discussed in Rodríguez et al. (1990), Cubas and Modolell (1992), and Van Doren et al. (1992).]

How are the complex patterns of *ac/sc* expression generated? A model has been inferred (Ruiz-Gómez and Modolell 1987) from the phenotypes of mutations associated with chromosomal breakpoints mapping in the 50 kb downstream of the *sc* structural gene (Fig. 1). These mutants showed that the closer a breakpoint was to the *sc* gene, the more macrochaetae of the head and notum were removed (Campuzano et al. 1985). A given breakpoint always removed at least the macrochaetae affected by more distal breakpoints, so that particular sequences of the *sc* downstream region seemed concerned with the development of specific macrochaetae. Key data were provided by the sc^6 mutation, an internal deletion of the *sc* downstream region (Fig. 1). This deletion suppressed the macrochaetae corresponding to the putative regulatory elements contained within the deleted DNA, but it did not affect those associated with the regulatory ele-

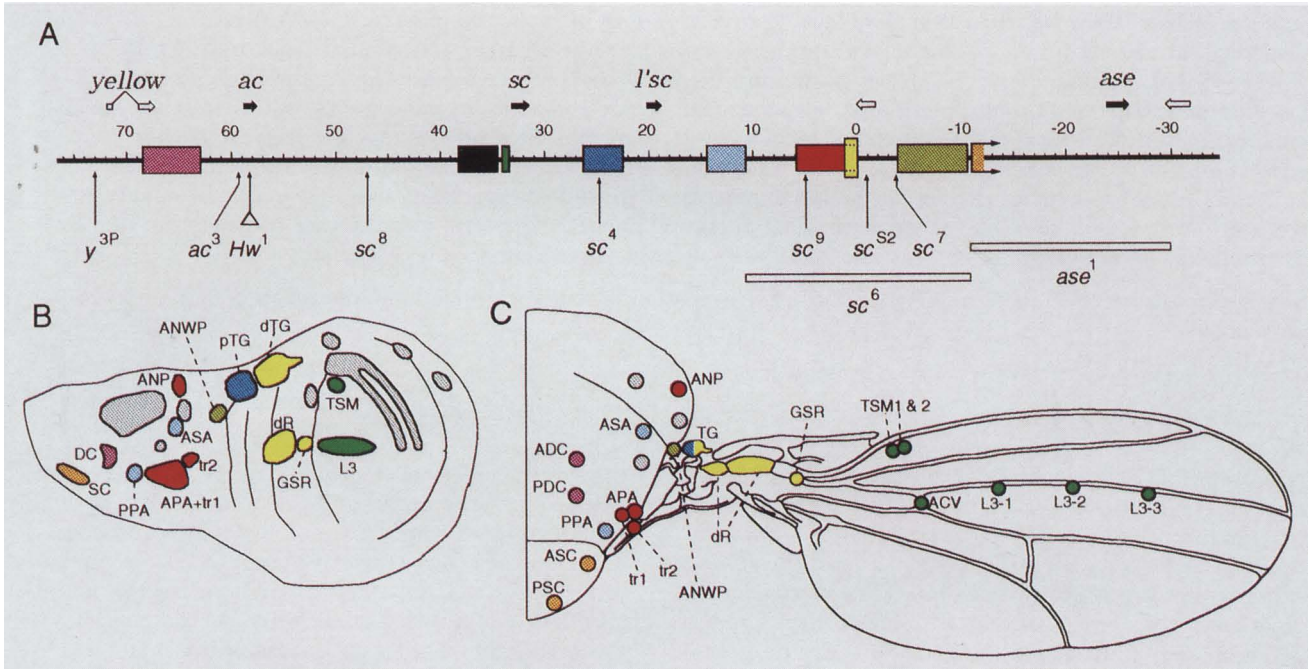

Figure 1. (*A*) Simplified physical map of the AS-C with indication of the regions containing known and presumed *ac/sc*-specific enhancers, shown as colored rectangles over the AS-C DNA line. Precise extent and position of the enhancer sequences within rectangles are unknown. Additional data (not shown) indicate that the L3/TSM enhancer is located between nucleotides −185 and −578 from the *sc* transcriptional start site. The positions of the enhancers corresponding to the ASA, PPA (light blue), and A/PSC (brown) macrochaetae and ANWP (light green) sensilla campaniformia are inferred from genetic data (see text). Extent of the A/PSC enhancer toward the *right* is undetermined. Position of an enhancer for expression in SMCs (Martínez and Modolell 1991) is shown in black. Transcription units corresponding to the four proneural genes of the AS-C are indicated by thick, solid, horizontal arrows. Position of other transcription units and the *yellow* gene are indicated by empty arrows. Coordinates on the AS-C DNA have been defined in Campuzano et al. (1985). Vertical arrows show positions of chromosomal breakpoints associated with the corresponding mutation. Arrow with triangle indicates insertion of the *gypsy* element associated with the Hw^1 mutation. Extents of the sc^6 and ase^1 deletions are indicated by elongated boxes. (*B*) Schematic drawing of an imaginal wing disc showing proneural clusters illuminated with colors matching the corresponding enhancers in *A* to illustrate their spatial specificity. The stippled area in some clusters indicates that positions of their enhancers are unknown. (*C*) schematic drawing of a heminotum and wing with indication of the position of macrochaetae and a few large sensilla campaniformia (circles), the dorsal radius (dR), and the tegula (TG), colored to match the corresponding proneural clusters and specific enhancers.

ments that would be located farther downstream of the deletion (Ruiz-Gómez and Modolell 1987). It was thus proposed that *cis*-acting, site-specific elements were scattered within this long stretch (~50 kb) of AS-C DNA (Ruiz-Gómez and Modolell 1987). The elements would respond to local specific combinations of factors [prepattern (Stern 1954)] and mediate *sc* activation, which subsequently would trigger development of a macrochaeta in the corresponding site. The model also explained the observation that breakpoints upstream of *sc* affected different sets of SOs than those downstream of it (Leyns et al. 1989).

In contrast, a similar phenotypic analysis performed with *ac* breakpoints suggested that the *ac* region of the AS-C had a simpler structure and contained fewer *cis*-controlling elements (Ruiz-Gómez and Modolell 1987). A problem thus arose to explain the essentially identical patterns of *ac* and *sc* expression. A solution was suggested by the expression in transgenic flies of a *lacZ* reporter gene driven by fragments of DNA containing either the *ac* or *sc* promoter regions (Martínez and Modolell 1991; Van Doren et al. 1992). It was found that the Ac and Sc proteins could activate, directly or indirectly, the transcription of *lacZ* mediated by the promoter of the reciprocal gene. Hence, it was proposed that *ac* and *sc* genes were first activated in complementary spatial domains in response to different *cis*-controlling sequences. Subsequently, each gene product would stimulate the expression of the other gene, thus generating similar patterns of expression. This proposal was supported further by the observation that deletion of one gene (together with large regions of contiguous AS-C DNA) led to the absence of both proneural gene products in the sites specified by its *cis*-regulatory sequences (Martínez and Modolell 1991; Skeath and Carroll 1991). In addition, this cross-activation could explain the paradox that although both genes were expressed in the corresponding proneural clusters, many SOs seemed dependent on only one of them for development (García-Bellido 1979). This "dependency" would simply indicate which gene was the "founder" of the corresponding proneural cluster, but it would not demonstrate a preference of the SO for either gene product. It is known that at least the *sc* gene expressed by means of a *hsp70* promoter can generate SOs that are typically dependent on the presence of the *ac* gene (Rodríguez et al. 1990).

In this work we have searched for the putative *cis*-regulatory elements in different regions of nontranscribed AS-C DNA. We have isolated several of them and shown, using a *lacZ* reporter gene fused in the leader sequences to fragments of the *ac* and/or *sc* promoter, that, as predicted by the model, they induce strong expression in sites corresponding to specific proneural clusters. However, with the help of new and classical AS-C mutations, we find that contrary to the above proposal to explain *ac/sc* coexpression (Martínez and Modolell 1991; Skeath and Carroll 1991), the endogenous *ac* and *sc* genes do not detectably cross-activate each other in most proneural clusters. Apparently, coexpression results from the activation of both genes by a single set of *cis*-regulatory elements, with each element possessing a unique spatiotemporal specificity.

Results

An enhancer-like element in the sc *promoter region*

A DNA fragment (3.7 kb) of the *sc* promoter region, which includes the transcription initiation site, directs strong expression of the *lacZ* reporter gene in two patches of the wing discs of third-instar larvae (Fig. 2B,C; Martínez and Modolell 1991). These patches are located at the proximal end of the presumptive anterior margin of the wing blade and at the dorsal part of the third vein. Two proneural clusters of *ac* and *sc* expression, those giving rise to the twin sensilla of the anterior wing margin (TSM) and to the anterior cross vein (ACV) and third vein (L3) sensilla campaniformia, colocalize with these two patches (Fig. 2, cf. A with B and C). We have found that the sequences extending 0.76 kb upstream from the nucleotide located 185 bp upstream of the *sc* transcriptional start (Fig. 1) also drive a similar expression, even when fused to the heterologous *hsp70* promotor (Fig. 2D). For the sake of simplicity, we will refer to this and other similar controlling elements as "enhancers," and to this particular element as the L3/TSM enhancer.

Isolation of enhancers in regions far removed from the ac *and* sc *transcribed sequences*

We searched for other enhancers that promoted expression at the wing imaginal disc in DNA fragments far removed from the AS-C–transcribed sequences. Some of the fragments chosen were delimited by chromosomal breakpoints that deleted different sets of notum macrochaetae. Other fragments were selected because they contained long, well-conserved sequences when compared, in heteroduplex analyses, with fragments located in equivalent regions of the AS-C from *Drosophila virilis* (Beamonte 1990). To maximize the chances of detecting controlling elements, assays were performed with the homologous *sc* promoter. Accordingly, each fragment was ligated upstream of the *3.7sc–lacZ* gene (whose expression is shown in Fig. 2B), and the resulting constructs were introduced into flies. The presence of the L3/TSM enhancer in these constructs was a positive control for expression of the transgene in each transformant line. The *sc* promoter fragment also carried sequences that drove expression in SMCs (Fig. 2B; Martínez and Modolell 1991). These provided topographical markers that helped assign the enhancer's localized expression to specific proneural clusters (Huang et al. 1991; Campuzano and Modolell 1992). Expression of the constructs was also examined in a ac^- sc^- ($In(1)sc^{10.1}$) background. This abolished expression in SMCs because these cells do not emerge in the absence of *ac/sc* (Romani et al. 1989), and consequently, it simplified the patterns of expression (Fig. 2, cf. B and C).

The phenotypes associated with a set of terminal deletions of the X chromosome suggested that an enhancer

for the dorsocentral (DC) cluster, which gives rise to the anterior and posterior DC macrochaetae (Fig. 1), is located approximately between 5 and 9 kb upstream of *ac* (Ruiz-Gómez and Modolell 1987). A 5.7-kb fragment encompassing this region [coordinates 62.9–68.6 of the AS-C molecular map (Fig. 1; Campuzano et al. 1985)] drove a strong expression in the DC area (Fig. 2E) that is concentric with that cluster (Fig. 2A). Additionally, it promoted weaker expression in relatively small adjacent areas of the presumptive prescutum and postnotum, two other sites of *ac/sc* expression.

In(1)sc^{9} and other chromosomal breakpoints to the left of it remove the anterior notopleural (ANP) macrochaeta, but *T(1;2)sc*^{S2} and the breakpoints to its right do not remove this bristle (Campuzano et al. 1985). Thus, a DNA fragment approximately spanning these breakpoints (coordinates 6.0–0.0, Fig. 1) was assayed for the ANP enhancer. This 6-kb fragment did promote expression in the ANP cluster and also in the clusters of the giant sensillum of the dorsal radius (GSR), the dorsal radius (dR), distal tegula (dTG), and the large cluster that gives rise to the anterior postalar (APA) macrochaeta and the tr1 and tr2 sensilla trichoidea (Fig. 2F). A 1.2-kb subfragment (Fig. 1; coordinates 1.2–0.0) directed expression in only the GSR, dR, and dTG clusters (Fig. 2G), which indicated that at least part of the sequences that promote expression in the ANP and APA/tr1/tr2 clusters are separable from those specific for the other sites. A 0.6-kb fragment from the central part of the 1.2-kb segment failed to promote expression (data not shown). We did not attempt to dissect this GSR/dR/dTG enhancer further.

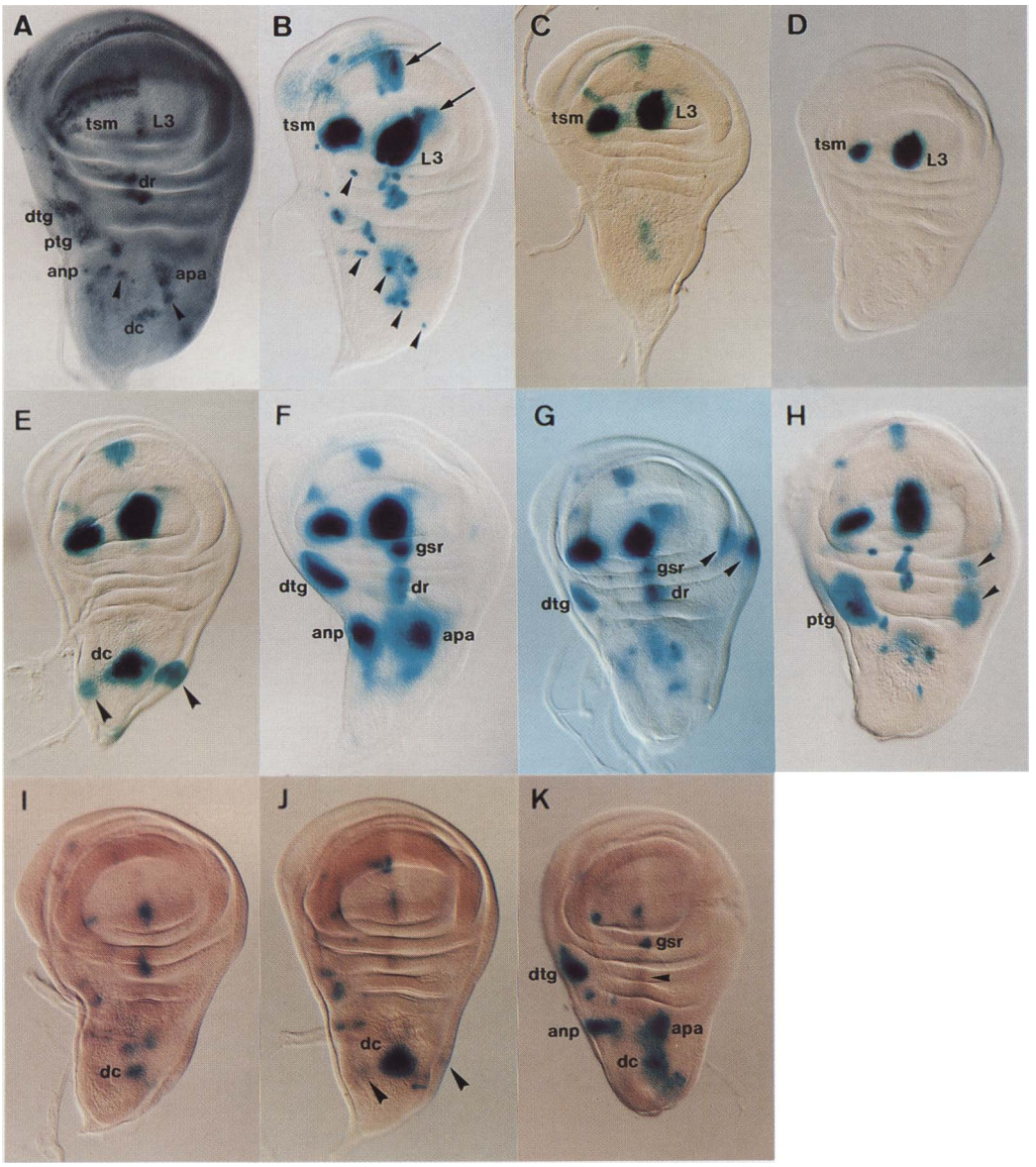

Figure 2. (*See facing page for legend.*)

The 12-kb region between the *sc* and *lethal of scute* (*l'sc*) structural genes was examined by interspecific heteroduplex analyses for regions of preferential sequence conservation. Under the moderately stringent conditions used, only two regions were identified (Beamonte 1990). One was contained within the 3.1-kb segment (coordinates 32.0–28.9) immediately downstream of *sc*. It did not drive any expression in the five lines assayed. The other was included in a 3.8-kb fragment (coordinates 26.3–22.5) and promoted expression in the proximal tegula (pTG; Fig. 2H).

In all experiments, expressions promoted by the L3/TSM and SMC enhancers were not detectably modified, suggesting the absence of interactions between these and the other enhancers assayed. Moreover, in all cases expression driven by the isolated enhancers was independent of the endogenous *ac* and *sc* genes (Fig. 2, cf. B with E and F; J.L. Gómez-Skarmeta and J. Culí, unpubl.). This demonstrates that the enhancers respond to cues other than the localized expression of *ac/sc*, possibly to constituents of the disc prepattern.

We have shown that the L3/TSM enhancer can drive a heterologous promoter (Fig. 2D). We examined further whether the isolated enhancers were also capable of driving the *ac* promoter by linking them upstream of a 0.8-kb *ac* promoter fragment fused to *lacZ* (Martínez and Modolell 1991). In the absence of enhancers, the *0.8ac–lacZ* transgene was weakly expressed in most proneural clusters and some SMCs because of activation by the endogenous Ac and Sc proteins (Fig. 2I; Martínez and Modolell 1991). The presence of the DC enhancer (coordinates 62.9–68.6) or the ANP and other enhancers contained in the 6-kb fragment between coordinates 6.0 and 0.0 (the only ones assayed) strongly activated the *ac* promoter in the proneural clusters specific for these enhancers (Fig. 2J,K). The only exception was the dR cluster, where expression was even weaker than in the control without enhancers (Fig. 2I).

Endogenous ac *and* sc *genes do not detectably cross-activate each other*

The *ac* and *sc* genes have similar patterns of expression in imaginal discs (Romani et al. 1989; Cubas et al. 1991; Skeath and Carroll 1991). This coexpression has been explained assuming that *ac* and *sc* are initially activated

Figure 2. Isolation of enhancer elements from the AS-C DNA. (*A*) Wild-type expression of *sc* as revealed by an anti-Sc antibody. Clusters for which an enhancer element has been isolated are named, except that for the GSR sensillum, whose cells no longer express *ac/sc* in late third-instar discs. In comparison with the disc of Fig. 4C, arrowheads point at the ASA and PPA clusters. (*B*) and (*C*) Expression of the *3.7sc–lacZ* transgene (Martínez and Modolell 1991) in a wild-type and an ac^- sc^- [$In(1)sc^{10.1}$] background, respectively. Note the very strong expression in the L3 and TSM regions and the weaker expression in other patches (arrows). Both are independent of the presence of *ac* and *sc*. Expression in individual cells in *B* is dependent on *ac/sc* and corresponds to SMCs (some are indicated by arrowheads). Most expression in the weaker patches (arrows) is ectopic and may be attributable to the presence in the constructs of sequences for activation unaccompanied by the sequences necessary for repression (Martínez and Modolell 1991). Other examples of ectopic expression observed with fragments of the AS-C are shown in *G* (arrowheads) and in Martínez et al. (1993). (*D*) Expression driven by the isolated L3/TSM enhancer. Note that the patches of β-galactosidase accumulation are larger than the corresponding proneural clusters of *sc* expression (cf. *A*). This is most likely because of the relatively slow turnover of β-galactosidase and to the fact that these clusters develop over a period of >24 hr at a time when the disc is growing at an average rate of approximately a cell doubling every 8–9 hr (García-Bellido and Merriam 1971). Hence, β-galactosidase can be present in cells long after its synthesis has ceased. Consistent with this explanation, the patches of *lacZ* mRNA are more compact (J. Culí, unpubl.), and the TSM and L3 proneural clusters generated by a *sc* minigene (Rodríguez et al. 1990) with upstream sequences (4 kb) similar to those of the *3.7sc–lacZ* transgene are of wild-type size (not shown). (*E*) Expression driven by the DC enhancer [$In(1)sc^{10.1}$ background]. This pattern of expression, although with different degrees of intensity, was observed in 11 of the 13 independent transformant lines obtained. Expression is strongest in the DC area and weaker in small areas of the presumptive prescutum and postnotum (arrowheads), two additional areas of *ac/sc* expression. In the wild type, no SMCs single out from the postnotum, probably because of the presence of high levels of the proneural antagonist Extramacrochaetae protein (Cubas and Modolell 1992). SMCs for microchaetae appear in the prescutum several hours after puparium formation (Hartenstein and Posakony 1989; Usui and Kimura 1993). (*F*) The fragment between coordinates 6.0 and 0.0 contains enhancers for the ANP, GSR, dR, dTG, and APA/tr1/tr2 proneural clusters [$In(1)sc^{10.1}$ background]. The same pattern has been found in five out of six independent transformant lines obtained. (*G*) The 1.2–0.0 fragment directs expression in only the wing group of proneural clusters, namely, the GSR, dR, and dTG. The same pattern was observed in two lines obtained. Arrowheads point at ectopic sites of expression characteristic of this construct. (*H*) The 3.8-kb (26.3–22.5) fragment promotes expression in the pTG area in six out of seven lines obtained. It also promoted expression in two areas of late *ac/sc* expression (arrowheads) that do not give rise to SOs. *G* and *H* correspond to discs in an ac^+ and sc^+ background, as the presence of SMCs facilitate unambigous identification of the dTG and pTG clusters. (*I*) The *0.8ac–lacZ* transgene, which contains the *ac* promoter region, expresses *lacZ* weakly in many proneural clusters because of activation by endogenous Ac/Sc proteins [Martínez and Modolell 1991; in the data shown in this reference, expression promoted by *0.8ac–lacZ* and other constructs appears much stronger because of overnight incubations with X-gal (instead of the 30 min to 2 hr used here) or to the highly sensitive detection of β-galactosidase mediated by a specific antibody]. (*J*) The isolated DC enhancer activates the *0.8ac* promoter similarly as it does to the *sc* promoter (cf. *E*) in six out of six lines tested. Expression in the small areas of the presumptive prescutum and postnotum (arrowheads) was particularly weak in this line but stronger in others (not shown), although in no line was as strong, relative to the DC cluster, as in *E*. (*K*) The 6-kb fragment (coordinates 6.0–0.0) drives the *0.8ac–lacZ* transgene in the same proneural clusters as the *3.7sc–lacZ* transgene, except at the dR (arrowhead). Interestingly, the presence of the enhancers increased the expression of *lacZ*, presumably dependent on Ac/Sc, in the DC cluster (cf. *I*).

in complementary spatial domains in response to different enhancer sequences and that each gene product stimulates expression of the other gene (Martínez and Modolell 1991; Skeath and Carroll 1991; Van Doren et al. 1992). However, coexpression can also be accomplished if each position-specific enhancer drives expression of both genes. To distinguish between these alternatives, we have examined the expression of *ac* and *sc* when the regions of the AS-C bearing these genes are far removed from each other, as in the *In(1)sc*^{8} chromosomal rearrangement (Fig. 1). If coexpression is mainly accomplished by activation mediated by the gene products, physical separation should have a minimal effect; but if it is accomplished by shared, position-specific enhancers, coexpression should disappear. The results clearly support the second alternative. The Ac protein present in large amounts in the DC cluster and to smaller extents in the dHCV and vHCV clusters (dorsal and ventral humeral cross-vein sensilla, respectively) (Fig. 3A) failed to activate *sc* expression at these sites (Fig. 3B); similarly, the Sc protein present in many clusters, like the L3, TSM, dR, dTG, pTG, anterior notal wing process (ANWP), ANP, posterior notopleural (PNP), APA, and scutelar (SC) (Fig. 3B), did not activate *ac* in any of them (Fig. 3A). Note the complementarity of the *ac* and *sc* patterns of expression, which suggests that most enhancers are not duplicated in the *ac* and *sc* regions of the complex. The main exception was the presumptive anterior wing margin (WM), where coexpression was maintained.

Support for the second alternative was also provided by the excess function *Hairy wing*^{1} (*Hw*^{1}) mutation. *Hw*^{1} is associated with a *gypsy* transposon inserted within the *ac*-coding sequences (Fig 1; Campuzano et al. 1986). The resulting protein, truncated 77 amino acids before its carboxyl terminus (Villares 1986), conserves the bHLH domain and is active in proneural function (Campuzano et al. 1986). The development of supernumerary SOs in ectopic positions is attributable to the generalized transcription of the *ac–Hw*^{1} gene in imaginal discs (Balcells et al. 1988). Figure 3C shows that this generalized transcription did not activate *sc* in ectopic positions. Moreover, *sc* was not expressed in the DC cluster except in a few isolated cells at this site and the surrounding area. These cells most likely correspond to extant and ectopic SMCs that have singled out under the influence of the Ac–Hw^{1} protein and express *sc* under the control of the SMC-specific activator.

It has been proposed that the *gypsy* transposon acts as a "barrier" for enhancer action and that this repressive effect requires its binding with the product of the *su(Hw)* locus (Geyer and Corces 1992). Thus, the absence of *sc* expression in the DC cluster of *Hw*^{1} discs could be attributable to the *gypsy*–Su(Hw) protein complex, located between the DC enhancer and the *sc* promoter, that would block enhancer action. This seemed to be the case. Removal of the Su(Hw) product, which suppresses both *ac–Hw*^{1} overexpression (Campuzano et al. 1986) and the Hw phenotype (Lewis 1949), largely restored *sc* expression in the DC cluster (Fig. 3D).

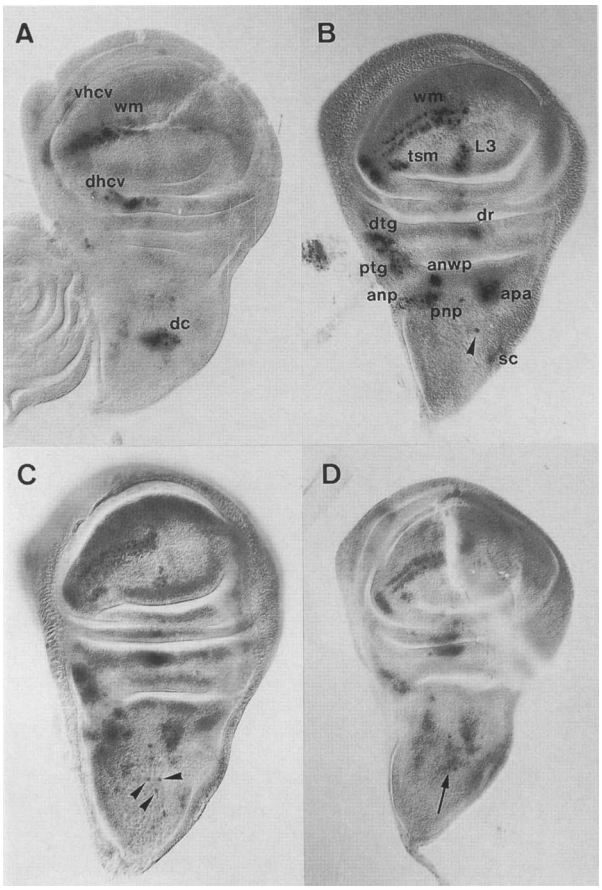

Figure 3. *ac* and *sc* do not cross-activate each other. Wing imaginal discs of the *In(1)sc*^{8} were stained with anti-Ac antibody (*A*) or anti-Sc antibody (*B*). Note that except for the prospective wing margin (wm), all of the remaining proneural clusters contain either Ac or Sc protein, but not both. Singled-out SMCs accumulate both Ac and Sc proteins. The arrowhead in *B* points to an Sc-stained SMC singled out of the *ac*-expressing DC cluster. (*C,D*) Sc protein accumulation in discs from *Hw*^{1} and *Hw*^{1}; *su(Hw)*^{2}/*su(Hw)*^{f} mutants, respectively. Despite the strong, ubiquitous expression of *ac* in *Hw*^{1} (Campuzano et al. 1986; Balcells et al. 1988), *sc* is not overexpressed and the DC proneural cluster fails to form. The single cells that accumulate Sc protein in the DC area (arrowheads) most likely correspond to extant and ectopic SMCs that have singled out under the influence of the Ac-Hw^{1} protein. The *su(Hw)* mutant condition restores the DC proneural cluster in *D* (arrow).

ac *expression is independent of* sc

As an additional test to discriminate between cross-activation and shared enhancers, we performed an extensive ethylmethane sulfonate (EMS) mutagenesis to obtain null *ac* or *sc* mutants with unbroken, *cis*-continuous AS-C DNA. In these mutants the expression of one gene would take place in the absence of the reciprocal gene product and without disconnecting the enhancer elements. Only one suitable mutation, *sc*^{M6}, was recovered. *sc*^{M6} harbors a nonsense codon in the *sc* gene that should terminate translation after a lysine residue lo-

cated at the start of the HLH domain (Murre et al. 1989). Therefore, the entire HLH domain, essential for bHLH factors to function (Murre et al. 1989; Davis et al. 1990; Hinz et al. 1994), and the carboxy-terminal part of the protein should be missing. A polyclonal anti-Sc antibody (Skeath and Carroll 1991) did not stain the sc^{M6} imaginal discs (Fig. 4A), although the mutated *sc* gene was normally transcribed (Fig. 4B). In spite of the absence of functional Sc protein, the Ac protein accumulated in all proneural clusters (Fig. 4C). Taken together, this and the above experiments permit us to conclude that most, if not all, *ac* expression is independent of the Sc protein.

Levels of Ac and Sc proteins in proneural clusters

If *ac*/*sc* coexpression is accomplished by shared enhancers rather than by cross-activation, it seemed pertinent to examine whether all proneural clusters accumulate similar relative levels of Ac and Sc proteins. Conceivably, a shared enhancer could activate the nearest gene most strongly and the farthest gene, to a lesser an extent. In general and in agreement with previous results (Skeath and Carroll 1991), the relative levels of these proteins, as detected with the help of double staining with anti-Ac and anti-Sc antibodies and confocal microscopy, were similar in most or all well-developed proneural clusters (Fig. 5D). This suggests that the enhancers activate *ac* and *sc* to similar relative extents, regardless of their proximity to each gene. However, in some cases, we detected a preferential accumulation of Sc, as at the posterior postalar (PPA) cluster during its initial stages (Fig. 5E) and at the most medial part of the SC cluster (Fig. 5F). These experiments also showed that within many clusters some cells were preferentially stained with either one of the antibodies, suggesting that they were relatively deficient in the reciprocal protein (Fig. 5A–C, E). Preferential accumulation of either protein also supports the conclusion that cross-activation mediated by the product of the reciprocal gene is not significant in regulating the expression of *ac* and *sc* in proneural clusters.

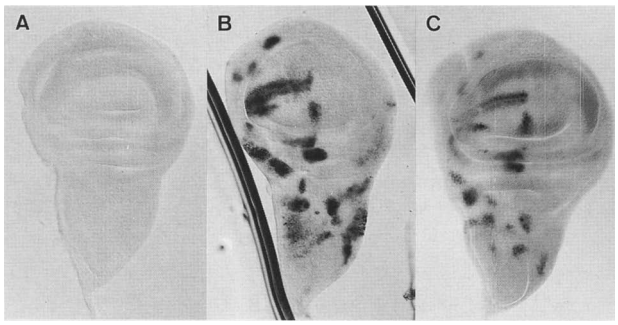

Figure 4. *sc* and *ac* expression in the sc^{M6} mutant. (*A*) An anti-Sc polyclonal antibody failed to detect Sc protein. (*B*) A DIG-labeled *sc* RNA probe shows that *sc* was transcribed normally in the sc^{M6} mutant. (The thick bars are artifacts of the microscopic preparation.) (*C*) An anti-Ac antibody revealed that the Ac protein accumulated in all proneural clusters of the mutant wing disc (cf. Fig. 2A; the sc^{M6} disc is younger than that in Fig. 2A, and its ASA and PPA clusters have not yet developed).

ac *and* sc *expression requires similar AS-C controlling regions*

We have shown that some of the isolated enhancers can drive expression of the *ac* and *sc* promoters (Fig. 2). In these experiments, enhancers and promoters have been placed next to each other and out of the AS-C context. Hence, it is still possible that in the intact AS-C enhancers might be specific for either *ac* or *sc*. To investigate this point we examined whether different regions of the right half of the AS-C containing putatively shared enhancers were equally necessary for the expression of *ac* and *sc*. It has been shown previously that the sc^{6} deletion and the sc^{7} and sc^{4} inversions (Fig. 1) remove *sc* expression from different sets of proneural clusters (Cubas et al. 1991). Figure 6 shows that these mutations also remove *ac* expression from the same sets of clusters. We thus conclude that, for at least some proneural clusters, similar AS-C regions are necessary to drive expression of *ac* and *sc* and that most likely the same enhancers promote expression of both genes.

Phenotype of the sc^{M6} *mutant*

The bristle phenotypes on the notum of sc^{M6}, ac^{-} $(Df(1)y^{3PL}sc^{8R})$ and sc^{-} $(Df(1)sc^{8L}sc^{4R})$ males are shown in Table 1. The macrochaetae suppressed by the sc^{M6} mutation are different from those removed by either deletion or by other *sc* or *ac* mutations (García-Bellido 1979). Thus, although the posterior dorsocentral (PDC), anterior dorsocentral (ADC), and posterior supra-alar (PSA) are affected similarly in sc^{M6} and in the *sc* deletion, many macrochaetae that are completely removed by this deletion [PNP, APA, presutural (PS), anterior supra-alar (ASA), and anterior and posterior scutelar (ASC and PSC)] are present in sc^{M6} individuals, some in all of them (PNP and PSC). This demonstrates that these macrochaetae, thought to depend on *sc* for development (García-Bellido 1979), can be generated by *ac* when expression of this gene is not impaired by the removal or blockade of enhancer elements. To our knowledge, sc^{M6} is the only available mutant in which the function of one AS-C gene is removed without simultaneous inactivation of enhancers. Moreover, the *ac* gene in the sc^{M6} mutant is most likely fully functional, as its product appears to be identical to that of the wild type (Materials and methods), accumulates at comparable levels (Fig. 4C), and complements the *sc* gene in chaetae development essentially as well as the *ac* gene in $Df(1)sc^{8L}sc^{4R}$ (Table 1, last two columns). Thus, the sc^{M6} phenotype may most closely represent the effect of the absence of *sc* function, without concomitant impairment of the *ac* function. Although *ac* and *sc* functions may be largely interchangeable (García-Alonso and García-Bellido 1986; Ruiz-Gómez and Modolell 1987; Balcells et al. 1988; Rodríguez et al. 1990; this paper), it seems clear

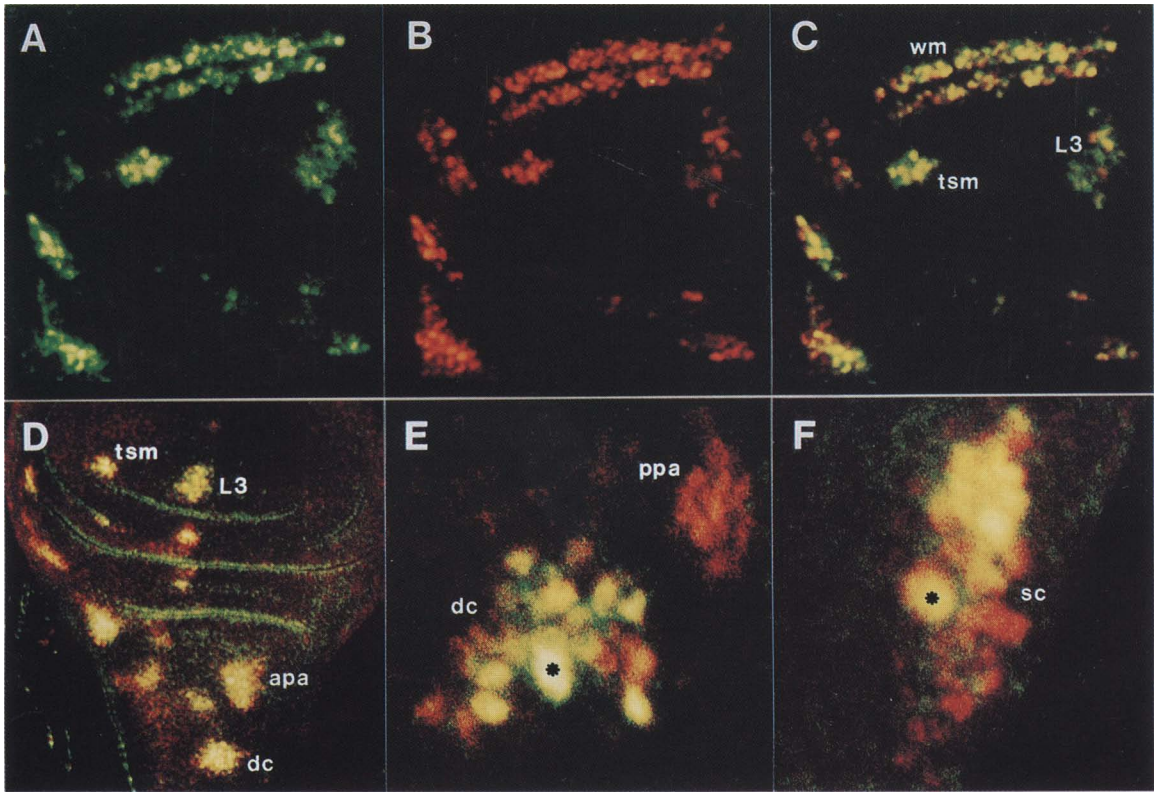

Figure 5. Ac and Sc proteins accumulate to similar levels in many, but not all, cells of proneural clusters. Discs doubly stained with anti-Ac and anti-Sc were examined under confocal microscopy. Ac is shown in green and Sc in red. Nuclei with strong staining with both antibodies appear yellow. (*A–C*) Separate Ac (*A*), Sc (*B*), and composite (*C*) views (intermediate power) of clusters at the wing pouch and dorsal wing hinge of a late third-instar disc; (wm) prospective wing margin. Ac and Sc distributions are similar but not identical. (*D*) Low-power view of a mid-third-instar disc. Most cells in different clusters accumulate similar relative levels of Ac and Sc proteins. Ac/Sc accumulation at the prospective wing margin has not yet started. (*E,F*) High-power views of DC and SC clusters, respectively. Note that in all intermediate and high-power images many nuclei were strongly stained with both antibodies but other nuclei were preferentially stained with one of the antibodies, indicating that the relative levels of Ac and Sc are not the same in all cells of a cluster. This is seen most clearly in the L3 (*C*), very early PPA (*E*), and the lower part of the SC clusters (*F*). The strongest staining nuclei in *E* and *F* correspond to SMCs (asterisks).

that *ac*, when fully expressed, is still insufficient to promote development of a full set of SOs.

Discussion

Position-specific enhancers drive ac/sc *expression in proneural clusters*

During the third-instar and early pupal stages, the *ac* and *sc* genes are coexpressed in the imaginal discs in many separate groups of cells, the proneural clusters (Romani et al. 1989; Cubas et al. 1991; Skeath and Carroll 1991). Each cluster has a characteristic size, shape, and developmental time of emergence and disappearance. Moreover, the pattern of accumulation of Ac and Sc proteins among the cells of a cluster is reproducible to some extent, as the highest accumulating cells always appear in the same position within a cluster. The SMCs are selected among these cells. Thus, *ac* and *sc* are expressed in complex and tightly controlled patterns that delimit the regions where SMCs will emerge. Here we show that as suggested by genetic data (Ruiz-Gómez and Modolell 1987; Leyns et al. 1989), expression in proneural clusters is driven by *cis*-controlling elements present within the large (~90 kb) nontranscribed regions of the complex. Relatively small fragments of AS-C DNA drive strong expression of a *lacZ* reporter gene, fused to the *sc* or *ac* promoter, in one or a few regions of the imaginal discs that correspond to specific proneural clusters. Thus, the highly complex patterns of *ac* and *sc* expression are constructed piecemeal by separable elements that promote expression in one or a few clusters.

Expression driven by these regulatory elements does not depend on *ac/sc*. As suggested previously (Ghysen and Dambly-Chaudière 1988), it is probably induced by the binding to these sequences of appropriate combinations of factors heterogeneously distributed [prepattern (Stern 1954)] in the disc epithelium. When isolated, these sequences promote expression at only one or a few sites,

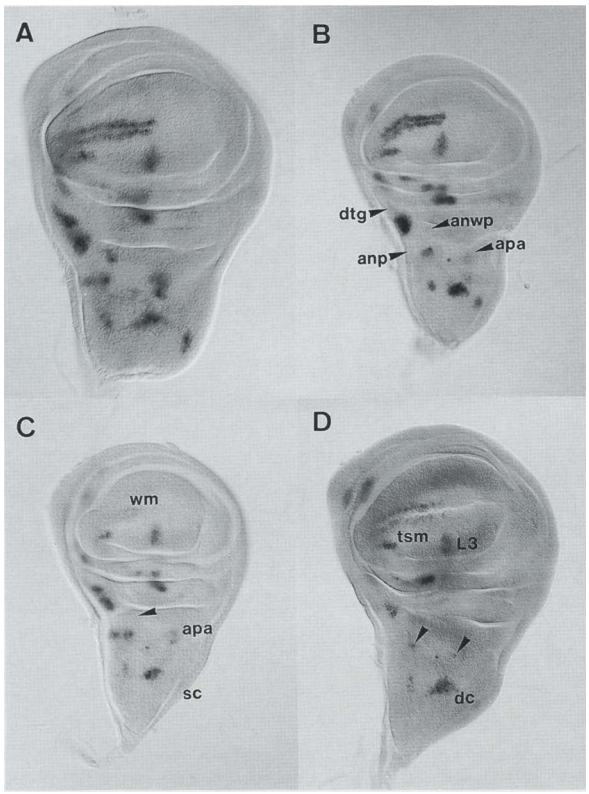

Figure 6. *ac* expression in wild-type and *sc* mutant discs of late third-instar larvae. (*A*) Wild-type distribution of Ac protein. (*B*) sc^6 mutant disc. Note the absence of Ac protein in the ANP, dTg, and ANWP clusters, and the strong reduction in the APA region. (*C*) $In(1)sc^7$ mutant disc. Note the absence of the SC and ANWP (arrowhead) clusters and the reduction of Ac protein accumulation in the APA and anterior wing margin (wm) regions. (*D*) $In(1)sc^4$ mutant disc. This inversion disconnects most of the *sc* downstream region, and Ac accumulation is absent or strongly diminished in many clusters. Note the wild-type level of Ac accumulation in the DC, L3, and TSM clusters, whose enhancers remain *cis*-connected to the *ac* gene (Fig. 1). In each of these mutants, Sc accumulation is decreased in the same clusters and to approximately the same extent as the Ac accumulation (see Fig. 5 of Cubas et al. 1991), which suggests that the same enhancers drive expression of *ac* and *sc*. Similarly as observed with *sc* expression (Cubas et al. 1991), the absence of a cluster of cells strongly expressing *ac* does not prevent development of the PNP and APA macrochaetae, and their SMCs are visible in *D* (arrowheads).

which indicates that enhancer sequences with different topological specificity require different combinations of prepattern factors. At present, most factors have not been identified. Exceptions are the *hairy* gene product, which binds near to the *ac* promoter and prevents ectopic expression of this gene in the early pupal stages (Skeath and Carroll 1991; Blair et al. 1992; Orenic et al. 1993; Ohsako et al. 1994; Van Doren et al. 1994), and the *iroquois* product, a homeo domain-containing protein that appears to bind to the L3/TSM enhancer (J.L. Gómez-Skarmeta, unpubl.). Another possible constituent of the prepattern is the Pannier protein (Ramain et al. 1993).

The removal or disconnection of the enhancers promoting expression in the L3, TSM, ANP, and GSR proneural clusters eliminates *ac/sc* expression and the corresponding SOs (Campuzano et al. 1985; Leyns et al. 1989; Cubas et al. 1991; Fig. 6; Gómez-Skarmeta et al., unpubl.). These enhancers are probably unique in activating *ac/sc* in these proneural clusters. Expression in some other regions of the wing disc may be promoted by more than one enhancer. Thus, deletion of the DC enhancer (and the *ac* gene), which prevents *sc* expression in the DC cluster and emergence of the corresponding SMCs (Cubas et al. 1991; Martínez and Modolell 1991; Skeath and Carroll 1991; Fig. 3B), does not completely eliminate the DC macrochaetae (Table 1). Most likely, the late occurring expression of *sc* in this and adjacent areas, which promotes emergence of the microchaetae precursors (Usui and Kimura 1993), partially rescues the DC macrochaetae. Similarly, removal of enhancers that drive expression in the pTG and/or dTG and dR clusters [$In(1)sc^4$ or sc^6 deletion] almost does not affect the sensilla campaniformia arising at these sites (D. Ferrés-Marcó, unpubl.), even though $In(1)sc^4$ reduces *ac/sc* expression very strongly in these clusters and sc^6 suppresses it in the dTG region in late third-instar discs (Fig. 6; Cubas et al. 1991). Two or more enhancers may also be responsible for expression at the prospective anterior wing margin (Fig. 3A,B). Alternatively, *ase*, another proneural gene of the AS-C (Fig. 1), which is expressed at the WM proneural cluster and in all SMCs (Brand et al. 1993; Domínguez and Campuzano 1993), may *trans*-activate

Table 1. *Presence (%) of notum bristles in* sc^{M6}, $ac^-[Df(1)y^{3PL}sc^{8R}]$ *and* $sc^-[Df(1)sc^{8L}sc^{4R}]$ *males, and* $sc^{M6}/ac^-[sc^{M6}/Df(1)y^{3PL}sc^{8R}]$ *and* $sc^-/ac^-[Df(1)sc^{8L}sc^{4R}/Df(1)y^{3PL}sc^{8R}]$ *females*

Bristle[a]	sc^{M6} [b]	ac^-	sc^-	sc^{M6}/ac^-	sc^-/ac^-
PDC	100	10	100	100	100
ADC	6	68	17	62	78
PSA	6	10	38	37	17
PNP	100	100	0	100	100
APA	89	100	0	100	100
PS	17	100	0	100	100
ASA	39	100	0	100	100
PPA	0	100	0	100	100
ANP	0	100	0	100	100
ASC	89	91	0	100	100
PSC	100	100	0	100	89
Microchaetae	84	53	100	71	93
N	18	22	24	8	18

[a] *N* equals number of heminota examined. Number of microchaetae per heminotum in Oregon-R individuals was 91 ± 6 (males) and 108 ± 4 (females). These figures were taken as 100%.
[b] sc^{M6} females did not survive, most likely because of the absence of sc^+ (= $sis\text{-}b^+$) function in the blastoderm (Torres and Sanchez 1989). Essentially identical results to those shown for $sc^{M6}/Df(1)y^{3PL}sc^{8R}$ were obtained with $sc^{M6}/Df(1)ac^3$ females (Materials and methods).

sc (and *ac*), as in the *ase*^{1} deletion *sc* expression is decreased strongly at this site and is restored by an *ase* transgene (Domínguez and Campuzano 1993). Finally, it is also possible that in the absence of enhancers, a basal transcription of *ac/sc* can generate some SOs in specific positions (Rodríguez et al. 1990).

Enhancers, and not cross-activation, promote coexpression of ac *and* sc

The current model for *ac* and *sc* regulation assumes that each of these genes is initially activated by different enhancers in complementary sets of proneural clusters, and then the product of each gene activates the reciprocal gene and coexpression is achieved (Martínez and Modolell 1991; Skeath and Carroll 1991; Van Doren et al. 1992). The model is based first on the ability of the Ac protein to stimulate expression of the *3.7sc–lacZ* transgene in the DC and PSA areas of the wing disc and of the Sc protein to activate *ac–lacZ* transgenes on most of the remaining clusters of this disc; and second on the concomitant loss of both *ac* and *sc* expression from complementary sets of proneural clusters when either *ac* or *sc* is deleted. In contrast, we find that the endogenous *ac* and *sc* genes do not stimulate each other detectably. This conclusion is based on several results. Thus, physical separation of the *ac* and *sc* regions of the AS-C prevents coexpression, so that under this condition either *ac* or *sc*, but not both, is expressed in most proneural clusters. The Ac–Hw^{1} protein, although present in large excess, does not stimulate *sc* in the DC cluster. In *sc*^{M6} mutant discs, the *sc* gene is normally transcribed but the Sc protein is undetectable, most likely because the gene has a stop codon at the beginning of the sequence encoding the HLH domain. The truncated protein without the HLH dimerizing domain should be nonfunctional (Murre et al. 1989; Hinz et al. 1994). Still, the *ac* gene is expressed in a seemingly wild-type pattern, which shows that its expression does not depend on Sc. Although *ac* and *sc* are coexpressed in all proneural clusters of the imaginal wing disc, double stainings with anti-Ac and anti-Sc antibodies reveal that a fraction of their cells accumulate different relative levels of the two proteins. Moreover, a large accumulation of Ac protein provided by an *upstream activating sequence* (*UAS*)–*ac* gene driven by a *HS–GAL4* gene (Brand and Perrimon 1993) does not activate the endogenous *sc* gene (I. Rodríguez and S. Sotillos, unpubl.), and reciprocally, the Sc protein provided by a *sc* transgene driven by a heat shock promoter (*HSSC*; Rodríguez et al. 1990) does not activate the endogenous *ac* gene (I. Rodríguez, unpubl.). Taken together, these data indicate that at most sites of the wing imaginal disc, coexpression of *ac* and *sc* is not mediated by reciprocal activation. How can this conclusion be reconciled with the activation (Martínez and Modolell 1991; Martínez 1992; Van Doren et al. 1992) of the *ac–* and *sc–lacZ* transgenes by Sc and Ac, respectively? Because the level of β-galactosidase accumulation attributable to Ac/Sc activation is much smaller than that promoted by the AS-C enhancers (Fig. 2, cf. I with E and J), it is possible that the transgenes reflect a weak cross-activation between the endogenous *ac* and *sc* genes. This cross-activation, undetectable with anti Ac and Sc antibodies, becomes detectable when a protein with a slow turnover like β-galactosidase is synthesized and, consequently, can accumulate to substantial levels. Alternatively, the cross-activation in proneural clusters observed with the *ac/sc–lacZ* transgenes may be another case of misexpression (see legend to Fig. 2) attributable to the absence from the transgenes of sequences concerned with restricting transcription. The cross-activation between *ac* and *sc* has been proposed to be a target for the interference of *extramacrochaetae* (*emc*), a negative regulator of *ac/sc* expression (Van Doren et al. 1992; Martínez et al. 1993). Our results suggest that *emc* would rather interfere with elements defining the prepattern.

Our findings indicate that enhancer-like elements drive *ac/sc* coexpression in the proneural clusters of the wing disc, as has been proposed for the proneural clusters of the embryonic neuroectoderm (Skeath et al. 1992; Ruiz-Gómez and Ghysen 1993; for review, see Skeath and Carroll 1994). Although not proven, we suggest that the same elements drive the expression of both genes. We have shown that several enhancers, located either upstream of *ac* or downstream of *sc*, drive expression of the *ac* and the *sc* promoters in our constructs. This suggests that these enhancers are not specific for either promoter. Moreover, the complementary patterns of *ac* and *sc* expression observed when the *ac* and *sc* regions are separated [*In(1)sc*^{8}] indicate that with the possible exception of the WM enhancers with similar spatial and temporal specificity are not found in both of these regions. The observation that mutations that disconnect different parts of the long *sc* downstream region [Fig. 1, *sc*^{6} deletion, *In(1)sc*^{7}, and *In(1)sc*^{4}] remove both *ac* and *sc* expression from proneural clusters (Fig. 6, Cubas et al. 1991) also argues for the same enhancers activating both genes. (Evidently, within an enhancer there could be sequences specific for the activation of each gene.) The nonduplication of enhancers in the *ac* and *sc* regions easily explains the second observation that led to the cross-activation model, namely, the concomitant loss of expression of both genes from complementary sets of proneural clusters when either *ac* or *sc* is deleted. The deletions used were large synthetic left–right deficiencies created by recombination between inversions [*Df(1)y*^{3PL}*sc*^{8R} and *Df(1)sc*^{8L}*sc*^{4R}; Martínez and Modolell 1991; Skeath and Carroll 1991]. They not only removed the *ac* or *sc* gene but they also deleted, or disconnected from the remaining gene, all of the enhancers with unique spatio-temporal specificities located at either the left or the right of the *sc*^{8} breakpoint, respectively (Fig. 1).

Near the origin of transcription of *ac* there are three E-boxes, putative interacting sites for bHLH proteins, that have been proposed to mediate *ac* self-stimulation and *sc trans*-activation, as measured in vivo with the help of *ac–lacZ* transgenes (Van Doren et al. 1992; Martínez et al. 1993). In a cell transfection assay, these E-boxes interact with Ac/Da or Sc/Da heterodimers and

activate an *ac–CAT* reporter gene. Moreover, they strongly potentiate the ability of an *ac* minigene to rescue in part the notum microchaetae (Van Doren et al. 1992). However, because there is no detectable *trans*-activation of the endogenous *ac* gene by Sc in all of the proneural clusters examined (with the possible exception of the anterior WM) these E-boxes probably mediate alternative regulatory interactions. One possibility is that they are concerned with *ac* self-stimulation (Martínez and Modolell 1991; Van Doren et al. 1992). Note, however, that self-stimulation seems unnecessary for *sc* to maintain high levels of transcription. Accumulation of *sc* mRNA does not depend on active Sc protein [sc^{M6} and $In(1)sc^{10.1}$ discs; Fig. 4; Cubas et al. 1991]. Moreover, a group of E-boxes are also present upstream of the *sc* promoter, and these probably mediate the high expression of this gene in SMCs (J. Culí, unpubl.). Thus, the *ac* E-boxes may similarly mediate *ac* expression in SMCs. Perhaps self- and cross-activation of *ac* and *sc* are restricted to SMCs and are necessary to attain the characteristic high accumulation of proneural proteins in these cells. This might explain the requirement for the E-boxes for the function of an *ac* minigene (Van Doren et al. 1992). The E-boxes may also facilitate binding of transcriptional controllers other than Ac or Sc, as their removal promotes expression in sites where *ac/sc* are not normally expressed (Martínez et al. 1993).

The present results and other data (Skeath et al. 1992; Ruiz-Gómez and Ghysen 1993) support a model in which single regulatory elements at either side of or in between *ac* or *sc*, two genes separated by ~25 kb of DNA, promote remarkably accurate coexpression of both genes in highly localized areas of the imaginal discs or the neurogenic regions of the embryo. A similar, albeit simpler, arrangement of genes and enhancers in *Drosophila* has been found for a pair of divergently transcribed neighboring genes, *yp1* and *yp2*, which encode yolk proteins (Logan et al. 1989; Logan and Wensink 1990). Transcription of this pair of genes is mediated by three enhancers, two interacting ones specific for certain lineages of ovarian follicle cells and the other for the fat bodies of adult females. One enhancer is located in the first exon of *yp2*, and the other two are in the intergenic region. It has been proposed that an equilibrium between alternative DNA loops allows *yp1* and *yp2* promoters to interact one at a time with, for instance, the fat body enhancer (Logan et al. 1989). Similar equilibria might allow the *ac* and *sc* promoters to interact alternatively with the site-specific enhancer that mediates activation in a particular proneural cluster and thus accomplish coexpression (Fig. 7). It is remarkable that nearly equal relative levels of Ac and Sc accumulation are achieved in many or most cells of different proneural clusters (Fig. 5). This suggests that the *ac* and *sc* promoters interact with similar efficiency with enhancers that are located with respect to both genes as differently as the DC (upstream of *ac*), L3/TSM (in between both genes but very close to *sc*), and ANP/PPA (downstream of *sc*, Fig. 7). We do not know of a similar case. The topological complexities of these interactions seem considerable but may be even greater during expression of the AS-C in the neurogenic region of the embryo. Here, *ac/sc* and *l'sc* are expressed simultaneously in partially overlapping patterns (Cabrera et al. 1987; Romani et al. 1987; Martín-Bermudo et al. 1991). *l'sc*, located proximally from *sc* and amid one of the regions containing *ac/sc*-specific enhancers (Figs. 1 and 7), is driven by another set of enhancers, also scattered within the AS-C DNA (Martín-Bermudo et al. 1993). Some of the enhancers may be shared by the three genes, whereas others are specific for either *ac/sc* or *l'sc*. The regulation of the AS-C thus poses a most interesting case of interactions between promoters and their *cis*-regulatory sequences.

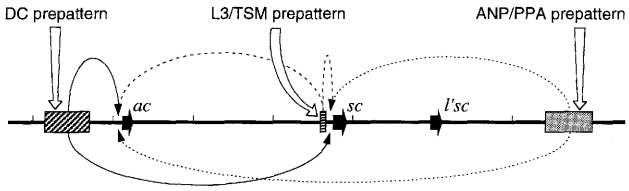

Figure 7. Model for the coexpression of *ac* and *sc* in proneural clusters of the imaginal wing disc. During disc growth and in response to the action of patterning genes and cell–cell interactions, heterogeneities develop that form a prepattern of factors that varies in time and among different regions of the disc. Prepattern factors bind to AS-C enhancers, and at the sites where appropriate combination of factors occur, e.g., at the DC region, a site-specific enhancer (the DC *cis*-element) and its complement of factors interact with the *ac* and *sc* promoters and activate transcription of both genes. The scheme is simplified in that only three enhancers are indicated, the DC and those that respond to L3/TSM and ANP/APA sets of prepattern factors. Presumably, to activate both the *ac* and *sc* genes, the enhancer with the full set of factors can reach an equilibrium between two loop conformations and alternatively interact with each of the two promoters (Logan et al. 1989). Regardless of their relative position with respect to the promoters, most AS-C enhancers seem capable of interacting with either gene and promote transcription with similar relative efficiencies.

Materials and methods

Drosophila *stocks and mutagenesis*

Drosophila stocks carrying mutations in the AS-C were from the collection of A. García-Bellido and are described in Campuzano et al. (1985), Villares and Cabrera (1987), and Lindsley and Zimm (1992). $y\ Hw^1\ w$ and $y\ Hw^1\ w;\ su(Hw)^2/TM6\ su(Hw)^f$ are described in Campuzano et al. (1986).

To obtain new *ac* mutations, 2- to 3-day old Oregon-R males were fed for 20–24 hr with 0.025 M EMS (Sigma) in 1% sucrose and crossed to $In(1)ac^3\ w^a$ females (3–5 days old), and the progeny scored for absence of notum and/or head bristles. From >28,000 chromosomes screened, only one mutation was recovered, and this turned out to be a *sc* mutation (sc^{M6}, see below and Results). The same mutagenized chromosomes yielded 0.3% *w*-independent mutations. $In(1)ac^3\ w^a/sc^{M6}$ females lacked the notum PSA and ADC macrochaetae (86% and 75% of heminota, respectively). The *ac*-transcribed region and part of its promoter [from G_{823} to G_{1615} (Villares and Cabrera 1987)] from the mutant allele was sequenced and found identical to the

wild-type Canton-S sequence. In contrast, the *sc* open reading frame was interrupted by a stop codon generated by the change of the wild-type C_{1000} (Villares and Cabrera 1987) to a T. Salivary chromosomes from sc^{M6} male larvae had normal morphology. We did not attempt to obtain additional *sc* alleles.

Plasmid constructions and fly transformations

AS-C DNA fragments assayed for the presence of *cis*-controlling sequences were 5.7-kb *Eco*RI (map location: 68.6–62.9, Fig. 1), 3.7-kb *Hin*dIII–*Hpa*II (37.2–33.5), 0.76-kb *Cla*I–*Xho*I (34.6–33.8), 3.1-kb *Bam*HI–*Eco*RI (32.0–28.9), 3.8-kb *Eco*RI (26.3–22.5), 6.0-kb *Bam*HI–*Eco*RI (6.0–0.0), 1.2-kb *Hin*cII–*Eco*RI (1.2–0.0), and 0.6-kb (0.9–0.3, prepared by PCR from the previous fragment). The *Hpa*II site at coordinate 33.5 is located within the *sc* leader sequence, 31 nucleotides upstream of the coding sequence (Villares and Cabrera 1987). The 3.7-kb *Hin*dIII–*Hpa*II (37.2–33.5) fragment containing this site was subcloned into plasmid pHSS7 (Seifert et al. 1986), to provide *Not*I sites, and introduced into transformation plasmid pLac20 (Schröder et al. 1988). In the resulting construct, the *sc* leader sequences were fused to *lacZ*. All fragments not containing the *Hpa*II (33.5) site, except 0.8-kb *Cla*I–*Xho*I (34.6–33.8), were first ligated upstream of the *sc* promoter fragment 3.7-kb *Hin*dIII–*Hpa*II (in pHSS7) and the resulting hybrid fragment introduced into pLac20. The 0.8-kb *Cla*I–*Xho*I (34.6–33.8) fragment, which does not contain the *sc* transcriptional origin (Villares and Cabrera 1987), was introduced into transformation plasmid HZ50PL (Hiromi and Gehring 1987), which contains a basal *hsp70* promotor fused to the *lacZ* gene. The 5.7-kb *Eco*RI and 6.0-kb *Bam*HI–*Eco*RI fragments described above were also ligated upstream of a 0.8-kb *Eco*RI–*Hae*II (59.6–58.8, Martínez and Modolell 1991) fragment that contained the *ac* promoter and the *ac* leader sequences (up to 47 nucleotides before the start of the *ac*-coding sequence; Villares and Cabrera 1987). The resulting hybrid fragments (in pHSS7) were introduced into pLac20. P-element-mediated transformation (Rubin and Spradling 1982) (ry^{506} stock) was performed with 0.3–0.4 mg/ml of DNA of each construct and 0.15 mg/ml of pΠ25.7wc DNA or pUChsΠΔ2-3 (Misra and Rio 1990).

Histochemistry

Imaginal discs were dissected in PBS, fixed in 1% glutaraldehyde for 2 min at 0°C, washed twice (5 min each) in PBS, stained with X-gal (0.2%) for 0.5–2 hr at 37°C, dehydrated in ethanol, and mounted in Canada balsam. Antibody staining of imaginal discs for observation with visible light was performed as in Cubas et al. (1991). Fluorescent double staining with monoclonal anti-Ac and preabsorbed (against *Drosophila* embryos, 10 hr) polyclonal rabbit anti-Sc antibodies (gifts from S.B. Carroll, University of Wisconsin, Madison) was performed similarly, except that secondary antibodies were anti-mouse-biotin (1/200, Amersham) for Ac, and anti-rabbit-FITC (1/40, Dako) for Sc. After 2 hr at room temperature in the dark, discs were washed four times in PBT (15 min each), and incubated (1 hr) with streptavidin/lissamine/rhodamine (1/400, Jackson). Finally, discs were washed in PBT as described above and mounted in Mowiol (Sigma). Images were acquired with a Zeiss LSM310 confocal microscope. In situ hybridization of whole mounts of imaginal discs was performed according to Tautz and Pfeifle (1989), as modified by Cubas et al. (1991) using a DIG-labeled probe prepared with a *sc* cDNA that spanned most of the corresponding gene-transcribed sequences (Campuzano et al. 1985).

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