Regulation of *achaete–scute* gene expression and sensory organ pattern formation in the *Drosophila* wing

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Adult *Drosophila* possess a large number of sensory organs, including large and small bristles and other types of sensilla, each arising from a single mother cell at particular positions in a reproducible pattern. Genetic studies have shown that sensory organ pattern formation is partly coordinated by a number of structurally similar, potential heterodimer-forming, helix–loop–helix (HLH) regulatory proteins. Here, by localizing regulatory gene expression during the development of normal and mutant imaginal discs, we show that two positive regulators of sensory neurogenesis, the proneural *achaete* and *scute* proteins, initially *trans*-activate each other and are transiently expressed in identical patterns, including clusters of wing ectodermal cells and the individual sensory mother cells that arise from them. Two negative regulators, *hairy* and *extramacrochaete*, suppress sensory neurogenesis by selectively repressing *achaete* and *scute* gene expression, respectively, but in different spatial domains and at different developmental stages. Surprisingly, we also find that the level of *achaete–scute* activity influences the level of *hairy* expression, thereby providing feedback control upon *achaete–scute* activity and sensory organ formation. Some or all of these interactions may involve specific dimerization reactions between different combinations of HLH proteins.

[*Key Words*: Neurogenesis; morphogenesis; imaginal discs; HLH proteins; *Drosophila*; pattern formation]

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Understanding the mechanisms that generate particular cell types at reproducible positions within an organism is a fundamental goal of developmental biology. In *Drosophila* the processes that create the precise and fixed pattern of the adult peripheral nervous system are amenable to genetic dissection. A number of mutations in *Drosophila* alter the pattern of sensory organs (SOs) (Stern 1954; Lindsley and Grell 1968; Garcia-Bellido and Santamaria 1978; Garcia-Bellido 1979; Botas et al. 1982), whereas a separate class of mutations alters the number but not the pattern of SOs, generating clusters of SOs in the place of a single SO (Dietrich and Campos-Ortega 1984; Simpson and Carteret 1989; for review, see Simpson 1990). Many studies have suggested that the pattern and number of SOs in the fruit fly are determined progressively (de la Concha et al. 1988; Brand and Campos-Ortega 1988; Ghysen and Dambly-Chaudiere 1988; Hartenstein and Posakony 1989; Romani et al. 1989). A prepattern of activators and repressors is postulated to restrict spatially the expression of the proneural *achaete* (*ac*) and *scute* (*sc*) genes (Ghysen and Dambly-Chaudiere 1989). Their mRNA patterns crudely foreshadow the adult SO patterns (Romani et al. 1989). In the simplest model, one cell from each proneural cluster is selected through an unknown mechanism to become a sensory mother cell (SMC). The SMC, presumably through cell communication, then inhibits its neighbors from becoming SMCs (lateral inhibition; for review, see Simpson 1990) and undergoes two differentiative divisions to give rise to a particular SO (Bate 1978; Hartenstein and Posakony 1989).

Genetic studies have identified several genes that regulate the number or position of SOs. The *ac–sc* complex (AS-C) contains four loci that influence larval and/or adult SO patterns. Loss-of-function mutations in the *ac* or *sc* genes remove particular adult SOs, while ac^-, sc^- flies lack most SOs altogether (Stern 1954; Garcia-Bellido and Santamaria 1978; Garcia-Bellido 1979; Ghysen and Dambly-Chaudiere 1988). Recessive mutations in the unlinked *hairy* (*h*) or *extramacrochaete* (*emc*) loci cause ectopic SOs to arise (Botas et al. 1982; Moscoso del Prado and Garcia-Bellido 1984a). In the case of *h* mutants, ectopic small bristles (microchaetes) arise on the notum and wing blade of flies bearing adult viable mutations of the gene (Moscoso del Prado and Garcia-Bellido 1984a,b; Ingham et al. 1985) or in clones of cells homozygous for embryonic lethal pair-rule alleles (Ingham et al. 1985). In *emc* mutants, ectopic large bristles (macrochaetes) arise on the notum of flies bearing hypo-

This paper is dedicated to the late Dr. J. Edward Skeath.

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morphic *emc* alleles (Botas et al. 1982). The SO patterns of *ac*, *sc*, *emc*, and *h* mutants are summarized in Figure 1. Gene dosage studies and double mutant analyses (Moscoso del Prado and Garcia-Bellido 1984a,b) have suggested that *h* and *emc* are negative regulators of the AS-C. Because the *ac* gene is epistatic to *h* and the *sc* gene is epistatic to *emc*, it has been proposed that *h* and *emc* are repressors that interact with *ac* and *sc*, respectively (Moscoso del Prado and Garcia-Bellido 1984a). The absolute specificity of this interaction is not certain, as some genetic studies have uncovered some effect of *emc* upon *ac* function (Garcia-Alonso and Garcia-Bellido 1988) in particular regions of the adult.

Molecular studies of all four genes have revealed that they each encode proteins that possess a helix–loop–helix (HLH) motif (Villares and Cabrera 1987; Rushlow et al. 1989; Ellis et al. 1990; Garrell and Modolell 1990) found in a number of proteins involved in transcriptional regulation and cell determination. The HLH domain is required for homodimerization/heterodimerization to occur among members of the HLH family (Murre et al. 1989b; Davis et al. 1990). Most HLH proteins, including *ac* and *sc*, also contain a basic region just to the amino-terminal side of the HLH domain that appears to confer DNA-binding specificity and transcription-activating properties on dimers of these basic HLH (B-HLH) proteins (Davis et al. 1990). In some members of the HLH family, the basic domain is absent (*emc*) (Ellis et al. 1990; Garrell and Modolell 1990) or altered (*h*) (Rushlow et al. 1989). Id, a mammalian HLH protein that lacks a basic domain, has been shown to associate specifically with three B-HLH proteins and to inhibit their ability to bind DNA (Benezra et al. 1990). These studies have suggested that heterodimer formation regulates the activity of HLH proteins (for review, see Olson 1990). It has been proposed, on the basis of their genetic interactions and their structure, that the *h* and *emc* gene products inhibit *ac* and *sc* activity by sequestering the *ac* and *sc* proteins in inactive heterodimers (Ellis et al. 1990; Garrell and Modolell 1990).

To investigate the respective roles and interactions among the *ac*, *sc*, *emc*, and *h* proteins during *Drosophila* SO development, we have developed antibody probes that localize the sites of *ac*, *sc* (J.B. Skeath, B.S. Thalley, and S.B. Carroll, in prep.), and *h* (Carroll et al. 1988) protein expression. By examining the level and relative patterns of these three proteins in wild-type and a variety of mutant wing imaginal discs, we have determined that (1) *ac* and *sc* are expressed in identical patterns that are created by mutual *trans*-activation; (2) SMCs are singled out from clusters of *ac*- and *sc*-expressing cells, and *ac/sc* protein disappears from the SMC before its first differentiative division; the non-SMCs gradually lose *ac* and *sc* expression without differentiating; (3) *h* and *emc* selectively repress *ac* and *sc* gene expression, respectively, but at distinct phases of sensory neurogenesis; (4) overexpression of *h* extinguishes *ac* expression only from non-SMCs, and not from SMCs, (*ac/sc* protein expression is therefore somehow qualitatively different in the SMC than in the surrounding epidermal cells); and (5) a previously undetected feedback loop exists between *ac/sc* and *h* where the level of *ac/sc* activity influences the level of *h* expression, probably through an indirect mechanism which, in turn, down-regulates *ac* expression.

Results

ac *and* sc *are expressed in the same cells*

ac and *sc* promote the development of largely complementary sets of SOs (Garcia-Bellido 1979; Dambly-Chaudiere and Ghysen 1987). The function of these genes has been studied most intensely in the *Drosophila* wing disc, one of the few adult structures for which a detailed fate map is known (Bryant 1975) (Fig. 2C). We used antibodies specific to the *ac* and *sc* proteins (J.B. Skeath, B.S. Thalley, and S.B. Carroll, in prep.), respectively, to determine the expression patterns of these proteins in the *Drosophila* wing disc during the late third-instar (LTI) stage (LTI-stage wandering larvae). The *ac* and *sc* proteins are expressed in complex but essentially identical patterns of ectodermal cells that largely correspond to the location of SMCs deduced from the wing fate map (cf. Fig. 2A–C; see Cubas et al., this issue). For example, *ac* and *sc* expression corresponds to the anterior, but not the posterior, wing margin (Fig. 2A and B),

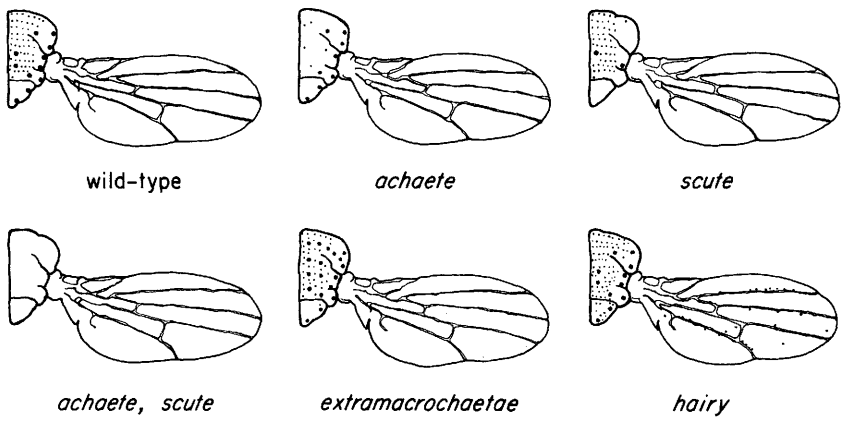

Figure 1. Adult SO patterns of regulatory mutants. The heminotum and wing blade chaetae patterns are diagramed. The large and small dots indicate the positions of macrochaetes and microchaetes, respectively. Note that *ac* mutations remove most microchaetes but only a few macrochaetes, while *sc* mutations remove most macrochaetes but few, if any, microchaetes. Double mutants lack all indicated sense organs. *emc* mutations increase the number of macrochaetes on the notum, while *h* mutations increase the number of microchaetes on the notum and wing blade but do not affect the macrochaete pattern. (Adapted from Moscoso del Prado and Garcia-Bellido 1984b.)

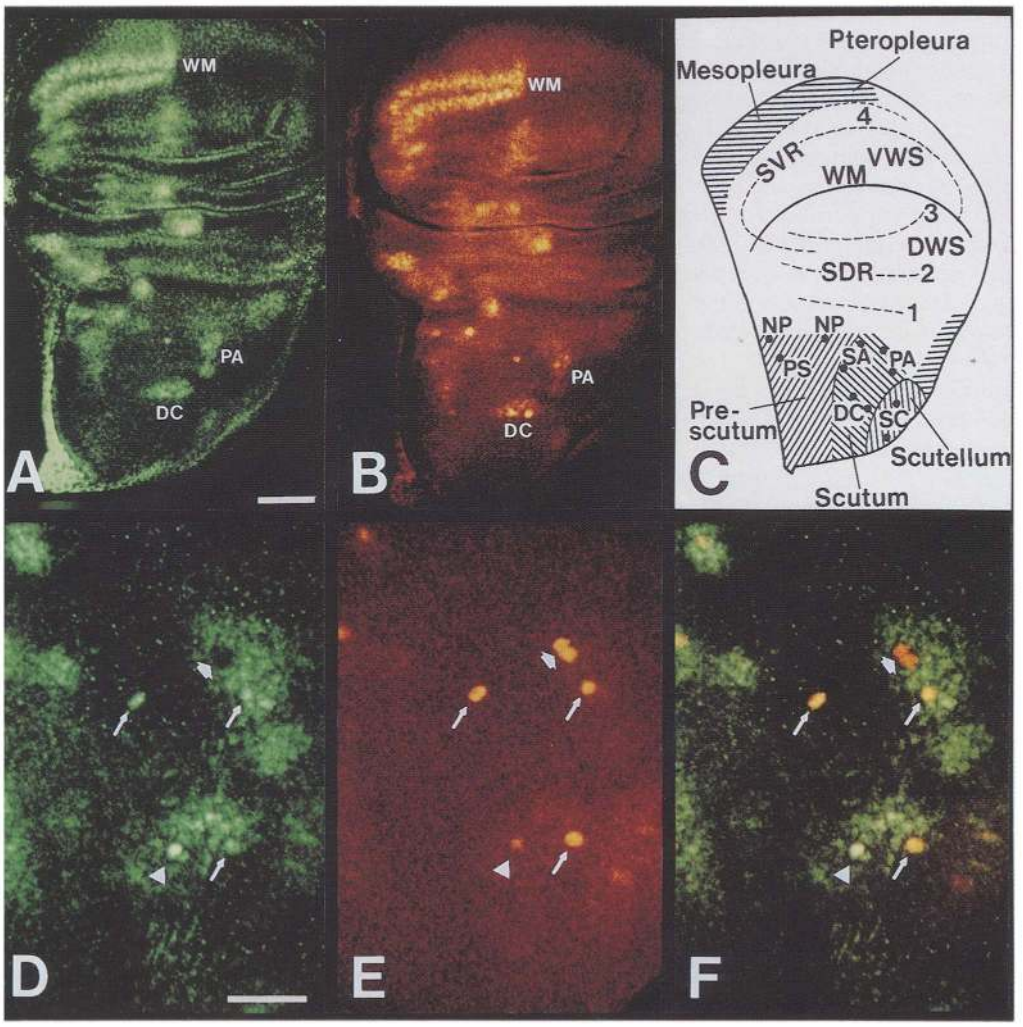

Figure 2. Coincident expression of the *ac* and *sc* proteins in the wing imaginal disc and the dynamics of SMC formation. Expression of the *sc* (*A*) and *ac* (*B*) proteins in a late third-instar stage wing imaginal disc visualized by double-label immunofluorescence and confocal microscopy. Bar, 50 μm. The proteins are expressed in identical clusters of ectodermal cells as well as single SMCs; note the bright cells in the dorsocentral (DC) cluster, which will give rise to two bristles, and the two rows of stained cells that mark the wing margin (WM). (*C*) A schematic of the general fate map of the wing disc indicating the location of presumptive regions for adult structures including various sensory organs; (for further details concerning the nomenclature of various structures and the individual chaetae, see Romani et al. 1989). The notum consists of the region below the fold marked *1*, and the development of the notal bristles will be dealt within subsequent figures. (*D–F*) The relationship between *sc* protein expression (*D*) and SMC differentiation (*E*) is illustrated by the correspondence between the pattern and level of *sc* protein (*D*) with the level of β-galactosidase protein (*E*) accumulating within SMC of flies carrying an enhancer trap expressed specifically in SMCs (A101.1F3). Double immunofluorescence staining reveals that cells expressing the highest level of *sc* (*D–F*, arrowhead) are just beginning to express the β-galactosidase SMC marker, whereas certain other cells in which *sc* expression is waning (*D–F*, long arrows) have much higher levels of β-galactosidase. SMCs that have lost *sc* expression entirely (short arrow) undergo two divisions, the first of which is illustrated by the pair of β-galactosidase-labeled cells in the cluster. (*F*) Merged image of *D* and *E* illustrating strong *sc*-positive cells (light green), weak *sc*/strong β-galactosidase cells (yellow–orange), and cells expressing only β-galactosidase (orange). Bar, 20 μm.

which carries a large number of innervated bristles in the adult. The identity of *ac* and *sc* expression and its correlation to the position of SMCs remains constant throughout development.

ac/sc *expression is highest in SMCs but is extinguished before they undergo division*

To determine more precisely the spatial and temporal relationship between *ac*/*sc* expression and SMC cell formation and differentiation, we have examined *sc* expression in wing discs of flies carrying a P-element *lacZ* enhancer trap, which is expressed early and exclusively in SMCs (for further details, see Cubas et al., this issue). Double-label immunofluorescence analysis demonstrates that the enhancer trap is activated in single cells with high levels of *sc* expression (Fig. 2D and E; arrow-

head), the SMC. As *sc* expression begins to wane in these cells, β-galactosidase expression becomes stronger (Fig. 2D and E; long arrows). Finally, *sc* expression disappears from the SMC before it divides, creating two β-galactosidase-labeled cells in the cluster shown in Figure 2, D and E (short arrow). Thus, *sc* (and *ac*; data not shown) is transiently expressed in SMCs, often at greater levels than surrounding cells of the proneural cluster, but not in their progeny. These observations are consistent with *ac/sc* expression playing a direct role in the decision of an ectodermal cell to become a sense organ but not in the final differentiation of the structure.

SMC commitment and proneural cluster extinction

Inspection of wing discs at several different times during LTI stage and early pupation reveals that SMCs arise from clusters of *ac/sc*-expressing cells, in particular spatial patterns and temporal sequences (for more detailed discussion, see Cubas et al., this issue). For example, the dorsocentral (DC) cluster consists of ~20 cells several hours before puparium formation (BPF) (Fig. 3a), ~10 cells shortly BPF (Fig. 3b), and only two cells, the putative SMCs of the DC bristles, by puparium formation (Fig. 3c). Finally, 2 hr later, only the SMC of the anterior DC bristle still expresses *ac* (Fig. 3d). Although the posterior postalar (PPA) cluster (Fig. 3) arises at a different time than the DC cluster, the expression of *ac* is similarly extinguished (Fig. 3a–d). At the times shown, *ac* and *sc* are expressed only in the putative SMC of the posterior supra-alar (PSA) bristle (Fig. 3), while the other notal SMCs exist within clusters of *ac*- and *sc*-labeled cells. A more detailed analysis of proneural cluster patterns and their differentiation is given by Cubas et al. (this issue), who have noted that the SMCs of certain clusters are surrounded by "halo"-like zones of reduced *sc* expression. These results suggest that the retention and high level of the *ac* and *sc* proteins in SMCs commit these cells to a neural fate and that the loss of *ac* and *sc* protein expression from the cells in the surrounding cluster reflects their loss of neural competency.

ac *and* sc *positively regulate each other*

Because the *ac* and *sc* genes are adjacent to each other in the genome (Campuzano et al. 1985) and are virtually certain to be related via some ancestral gene duplication, their identical expression patterns could occur due to the possession of common *cis*-acting elements to which each gene responds or by mutual *trans*-activation between the two genes. To differentiate between these possibilities, the expression of each gene was determined in wing discs of animals mutant for the other locus. The results are described most easily for the notal region of the disc. The adult thoracic notum bears 11 macrochaetes (mechanosensory bristles): 8 depend solely on *sc* ac-

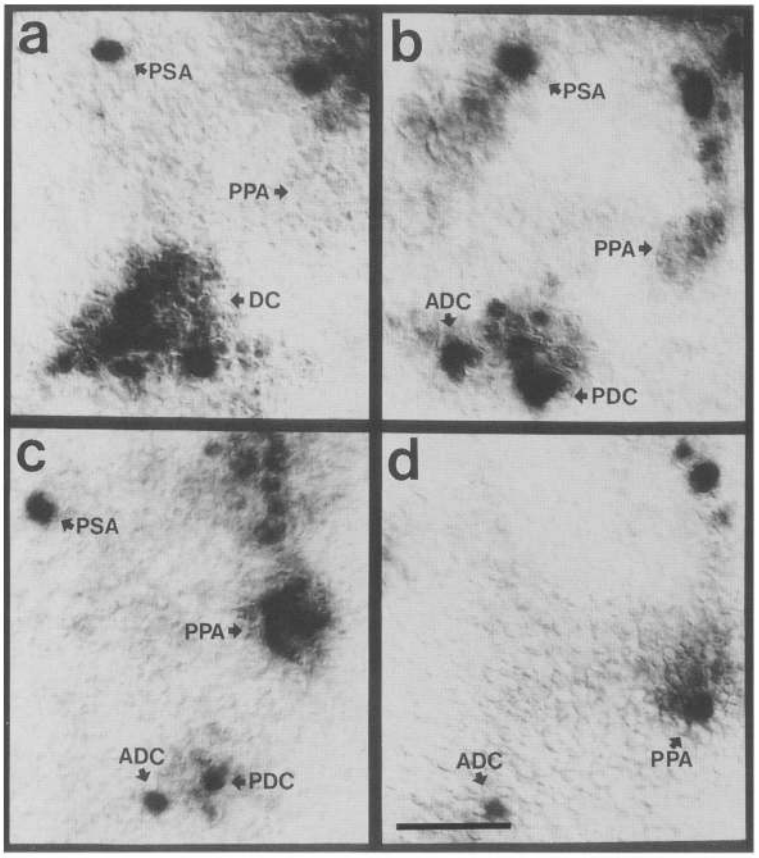

Figure 3. Dynamics of proneural gene expression and its extinction during wing disc development. The pattern of *ac* protein expression in the wild-type notum at four different points during wing disc development reveals that different clusters arise, refine, and differentiate in a particular and reproducible temporal sequence. The DC cluster progresses from a group of ~20 cells several hours BPF (*a*), to 10 cells shortly BPF (*b*), to just 2 putative SMCs by puparium formation (*c*), and just 1 SMC by 2 hr after puparium formation (APF) (*d*). Note that although the PPA cluster arises temporally after the DC cluster, it is similarly refined. Also, at the times shown *ac* and *sc* are only expressed in the putative SMC of the PSA bristle. Bar, 20 μm.

tivity; 2, the DCs, largely require *ac*; and 1, the PSA, depends on both *ac* and *sc* (for review, see Ghysen and Dambly-Chaudiere 1988). In the wild-type notum the patterns of *ac* and *sc* coincide with the locations from which all 11 bristles arise (Figs. 2 and 4a,c). Interestingly, the $In(1)ac^3$ mutation (no detectable *ac* protein accumulation occurs in flies carrying this mutation; J.B. Skeath and S.B. Carroll, unpubl.) removes *sc* expression from the cell, and from the cell cluster from which the *ac*-dependent PSA and DC bristles normally arise (Fig. 4b, arrowhead and arrow). Reciprocally, the $Df(1)sc^{8L4R}$ mutation, which removes the *sc* locus, eliminates *ac* expression from the notal clusters from which *sc*-dependent bristles arise (Fig. 4d). Additionally, the $Df(1)sc^{8L4R}$ mutation removes *ac* expression from specific regions of other imaginal discs, which give rise to *sc*-dependent SOs (data not shown). The reciprocal nature of these interactions, combined with recent evidence that *ac* and *sc* are initially activated in complementary spatial domains in response to different *cis*-controlling sequences (Martinez and Modolell 1991), suggests that the identical patterns of *ac* and *sc* result from two sequential processes. First, *ac* and *sc* are activated in complementary regions of the wing disc. Each gene product then stimulates expression of the reciprocal gene, generating identical expression patterns of the two genes.

emc *and* h *negatively regulate* sc *and* ac *gene expression*

Loss-of-function mutations in the *h* and *emc* genes cause ectopic SOs to arise on the notum and wing of adult flies (Botas et al. 1982). Genetic evidence has suggested that *h* and *emc* regulate sensory neurogenesis by repressing *ac* and *sc* function, respectively (Moscoso del Prado and Garcia-Bellido 1984a; Garcia-Alonso and Garcia-Bellido 1986, 1988). We find that the *ac* and *sc* protein patterns are subtly altered in the wing disc during the LTI stage in emc^{Pel}/emc^{E12} mutants (Fig. 5) and *ac* expression is modified in the wing blade from 5 to 6 hr after puparium formation (APF) in h^{R47}/h^{7H94} mutants (Fig. 6).

There are a number of important features to *sc*/*ac* expression in the notal region of *emc* mutant wing discs: novel sites of expression do not occur in clusters of cells but only in single cells, the locations of which correspond to the position of future ectopic SOs (Fig. 5b and

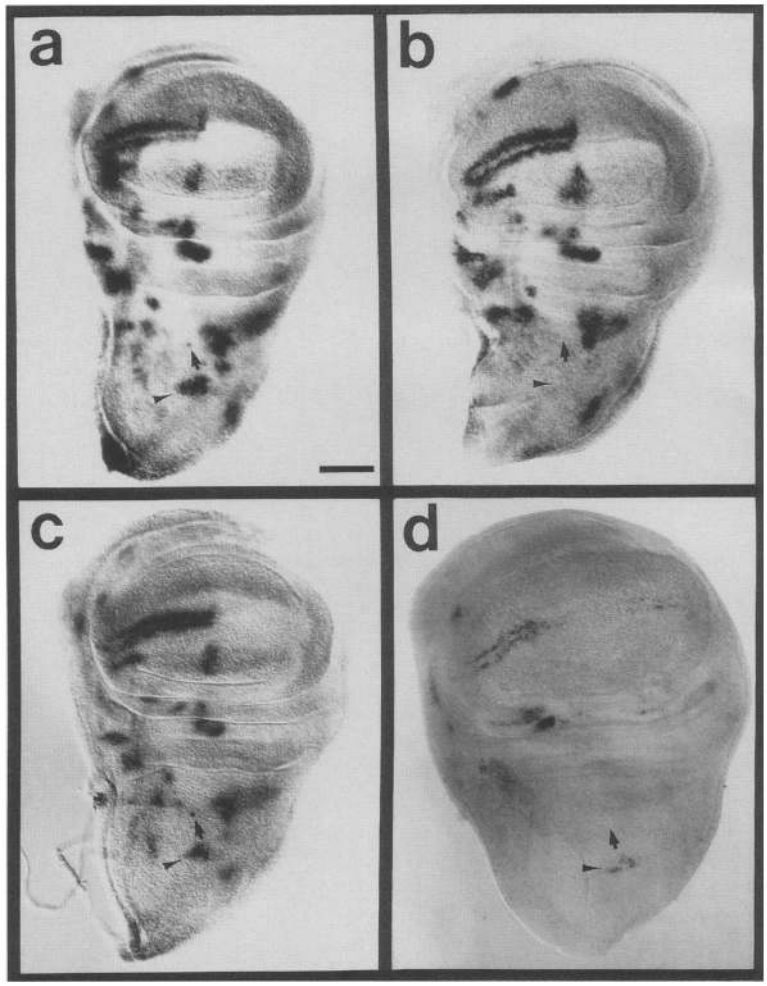

Figure 4. Regulatory interdependence of *ac* and *sc*. The expression of *sc* (*a*,*b*) and *ac* (*c*,*d*) in mutants lacking the other gene shows that in ac^3 homozygotes or hemizygotes, *sc* expression is lost from the region of the notum that gives rise to *ac*-dependent bristles (*b*, arrowhead; cf. the wild-type pattern in *a*). Conversely, in wing discs from sc^- animals, *ac* protein expression is lost from virtually all *sc*-dependent regions but remains in the *ac*-dependent regions (*d*, arrowhead; cf. wild-type *ac* pattern in *c*). In addition, note that the SMC that gives rise to the *ac*- and *sc*-dependent posterior PSA bristle does not arise in either the ac^- or the sc^- animals (*c*,*d*, arrow; cf. wild-type patterns in *a*,*b*). Bar, 50 μm.

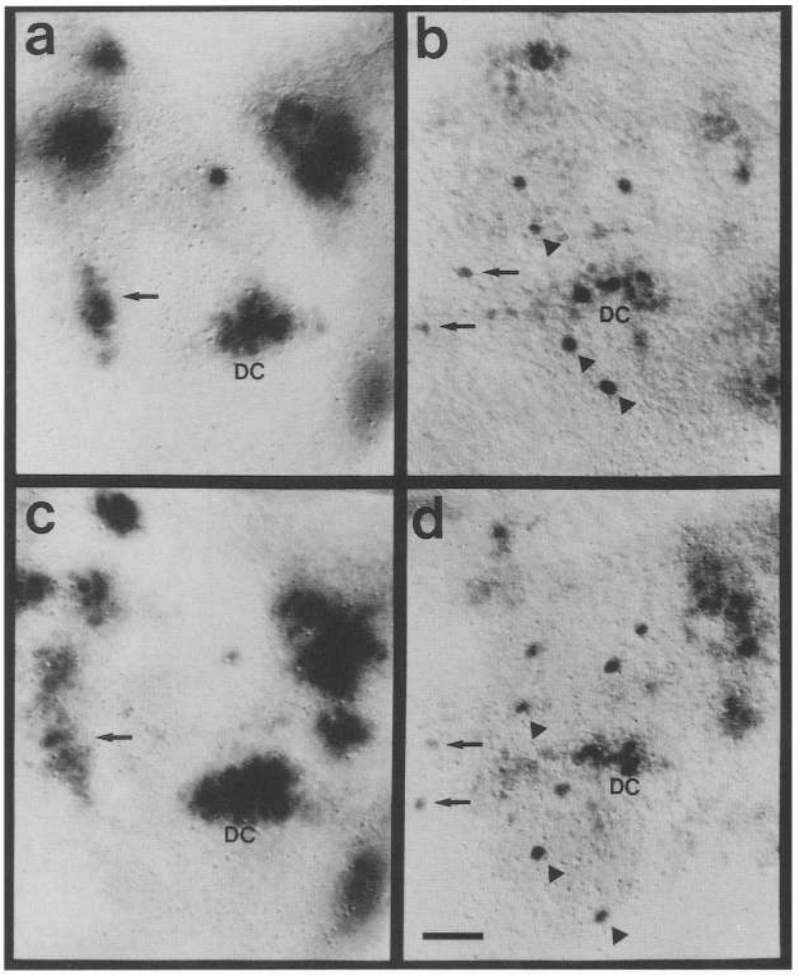

Figure 5. Derepression of proneural gene expression in *emc* mutants. (*a*) Notum region of wild-type wing disc stained with *ac* antibody. (*b*) Same region of emc^{Pel}/emc^{E12} wing disc, stained with *ac* antibody; note the numerous ectopic cells expressing *ac* (arrowheads) and the single cells expressing *ac* in place of the cluster, which does not give rise to a sense organ in wild-type flies (arrows). (*c*) Same region of wild-type wing disc stained with *sc* antibody. (*d*) emc^{Pel}/emc^{E12} wing disc stained with *sc* antibody; again note the ectopic cells expressing *sc* (arrowheads) and the cells expressing *sc* in single cells instead of in the "inactive" cluster (arrows). Note also the similarity between the *ac* and *sc* patterns, even in the mutant wing discs. Bar, 20 μm.

d); ectopic expression coincides temporally with wild-type expression (Fig. 5); and high-level expression also occurs in single cells in the notal region where a cluster normally exists but from which a macrochaete does not normally arise (Fig. 5) (in *emc* mutants macrochaetes arise from this area). In addition, Cubas et al. (this issue) have noted an increase in background levels of *sc* protein expression in *emc* mutants. These results suggest that *emc* inhibits ectopic SMC formation by repressing the expression of the *ac* and *sc* gene products. In this capacity *emc* may function as one of the elements required to set up the prepattern to which *sc* and *ac* respond.

Mutations in *sc* but not *ac* suppress the *emc* phenotype (Moscoso del Prado and Garcia-Bellido 1984a). Thus, it was unexpected that *ac* would be expressed ectopically in *emc* mutants (Fig. 5b). To determine whether any of the alterations in *sc* expression depend on *ac* we constructed an $In(1)ac^3/Y;emc^{Pel}/emc^{E12}$ double-mutant fly. In the absence of *ac* no change to the *emc* phenotype or to ectopic *sc* expression was observed (data not shown). This result, combined with the observed interdependence between *ac* and *sc*, shows that the *emc*-induced alterations in *sc* expression do not depend on *ac* function. Thus, it is likely that *emc* acts through *sc* and alterations of *ac* expression in *emc* mutants most likely depend upon *sc* function.

The strong adult viable *trans*-heterozygote h^{R47}/h^{7H94} exhibits inappropriate *ac* expression in regions of the imaginal wing blade, which give rise to ectopic SOs in the adult. In the h^{R47}/h^{7H94} mutant, the loss of *h* expression from the longitudinal preveins L3 and L5 (Fig. 6a and c) coincides with the ectopic appearance of *ac* along these preveins (Fig. 6b and d) ~5 hr APF. In contrast with the changes observed in *emc* mutants, ectopic *ac* expression in h^{R47}/h^{7H94} mutants occurs ~2 hr after wild-type *ac* expression has disappeared from along L3 (data not shown). These results suggest that *h* acts after the initial SO patterning process to inhibit inappropriate sensory neurogenesis by maintaining the *ac* locus in an inactive state.

Ectopic expression of h *represses* ac *expression*

We further investigated the regulatory role of *h* upon *ac* expression using a heat-shock-inducible construct of the *h* gene (HSH) (Ish-Horowicz and Pinchin 1987). It has been observed that generalized overexpression of *h* via a hsp70 promoter during pupal development has, surpris-

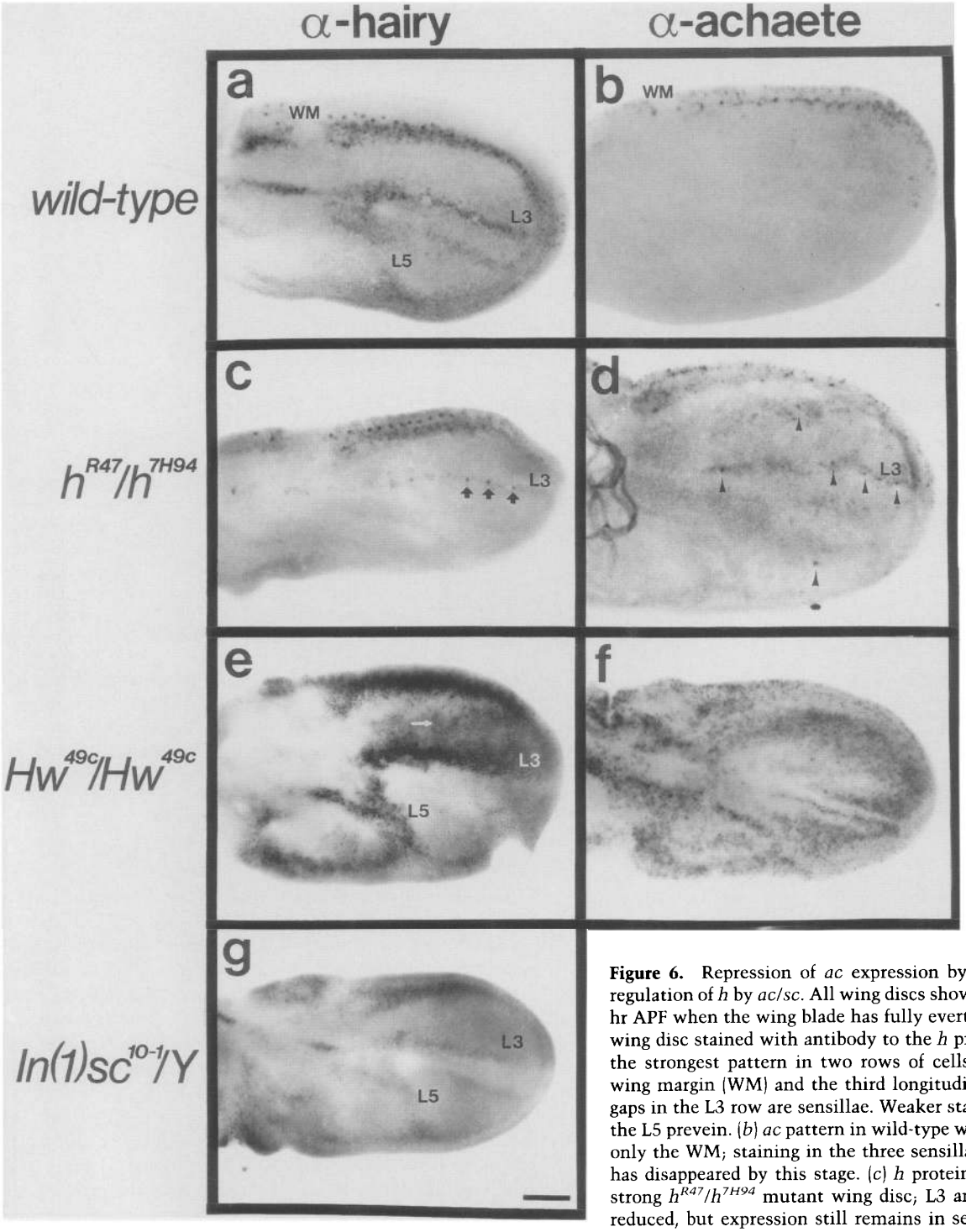

Figure 6. Repression of *ac* expression by *h* and feedback regulation of *h* by *ac/sc*. All wing discs shown (*a–g*) are ~5–6 hr APF when the wing blade has fully everted. (*a*) Wild-type wing disc stained with antibody to the *h* protein. Note that the strongest pattern in two rows of cells comprising the wing margin (WM) and the third longitudinal prevein (L3); gaps in the L3 row are sensillae. Weaker staining runs along the L5 prevein. (*b*) *ac* pattern in wild-type wing disc involves only the WM; staining in the three sensillae of the L3 vein has disappeared by this stage. (*c*) *h* protein expression in a strong h^{R47}/h^{7H94} mutant wing disc; L3 and L5 staining is reduced, but expression still remains in several cells along L3, which appear to be sensillae (arrows). (*d*) *ac* protein pattern in h^{R47}/h^{7H94} discs exhibits an ectopic pattern along L3, L2, and L5 (arrowheads). (*e*) *h* protein expression in Hw^{49c}/Hw^{49c} mutant wing disc is greatly elevated with the WM, L3, and L5 rows all wider and stronger, and intervein staining is much more pronounced (arrow). (*f*) *ac* is broadly expressed in this Hw^{49c}/Hw^{49c} disc, involving most of the structure except the more proximal part of the L3 region (unstained area near center). (*g*) *h* expression in $ac^{-}sc^{-}$ double-mutant wing disc [$In(1)sc^{10-1}/Y$] is greatly reduced from that of wild-type disc (*a*) and far below that observed in the Hw^{49c} disc (*e*). Bar, 50 μm.

ingly, no effect on normal SO development but does remove the ectopic bristles found in hypomorphic *h* mutants (Rushlow et al. 1989). To understand the direct effect of ectopic *h* expression on proneural gene expres-

sion, we examined the *ac* and *sc* patterns in the notal region of LTI stage larvae that overexpressed *h* when heat shocked. In heat-shocked wild-type larvae the level of *ac* and *sc* expression was reduced (this appears to be a nonspecific heat-shock effect as other proteins exhibit similar reductions in expression after heat shock, (N. Brown, pers. comm.), but no alteration to their patterns was observed (Fig. 7a; data not shown). In contrast, the notal patterns of *ac* and *sc* expression are modified in HSH larvae (Fig. 7c and d). Larvae that are heat shocked, either once for 1 hr or sequentially for 4.5 hr, exhibit identical modified *ac* patterns: the premature singling out of one or both of the SMCs of the *ac*-dependent DC cluster (Fig. 7c; data not shown) but no reproducible refinement of the other clusters (Fig. 7c). Refinement of *sc* expression lags behind that of *ac*. After a 1-hr heat shock period, *sc* expression in the DC cluster is incompletely refined (Fig. 7d) but is completely refined after the 4.5-hr sequential heat shock (data not shown). Thus, only the cells that surround the SMC of *ac*-dependent proneural clusters are sensitive to overexpression of *h*. This suggests that the nature of the *ac* and *sc* proteins and/or the regulation of their expression is different in SMCs than in the surrounding cells of the proneural cluster.

ac/sc *activity regulates* h *expression*

We have noted previously how *h* expression in wild-type imaginal discs often surrounds differentiated SOs and appears to increase in intensity APF (Carroll and Whyte 1989). This suggested to us a possible connection between SO formation and the regulation of *h* expression. Therefore, we examined *h* expression in both loss- and gain-of-function mutations in the *ac* and *sc* genes. We found that the level of *ac/sc* activity influences the expression level of *h*. The Hw^{49c} mutation, an inversion in the AS-C, widely overexpresses both the *ac* and *sc* proteins (Fig. 6e; J.B. Skeath, unpubl.) and causes ectopic SOs to form on the notum and wing of the fly (Balcells et al. 1988). In Hw^{49c} mutants the global pattern of *h* expression in the wing is not altered appreciably but the level of *h* expression dramatically increases, most noticeably along the preveins to L1, L3, and L5 (Fig. 6a and e) and in the interveins between L1 and L3 (Fig. 6e). Also, note how the *h* and *ac* patterns appear to complement each other, especially in the proximal region of L3, where *h* levels are high and *ac* expression is absent, reinforcing the suggestion that *h* suppresses *ac* expression. Hw^{1} mutants also transcribe *ac* in a generalized manner (Campuzano et al. 1986) and exhibit a similar increase in the *h* expression level (data not shown). Reciprocally, the pattern of *h* remains unaltered but its level is reduced, most noticeably along L3 (Fig. 6g), in the *ac/sc* double loss-of-function mutant, $In(1)sc^{10\text{-}1}$. The observations that the level (but not the spatial pattern) of *h* expression responds to the level of *ac/sc* activity, and that *h* in turn

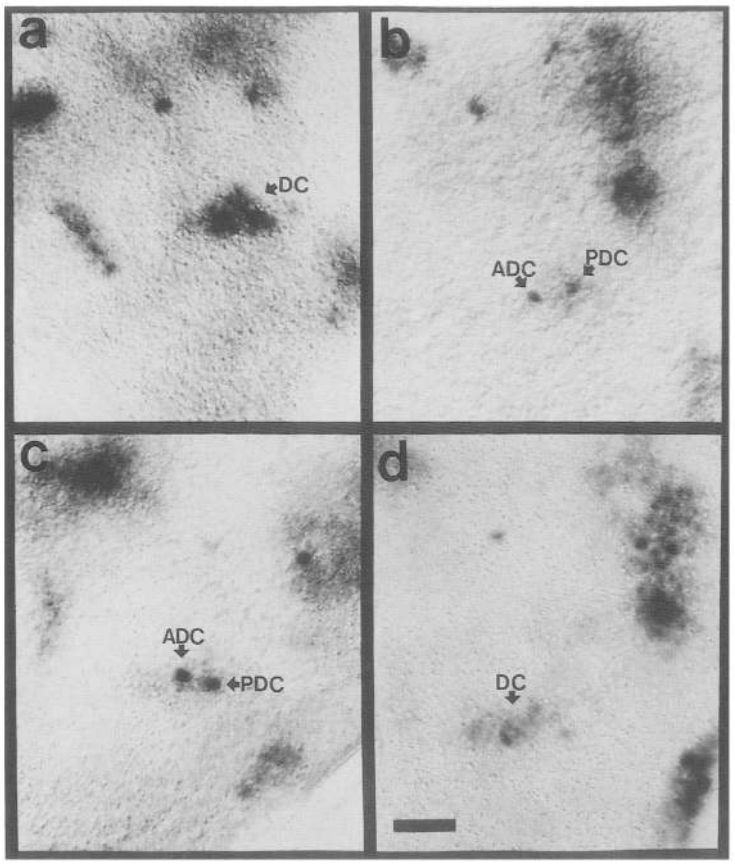

Figure 7. Ectopic expression of *h* represses *ac* expression but not in the SMC. (*a*) Wild-type LTI-stage wing disc after heat shock; *ac* expression is generally lower because of the nonspecific effects of heat shock. (*b*) A wild-type wing disc at puparium formation stained with *ac* for comparison with *c*. At this stage, the DC cluster is normally reduced in size. (*c*) *hsp-70* (HSH) LTI-stage wing disc after heat shock. The DC cluster has been reduced prematurely to two cells; the surrounding cells, which normally express *ac*, have been shut off. (*d*) HSH LTI-stage wing disc after heat shock stained with *sc* antibody. *sc* expression in the DC cluster appears to be reduced, though not as severely as *ac* expression (*c*). The effect on the DC cluster may be due to the dependence of *sc* on *ac* expression in this region. Bar, 20 μm.

represses *ac* expression, strongly suggest that there is a regulatory feedback loop between *ac/sc* and *h*.

Discussion

Expression and regulation of proneural genes during SO pattern formation

Our results strongly support prior general models concerning the relationship of proneural gene expression to SO pattern formation (Ghysen and Dambly-Chaudiere 1989; Romani et al. 1989; Simpson 1989). Specifically, we have shown that the *ac* and *sc* proteins are initially distributed in clusters of ectodermal cells from which one or two SMCs are singled out (see also Cubas et al., this issue). The remaining cells lose *ac/sc* expression and presumably become epidermal cells. *ac/sc* protein expression then disappears from the SMC even before the first differentiative division occurs. Thus, the correlations between the positions of imaginal wing cells expressing *ac/sc* and the position of adult SOs, and the dynamics of *ac/sc* protein expression and the inferred steps of SO differentiation, completely support the view that the *ac/sc* regulatory proteins are at the center of the pathway leading to SO formation.

The regulation of SO pattern clearly focuses on the spatial regulation of *ac/sc*. In this regard, our results have built on prior genetic studies (Garcia-Bellido 1979; Botas et al. 1982; Moscoso del Prado and Garcia-Bellido 1984a,b; Garcia-Alonso and Garcia-Bellido 1986, 1988) to help clarify the respective roles of *emc* and *h*, and of *ac–sc* themselves. Specifically, we have shown that (1) the identical patterns of *ac* and *sc* protein distribution, which outline the SO pattern of the adult mesothorax, result from mutual *trans*-activation between *ac* and *sc*; (2) the effect of *emc* on the notal macrochaete pattern reflects a selective negative control of the *sc* gene by *emc* at the time of notal macrochaete precursor formation; (3) the effect of *h* on the wing microchaete pattern reflects a selective negative control of the *ac* gene by *h* after most wing SOs have been determined; (4) inappropriate expression of *h* preferentially affects *ac* expression but only in the cells fated to become epidermal cells and not in the SMC, that is, proneural gene expression is qualitatively different in the SMC from that in the surrounding non-neural cells; and (5) *ac/sc* act indirectly to suppress *ac–sc* expression in cells outside of the SMC, that is, the level of *h* expression responds to the level of *ac/sc* activity and, in turn, represses *ac* function, perhaps in a manner similar to the postulated mechanism of lateral inhibition.

In addition to these four HLH-type proteins that we have discussed in detail, there is a requirement for a fifth HLH-type protein, *daughterless* (*da*) (Caudy et al. 1988; Dambly-Chaudiere et al. 1988), to cooperate with *ac/sc* in SO formation. *da* is expressed apparently in all cells and may function in heterodimers with *ac* or *sc* to regulate gene expression. The genetic circuitry guiding ASC gene expression and SO pattern and differentiation (including the so-called prepattern genes, see below) is shown schematically in Figure 8.

Additional genes are required for SO patterning

Although we have determined more precisely the place of *h* and *emc* in the process of *ac/sc*-dependent SO formation, these genes are clearly not sufficient to explain SO patterning. *emc* mutations add bristles to, but do not alter, the basic SO pattern found on the adult notum (Botas et al. 1982; Moscoso del Prado and Garcia-Bellido 1984a). Thus, mutations in *emc*, a ubiquitously expressed gene (Ellis et al. 1990; Garrell and Modolell 1990), may not alter the prepattern but the response of *sc* to the prepattern, that is, mutations in *emc* may lower the threshold at which different elements of the prepattern activate *sc*. Other regulators expressed in distinct regions within the imaginal discs most likely define the prepattern to which *ac* and *sc* respond. For example, the exclusion of *ac* and *sc* expression from the posterior compartment of the imaginal wing disc (Figs. 2 and 3) may be mediated by the segment polarity gene *engrailed*, which is expressed in the posterior region of all imaginal discs (Kornberg et al. 1985). Thus, segment polarity genes are likely candidates for these regulators as they are expressed in spatially restricted domains in the imaginal discs and function in adult development (Kornberg et al. 1985; Baker 1988a,b). In addition, it has been shown that the capacity of epidermal primordia to form SOs is temporally regulated, indicating that the regula-

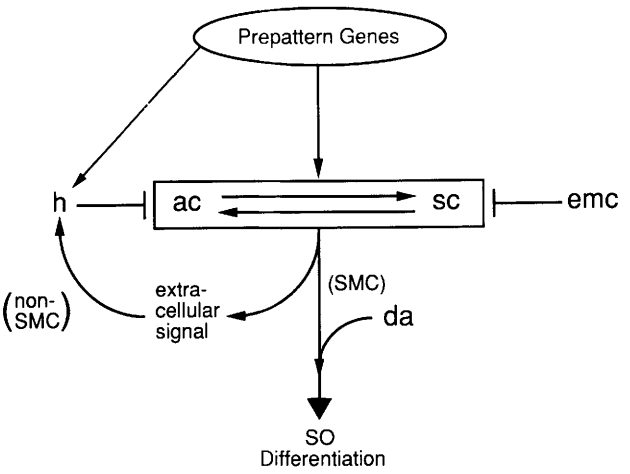

Figure 8. Gene interactions controlling SO pattern formation. A prepattern of regulatory genes specifies the proneural cluster pattern of the *ac* and *sc* proteins and the spatially restricted pattern of the *h* protein. *sc* is activated where *emc* levels are insufficient to suppress its expression. *ac* and *sc* act in *trans* (1) to positively regulate each other's expression; (2) in conjunction with *da*, another HLH-type regulator, to activate those genes necessary for sensory organ differentiation; (3) to activate a signal for non-SMCs already expressing *h* to increase their level of *h* expression; and (4) to activate a lateral inhibition signal to all non-SMCs of the cluster to suppress *ac/sc* expression (not shown).

tory properties of the prepattern change during disc development (Rodriguez et al. 1990).

In the wing blade the level of *ac/sc* activity controls the level of *h* expression which, in turn, represses *ac* expression. Because *ac/sc* activity appears to influence the level of *h* expression outside of the cells that express *ac–sc* at high levels, we suspect that other genes must function to link the activity of *ac/sc* in proneural clusters of SMCs to the level of *h* expression in surrounding cells. Genes such as *Notch* and *shaggy* appear to function in the lateral inhibition process in the notum (Dietrich and Campos-Ortega 1984; Simpson and Carteret 1989) to repress *ac* and *sc* function; they may also act in the wing blade to link the level of *ac/sc* activity to the level of *h* expression. *h* may then act directly to repress *ac* function perhaps by sequestering transcriptional activators of *ac* or the *ac* protein itself in inactive heterodimers.

During SMC formation in the larva and pupa, a number of genes are thought to function in a cell communication pathway to remove neural competency from the cells surrounding the SMC in proneural clusters (lateral inhibition) (for review, see Simpson 1990). We and others have argued that the gradual loss of *ac* and *sc* expression from these cells causes their loss of neural competency. Thus, the final effect of lateral inhibition may be the removal of the *ac* and *sc* proteins from cells not chosen to become SMCs. Although there is no genetic evidence to suggest that *h* acts in the lateral inhibition process, we do show that overexpression of *h* can prematurely mimic the natural refinement of *ac* and *sc* expression in certain proneural clusters. Perhaps, HLH proteins similar to *h* function in vivo to carry out the last step of lateral inhibition and remove *ac* and *sc* expression from cells initially competent but not selected to become SMCs. For example, the *E(spl)* complex appears to act in the last step of lateral inhibition (de la Concha et al. 1988) and encodes several HLH proteins similar to *h* (Klambt et al. 1989). These may be the in vivo regulators of *ac/sc* in the lateral inhibition process that overexpression of *h* mimics. Whatever the true mechanism of lateral inhibition is, the observation that overexpression of *h* has no effect on *ac/sc* expression in the SMC suggests that there is some key difference in either the regulation of *ac/sc* expression and/or the nature of the *ac/sc* proteins in the SMC that makes them refractory to *h* action. Indeed, Martinez and Modolell (1991) have shown that the *cis*-elements that drive *sc* expression in the SMC may be distinct from those that regulate expression in the proneural cluster.

HLH protein interactions and the specification of cell fate

The ability of *h* to repress *ac* (and, indirectly, *sc*) gene expression in one cell type but not in another argues that the context in which HLH proteins are found within a cell may determine how they function. A number of studies focusing on B-HLH proteins and, in particular, the myogenic determination gene, MyoD, have shown that homodimers and heterodimers of these proteins are capable of binding DNA and activating transcription (Tapscott et al. 1988; Murre et al. 1989a,b; Davis et al. 1990). Id, an HLH protein that lacks a basic domain, can specifically associate with three mammalian B-HLH proteins (MyoD, E12, and E47) and inhibit their ability to bind DNA (Benezra et al 1990). Thus, one mechanism by which negatively acting HLH proteins mediate their repressive actions on B-HLH proteins may be to sequester them in "poisoned" heterodimers incapable of binding DNA (Benezra et al. 1990; Ellis et al. 1990; Garrell and Modolell 1990). Another possibility is that these heterodimers bind DNA but the negatively acting HLH protein quenches the activation capability of the B-HLH protein. Preferential interactions may occur between different *Drosophila* HLH proteins (e.g., *emc* and *sc*; *h* and *ac*) that could determine the specificity of each protein. Modifications of HLH proteins also could modulate their interactions and function. A possible role for post-translational modification of *l'sc*, a member of the AS-C, in the neuroblasts of the embryonic central nervous system has been discussed recently (Cabrera 1990). Similarly, modifications to the *ac* and *sc* proteins in the SMC could account for their ability to withstand overexpression of *h*. Clearly, biochemical studies of the *Drosophila* HLH proteins will be crucial to understand how the combination, relative proportion, and nature of different HLH proteins within a cell control gene expression and cell fate.

Materials and methods

Antibodies

Antibodies were raised against synthetic peptides and recombinant forms of the *ac* and *sc* proteins, as will be described elsewhere (J.B. Skeath, B.S. Thalley, and S.B. Carroll, in prep.). For this work, *ac* protein was localized using a mouse monoclonal antibody and *sc* protein was localized using an affinity-purified rabbit antibody directed against a synthetic peptide. Antibodies to the *h* protein were produced as described in Carroll et al. (1988). Antibodies to β-galactosidase were from Boehringer Mannheim.

Immunohistochemistry

Wing discs were dissected from larvae or pupae as part of larger imaginal complexes and fixed as described previously (Carroll and Whyte 1989). For single-labeling studies, the mouse anti-*ac* or rabbit anti-*sc* antibodies were incubated with biotinylated goat anti-mouse (Vector) or biotinylated goat anti-rabbit (Vector), washed, and then incubated with streptavidin–horseradish peroxidase conjugate (BRL). After washing, the stain was developed with 0.5 mg/ml of diaminobenzidine (DAB) and 0.03% (wt/vol) Co^{2+} and Ni^{2+} ions. Discs were then mounted in 50 mM Tris (pH 8.8) containing 10% glycerol and viewed by either bright-field or Nomarski (DIC) optics.

For double-labeling studies, the same primary antibodies or mouse anti-β-galactosidase were followed by biotinylated goat anti-rabbit IgG and rat anti-mouse IgG, and then by fluorescein-conjugated streptavidin and Texas Red-conjugated goat anti-mouse antibodies resulting in detection of *sc* on the green channel and *ac* or β-galactosidase on the red channel. The discs were then mounted in 50 mM Tris (pH 8.8) containing 10% glycerol

and 0.5 mg/ml of *p*-phenylenediamine to prevent quenching. Microscopy was on a Nikon Optiphot equipped with a Bio-Rad MRC600 Lasersharp Confocal system.

Heat shock induction of h

Heat shocks were performed using a strain carrying a P-element containing the *h*-coding region fused to the *hsp70* promoter (HSH) (generously provided by David Ish-Horowicz); this construct can suppress the h^2/h^2 phenotype (Rushlow et al. 1989). Heat shocks were performed in quart-sized bottles containing third-instar larvae. Bottles were submerged in a 37°C water bath for varying periods of time. Wild-type and HSH LTI-stage wing discs were dissected from larvae immediately after either a continuous 1-hr or a 4.5-hr heat shock period (three 30-min heat shocks, the first two followed by a 90-min rest period at 25°C). Discs were stained with antibodies as described above with one modification—due to the nonspecific reduction of protein expression observed with heat shocks the discs were preincubated for 3 min in 0.1 M Tris-HCl (pH 6.8), 0.5 mg/ml of DAB, and 0.03% Co^{2+} and Ni^{2+} prior to addition of 0.03% H_2O_2 to increase the sensitivity of the staining.

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