The spatial organization of epidermal structures: *hairy* establishes the geometrical pattern of *Drosophila* leg bristles by delimiting the domains of *achaete* expression

Teresa V. Orenic¹, Lewis I. Held, Jr², Stephen W. Paddock¹ and Sean B. Carroll¹,*

¹Howard Hughes Medical Institute, Laboratory of Molecular Biology, University of Wisconsin-Madison, Madison, WI 53706, USA
²Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409, USA

*Author for correspondence

**SUMMARY**

The spatial organization of *Drosophila melanogaster* epidermal structures in embryos and adults constitutes a classic model system for understanding how the two dimensional arrangement of particular cell types is generated. For example, the legs of the *Drosophila melanogaster* adult are covered with bristles, which in most segments are arranged in longitudinal rows. Here we elucidate the key roles of two regulatory genes, *hairy* and *achaete*, in setting up this periodic bristle pattern. We show that *achaete* is expressed during pupal leg development in a dynamic pattern which changes, by approximately 6 hours after puparium formation, into narrow longitudinal stripes of 3-4 cells in width, each of which represents a field of cells (proneural field) from which bristle precursor cells are selected. This pattern of gene expression foreshadows the adult bristle pattern and is established in part through the function of the *hairy* gene, which also functions in patterning other adult sense organs. In pupal legs, *hairy* is expressed in four longitudinal stripes, located between every other pair of *achaete* stripes. We show that in the absence of *hairy* function *achaete* expression expands into the inter-stripe regions that normally express *hairy*, fusing the two *achaete* stripes and resulting in extra-wide stripes of *achaete* expression. This misexpression of *achaete*, in turn, alters the fields of potential bristle precursor cells which leads to the misalignment of bristle rows in the adult. This function of *hairy* in patterning *achaete* expression is distinct from that in the wing in which *hairy* suppresses late expression of *achaete* but has no effect on the initial patterning of *achaete* expression. Thus, the leg bristle pattern is apparently regulated at two levels: a global regulation of the *hairy* and *achaete* expression patterns which partitions the leg epidermis into striped zones (this study) and a local regulation (inferred from other studies on the selection of neural precursor cells) that involves refinement steps which may control the alignment and spacing of bristle cells within these zones.

Key words: *hairy*, *achaete*, *Drosophila* leg bristle, pattern formation

**INTRODUCTION**

Many multicellular organisms display epidermal structures that are arranged in ordered spatial patterns, for example, the feathers of birds, the colored scales on butterfly wings and denticles on the cuticle of *Drosophila* embryos. Because the epidermis is easily accessible to experimental manipulation, the regulatory mechanisms that generate ordered arrays of epidermal structures have been analyzed extensively. In this paper, we focus on the genetic regulatory events that establish bristle pattern in the cuticle of the legs of *Drosophila melanogaster*, an animal that is particularly amenable to genetic studies of pattern formation. The cuticle of the *D. melanogaster* adult is covered with an array of small and large bristles (microchaetes and macrochaetes) which are generally arranged in stereotypical patterns. This is exemplified by the periodic arrangement of microchaetes on the legs. On most leg segments the microchaetes are arranged in straight longitudinal rows located at fixed positions, although the number and positions of the bristles within each row are variable (reviewed by Held and Bryant, 1984). A function for the precise order of leg bristle rows is not known; however, the same arrangement of bristle rows is observed in a number of *Drosophila* species, suggesting conservation of patterning mechanisms (Held, 1979a).

Most bristles, such as the leg microchaetes, are innervated sense organs (SO) that stem from a single precursor cell, the sensory mother cell (SMC; Bate, 1978; Hartenstein and Posakony, 1989). Patterning of certain SMCs, such as the thoracic macrochaetes, is a multistep process (Ghysen and Dambly-Chaudiere, 1989; Held, 1991); supposedly, a small cluster of cells (proneural cluster), all of which are competent to produce an SMC, is chosen at a specific site
and then via a cell-cell communication process (Heitzler and Simpson, 1991), a single cell from this cluster ultimately emerges as the SMC. The SMC through a process known as lateral inhibition then inhibits the surrounding cells from following a neural pathway (Stern, 1954; Wigglesworth, 1940; reviewed by Simpson, 1990). The neural precursor cells of the CNS, the neuroblasts (NBs), are selected through a similar mechanism (reviewed by Campos-Ortega, 1991). Several classes of genes have been identified that function in different steps of SMC or NB patterning. For example, the proneural genes such as those of the achaete-scute complex (AS-C), which are required for the differentiation of most adult SOs, are expressed in the cells of the proneural clusters (Cabrera et al., 1987; Romani et al., 1987, 1989; Cubas et al., 1991; Skeath and Carroll, 1991, 1992) and are thought to bestow upon a cell the potential to follow a neural pathway (Stern, 1954; Ghysen and Richelle, 1979; Garcia-Bellido and Santamaría, 1978; Garcia-Bellido, 1979). The cell-cell communication processes that are involved in the refinement of the cluster to a single SMC or NB are mediated by the neurogenic genes (Lehmann et al., 1983; Hartenstein and Posakony, 1990; Campos-Ortega, 1991; Heitzler and Simpson, 1991; Goriely et al., 1991).

The organization of adult sense organs is thought to be largely determined by the original pattern in which SMCs arise within the primordia of adult structures, the imaginal discs. For example, in the wing disc SMCs arise at specific positions in a pattern that presages the final wing and thoracic SO arrangement (Huang et al., 1991; Blair et al., 1992). The expression of the proneural genes achaete (ac) and scute (sc) correlates with the sites of SMC formation in the wing disc and NB formation in the embryonic CNS, and it has been suggested that the arrangement of sense organs in the wing and notum of the adult and NBs in the embryo is determined by the pattern of proneural gene expression (Cabrera et al., 1987; Romani et al., 1989; Cubas et al., 1991; Skeath and Carroll, 1991, 1992). Thus, deciphering of the mechanisms involved in patterning of proneural gene expression should lead to an understanding of the patterning of adult SOs and embryonic NBs. In the embryonic CNS, the pattern of the first wave of proneural gene expression is established mainly through the function of the dorsal/ventral genes and the pair-rule genes, which are genes that specify pattern along two different axes within the embryonic epidermis (Martin-Bermudo et al., 1991; Skeath et al., 1992). The pattern of proneural gene expression in the wing disc is determined in part via the function of the extramacrochaetae (emc) gene, which is expressed in a complex pattern in the wing disc. In certain cases, there is an inverse correlation between the levels of emc expression and levels of sc expression or the propensity to produce an SMC (Cubas and Modolell, 1992).

The ac and sc genes are required not only for SO formation in the wing disc, but also for the differentiation of leg microchaetae (Held, 1990). We find that within most pupal leg segments ac is expressed in narrow longitudinal stripes that appear to foreshadow the arrangement of leg microchaetae. Thus, as in the wing disc, the pattern of proneural gene expression correlates with final leg SO pattern, and insight into the process of patterning leg bristles can be gained by understanding the regulation of the ac pupal leg pattern. One gene that could potentially function in patterning ac leg expression is the hairy (h) gene, a known negative regulator of ac function in the adult PNS (Botas et al., 1982; Moscoso del Prado and Garcia-Bellido, 1984a,b; Skeath and Carroll, 1991) which in leg imaginal discs is expressed in a pattern somewhat similar to that of ac. In pupal legs, h is expressed in 4 longitudinal stripes along each leg and in transverse stripes that encircle each tarsal segment (Carroll and Whyte, 1989). Absence of h function during adult development results in the formation of ectopic bristles in the wing, thorax and head of the adult (Ingham et al., 1985; Moscoso del Prado and Garcia-Bellido, 1984a), and slight increases in bristle number have also been observed in legs from adults homozygous for h1, a hypomorphic h allele (Held, 1990). The similarity between the h and ac patterns of expression, the function of h in patterning bristles in a number of adult structures and the known role of h as a regulator of ac led us to investigate the role of h in patterning leg bristles.

To elucidate the possible roles of h and ac in patterning leg microchaetae we have examined the correlation between h and ac expression, the effect of loss of h function on the leg bristle pattern and the potential regulation of the ac leg pattern by h. Our results show that the longitudinal h stripes are expressed between alternating pairs of ac stripes. In the absence of h function, the leg bristle rows become disorganized, most likely because the periodicity of ac expression is reduced; the narrow stripes are merged into wider stripes. This suggests that the h longitudinal stripes of expression specify positional cues in pupal leg discs that results in the production of half of the ac interstripes. This function of h in the leg is significantly different from its well known function in the wing where it is not involved in the initial patterning of ac expression but rather in suppressing its ectopic expression after the wild-type SMCs have been selected (Skeath and Carroll, 1991; Blair et al., 1992).

## MATERIALS AND METHODS

### Fly strains

The following fly strains were used: Oregon R, cosh;Il79K;h507 TM6 Ubx (cosh is a 28 kb cosmid from the h region that has been transformed into flies and complements h embryonic function), kC1/TM3, h7094/TM3, hK1/TM3, hR37/h7694 and hL43a/TM3 (see Lindsley and Zimm, 1992). The cosh;Il79K;h507 TM6 Ubx and hL43a/TM3 lines were the kind gifts of D. Ish-Horowicz (Rushlow et al., 1989) and L. Fasano (Fasano et al., 1988) respectively. The following stocks were made by standard genetic methods for this study: cosh;Il79K;h7094, cosh;Il79K;hC1, cosh;Il79K;h7694, cosh;Il79K;hR37, cosh;Il79K;hL43a. These heteroallelic combinations were used because in general cosh does not rescue the homozygotes of any of the alleles. This is likely due to the fact that these alleles have been maintained over balanced chromosomes for many generations, allowing second site lethal mutations to accumulate.

### Cloning and transformation

The fragments tested for enhancer activity were first subcloned into Bluescript (Stratagene) and then into the KpnI-NorI sites of
the vector HspCasper, which has the basal promoter of hsp70 fused to the bacterial lacZ gene (kind gift of H. Nelson). These constructs were then introduced into the Drosophila germline by standard P-element-mediated transformation (Rubin and Spradling, 1982).

**Immunohistochemistry and microscopy**

Imaginal disc complexes were dissected from larvae or pupae, fixed and blocked as described previously (Carroll and Whyte, 1989) except that fixation times were extended to 1 hour for experiments involving the ac antibodies. For single-label experiments, the discs were incubated in primary antibody, either mouse monoclonal anti-h diluted 1:5 (Gates and Carroll, unpublished data) or mouse monoclonal anti-ac diluted 1:1 (Skeath and Carroll, 1991), overnight. After washing, they were incubated in biotinylated horse-anti-mouse IgG, washed, then incubated in Avidin DH and biotinylated horseradish peroxidase H (20 µl/ml of each; Vectorstain Elite ABC Kit, Vector). Individual discs were dissected from the complexes, mounted and viewed by Normarski (DIC) optics.

For double-label experiments, discs were first incubated in primary antibody, either mouse monoclonal anti-ac, mouse monoclonal anti-en (concentrated 4-fold; Patel et al., 1989), rabbit anti-sc (0.5 µg/ml; S. Carroll, unpublished). The binding of the mouse monoclonal antibodies was detected with rat anti-mouse IgG (this and all other secondary and tertiary reagents were diluted 1:200; Boehringer Mannheim) followed by fluorescein-conjugated donkey anti-rat IgG (Jackson) or rhodamine-conjugated goat anti-rat IgG (Cappel). Rabbit primary antibodies were detected with biotinylated goat anti-rabbit IgG (Vector) followed by streptavidin-fluorescein (Boehringer Mannheim) or streptavidin-rhodamine (Jackson). After dissection from the complexes, discs were mounted and viewed on a Zeiss IM35 microscope equipped with a BioRad MRC600 Lasersharp confocal system. The images were merged and displayed as described by Paddock et al., 1993.

**Mounting and microscopy of adult tissue**

Legs were dissected from adult flies and mounted in Faure’s solution (Lee and Gerhart, 1973) between cover slips. Cuticular abnormalities were scored using an Olympus BH-2 compound microscope at 400× magnification. Bristles were counted with the aid of an ocular 10×10 grid square. Legs for photography were critical-point dried, mounted on stubs, gold coated and examined with a Hitachi S-570 scanning electron microscope.

**RESULTS**

A novel h regulatory mutant retains h embryonic expression but lacks most h imaginal disc expression

In order to examine the effect of a loss of h expression in imaginal tissues we sought to create a fly strain that had normal h embryonic function but lacked h imaginal function. This was facilitated by utilization of a cosmid construct (cosh) of a large portion of the h locus (Fig. 1; Rushlow et al., 1989). This 28 kb cosmid rescues the h embryonic segmentation defect, and the flies survive to adulthood. However, the cosmid does not rescue the h adult wing defect. To test whether the cis-regulatory sequences that drive h expression in imaginal discs other than the wing disc are contained within this construct, it was crossed into genetic backgrounds containing h alleles that do not produce detectable levels of functional h protein: cosh; hC/cosh, cosh; hC/hIL79K and cosh; h7h94/hIL79K (Carroll et al., 1988). The hC, h7h94 and hIL79K alleles encode truncated proteins of 42, 71 and 113 amino acids respectively (Wainwright et al., 1992), so a monoclonal antibody that recognizes an epitope C-terminal to these truncations detects only h protein produced by the cosmid (Gates and Carroll, unpublished data). Through analysis of h expression in imaginal discs of these genotypes and in discs from two breakpoint mutants, we found several different elements responsible for different aspects of h disc expression.

Imaginal discs were dissected from larvae and pupae of the cosh; hC1/h7h94, cosh; hC1/hIL79K and cosh; h7h94/hIL79K genotypes and the wild type and stained with anti-h antibody. h exhibits distinct patterns of expression in the pupal leg, pupal wing and larval eye/antennal discs (Carroll and Whyte, 1989; Fig. 2A,D and G respectively). In the pupal leg disc, h is expressed in discontinuous rings of expression (transverse stripes) around each tarsal segment and in 4 longitudinal stripes along the leg (Fig. 2A). In cosh;h7h94/hIL79K pupal leg discs there are no longitudinal stripes of expression, although the transverse stripes are still present (Fig. 2B). In the pupal wing, h is normally expressed along the presumptive wing veins (Fig. 2D) but its expression is drastically reduced in cosh;h7h94/hIL79K

---

**Fig. 1.** Map of the h region. The top line is a restriction map of the h region (Rushlow et al., 1989; Howard et al. 1988) depicting the hK1 breakpoint and the site of the L43a enhancer trap insertion. The open rectangles below the map are fragments that were tested in lacZ reporter constructs for the ability to drive lacZ expression in the h imaginal disc pattern of expression. The rough locations of the putative h imaginal disc enhancer elements are also shown. The lower map shows the region spanned by the h cosmid (cosh; Rushlow et al., 1989). R1, EcoRI; R; HindIII; X, XhoI; K, KpnI; N, NotI; Xb, XbaI.
pupal wing discs (Fig. 2E). The most prominent component of h expression in eye/antennal discs is a stripe anterior to the morphogenetic furrow (Fig. 2G). In discs of the cosh; h^{7h94}/h^{IL79K} (Fig. 2H) genotype as well as those of the cosh; h^{C1}/h^{IL79K} genotypes (not shown), no expression of h protein is observed in the eye disc. The reduction or absence of h protein expression in imaginal discs suggests that the cis-regulatory elements that regulate h expression in imaginal discs, except for those that drive the transverse stripes in the leg, lie outside the region encompassed by the cosmid.

To determine whether the h disc enhancer elements lie 3′ to the sequences spanned by cosh, we examined h protein expression in imaginal discs from larvae carrying a breakpoint mutation in the h region, h^{K1}. h^{K1} is an embryonic lethal allele with an inversion breakpoint approximately 2.5 kb 5′ to the start of transcription (Ingham, 1985; Howard et al., 1988, Fig. 1). We tested whether the sequences 3′ to the h^{K1} breakpoint drive h imaginal disc expression by crossing h^{K1} into a cosh background to complement the h embryonic function. Imaginal discs other than the wing disc from cosh; h^{K1}/h^{IL79K} express h in the
wild-type pattern, although in some cases at lower levels (Fig. 2C,F,I). This result and the fact that the cosh insert cannot drive the expression of h in imaginal discs suggest that the leg longitudinal stripe and eye disc pattern enhancer elements lie 3′ to the region encompassed by the cosmid (at least 14 kb 3′ to the start of transcription). Below the map in Fig. 1 the open rectangles represent fragments that have been tested in reporter constructs for the ability to drive lacZ expression in h imaginal disc expression patterns. None of these constructs express lacZ in imaginal discs. Combined with the results on h expression in discs from the breakpoint mutants and cosh, these observations suggest that some or all the wing elements are located more than 15 kb 5′ to the start of transcription and that the leg transverse elements are likely located within the 7 kb XbaI transcription unit (depicted in Fig. 1).

Legs from cosh;hnull/hnull adults show a bristle patterning defect that correlates with the expression pattern of h in pupal leg imaginal discs

The lack of the h longitudinal stripes of expression in cosh;hnull/hnull pupal legs allowed us to assess the function of these stripes in normal leg development. Bristle counts on cosh;h7h94/hIL79K and cosh;hC1/hIL79K reveal that there are mild increases in bristle number for most segments of both genotypes (approx. a 10% increase for the whole leg; Table 1). Furthermore, the longitudinal rows of bristles are disorganized (Fig. 3B). The basitarsal segment of the wild-type second leg has 8 rows of bristles. Rows 1 and 8 are very straight, and tiny hairs, but never bristles, are found between them (Fig. 3A; Hannah-Alava, 1958; Held, 1979a, 1990). In the cosh;h7h94/hIL79K legs, the bristles in rows 1 and 8 are arranged in a zigzag pattern (Fig. 3B), as are other rows elsewhere on the legs, and ectopic bristles are frequently found between these rows. Similar abnormalities characterize cosh;h7h94/hC1 flies. These results demonstrate a definite cuticular phenotype associated with lack of h longitudinal stripes of expression in pupal legs and therefore are suggestive of a direct role for h in the patterning of leg bristles.

h expression in pupal leg discs is adjacent to but excluded from ac expressing cells

To understand the basis for the leg phenotype in cosh;hnull/hnull flies, it was necessary to determine the expression of h relative to the bristle primordia in developing pupal legs. The earliest indication of a cell’s competence to become a bristle cell should be its expression of the proneural genes, ac or sc. We therefore examined the

<table>
<thead>
<tr>
<th>Segment</th>
<th>Wildtype</th>
<th>cosh;h7h94/hIL79K</th>
<th>cosh;hC1/hIL79K</th>
</tr>
</thead>
<tbody>
<tr>
<td>femur</td>
<td>112.0±3.08</td>
<td>125.2±7.91</td>
<td>123.8±3.03</td>
</tr>
<tr>
<td>tibia</td>
<td>145.4±4.51</td>
<td>155.8±3.35</td>
<td>146.4±0.95</td>
</tr>
<tr>
<td>basitarsus</td>
<td>76.4±3.21</td>
<td>88.6±4.51</td>
<td>88.6±0.07</td>
</tr>
<tr>
<td>tarsus2</td>
<td>38.8±1.14</td>
<td>39.6±1.14</td>
<td>38.5±1.14</td>
</tr>
<tr>
<td>tarsus3</td>
<td>22.2±1.30</td>
<td>25.0±2.65</td>
<td>22.8±1.79</td>
</tr>
<tr>
<td>tarsus4</td>
<td>19.6±0.89</td>
<td>24.2±1.10</td>
<td>23.2±1.30</td>
</tr>
<tr>
<td>tarsus5</td>
<td>23.4±0.55</td>
<td>23.8±1.79</td>
<td>23.6±2.51</td>
</tr>
<tr>
<td>Total</td>
<td>465.4±9.02</td>
<td>520.0±25.67</td>
<td>503.0±12.94</td>
</tr>
</tbody>
</table>

*Bristle numbers are given as the mean±standard deviation. n=5 2nd legs for all genotypes.

and 8 are arranged in a zigzag pattern (Fig. 3B), as are other rows elsewhere on the legs, and ectopic bristles are frequently found between these rows. Similar abnormalities characterize cosh;h7h94/hC1 flies. These results demonstrate a definite cuticular phenotype associated with lack of h longitudinal stripes of expression in pupal legs and therefore are suggestive of a direct role for h in the patterning of leg bristles.

h expression in pupal leg discs is adjacent to but excluded from ac expressing cells

To understand the basis for the leg phenotype in cosh;hnull/hnull flies, it was necessary to determine the expression of h relative to the bristle primordia in developing pupal legs. The earliest indication of a cell’s competence to become a bristle cell should be its expression of the proneural genes, ac or sc. We therefore examined the ectopic bristles can be seen between rows 8 and 1 (arrowheads), a region where bristles normally never form. Scale bar for A and B, 25 µm. (C) Expression pattern of the ac gene in a 6-hour APF pupal leg. Within the leg segments, ac is expressed in longitudinal stripes 3-4 cells wide. The continuity of the stripes is disrupted at the junctures between the leg segments. Scale bar, 50µm.
Fig. 4. Immunohistochemical staining to show complementary patterns of h and ac expression throughout pupal leg development. In all panels, staining within the tarsal segments is shown and proximal is to the left. (A) ac expression in a pupal leg disc at 4 hours APF. The predominant expression pattern at this stage is discontinuous transverse stripes that encircle the tarsal segments. The transverse stripes are observed in different focal planes showing that the higher degree of folding in this 4-hour leg disc (relative to the 6-hour disc, see G-I) does not obscure a possible striped pattern which we observe at 6 hours (see G, H). Scale bar (in A) for all panels except C,F,I and L, 50 µm. (B) Same leg as in A showing the h pattern of expression. h is expressed in the same pattern throughout pupal development, in transverse stripes that encircle the tarsal segments and in 4 longitudinal stripes. (C) Superimposition of the ac (green) and h (red) expression (overlap in yellow) shown in A and B. ac and h expression show little overlap. The ac transverse rings are disrupted by the transverse and longitudinal h stripes. Scale bar (in C) for C,F,I and L, 50 µm. (D) Expression of ac in a 5-hour pupal leg. ac is still expressed in transverse stripes, but longitudinal rows expressing ac are beginning to appear proximally. (E) h expression in the leg shown in D. (F) Superimposition of ac and h expression at 5 hours. As seen at 4 hours, h and ac expression are largely noncoincident. (G) By 6 hours APF, the transverse stripes of ac expression are no longer present and the predominant pattern is one of ac in longitudinal rows of cells. (H) h expression at 6 hours. (I) Superimposition of ac and h expression at 6 hours. The h stripes are positioned between every other pair of ac stripes with little overlapping expression. (J) Expression of ac in cosh:hL413a/hIL79K at 6 hour APF. hL413a is an enhancer trap insertion in h which is an h null allele and exhibits the same phenotypic effects in adult legs as do other h alleles. The lacZ expression from this enhancer trap in pupal legs is identical to that of the h protein, in both wild-type and mutant discs. In this mutant and in
expression of the *ac* gene at several stages of pupal development. At approximately 6 hours after puparium formation (APF), *ac* is expressed in a pattern of longitudinal stripes (which are still disrupted longitudinally probably because the *h* transverse stripes are present in the mutant) as opposed to the normal narrow stripes of expression. (K) lacZ expression of *hd-1* enhancer trap in a *cosh;hL43a/hIL79K* leg. Although the endogenous *h* longitudinal stripes are missing in *cosh;hnull/hnull* flies, the enhancer trap insert can be used to detect where the *h* protein would have been expressed and thus, this expression can be compared to the altered expression of *ac*. (L) Overlap of *ac* and lacZ expression in the 6-hour *cosh;hL43a/hIL79K* mutant leg. The lacZ-*h* stripes run down the middle of the wide *ac* stripes. This suggests that the *h* stripes specify the *ac* interstripes.
identifies the proneural fields where the leg microchaetes will arise.

The pattern of ac expression during early pupal development is quite dynamic. The predominant expression from 3-5 hours APF is in discontinuous transverse stripes that encircle the tarsal segments (Fig. 4A; tarsal segments are shown throughout Fig. 4). At approximately 5 hours the longitudinal stripes begin to appear in conjunction with the transverse stripes (Fig. 4D) and by 6 hours the longitudinal stripes in each segment (eight longitudinal stripes in each tarsal segment) replace the transverse stripes (Fig. 4G).

Monitoring of gene expression beyond 6 hours APF was hindered by the formation of pupal cuticle. To examine the relationship between h and ac expression, discs were double labelled with antibodies against both ac and h. The h (red) and ac (green) expression patterns are largely nonoverlapping during pupal development (Fig. 4C,F,I). Note in particular that at 6 hours APF (Fig. 4I), the h stripes are situated between every other pair of ac stripes with little overlapping expression. The h longitudinal stripes are present from early pupal development, well before the ac stripes are formed.

**h delimits one half of the boundaries of the ac stripes**

The expression of h stripes relative to ac stripes in the leg suggests that h represses ac expression in every other interstripe region. In order to test this hypothesis, we examined ac expression in two different heteroallelic combinations of h in a cosh background: cosh;hIL79K/h794 and cosh;hL43a/hIL79K. hL43a is an embryonic lethal enhancer trap insertion (Fasano et al., 1988; see Fig. 1) in the h gene and cosh;hIL79K/h794 flies exhibit the leg phenotype. This insertion expresses lacZ in the wild-type h pattern in both wild-type and mutant imaginal discs. Thus, h-lacZ expression marks, in a mutant disc, the regions where h is normally expressed in a wild-type leg disc and provided a landmark that allowed us to determine clearly if the ac pattern is altered in the mutant leg disc. In the 6-hour pupal leg of both cosh;hIL43a/h794 (Fig. 4J,K,L) and cosh;hIL79K/hL43a (not shown) mutants, the ac pattern appears as four extra-wide stripes rather than the normal eight stripe ac pattern found in the tarsal segments. The segmental interruptions in the ac longitudinal stripes are still observed in the mutant presumably because the h transverse stripes are still present in cosh;hnull/hnull mutants. The altered ac expression occurs because the two ac stripes that border each h stripe fuse due to the expansion of ac expression into the h domain. This is particularly evident in the mutant leg disc doubly labelled for ac (green; Fig. 4L) and h-lacZ (red; Fig. 4L). Note that the normal realm of h expression runs through the center of each wide ac stripe. This result clearly implies that the h longitudinal stripes function to repress ac expression in every other ac interstripe.

In a h mutant, ectopic bristles arise in the region between bristle rows 1 and 8 (Fig. 3). It is likely that this region corresponds to one of the ac interstripes which normally expresses h. To confirm this, double-label experiments were performed with anti-sc/anti-en (anti-sc was used for this experiment because it has an identical expression pattern to that of ac) in pupal legs and this antibody was produced in rabbits, making it possible to perform a double-label experiment with an en monoclonal antibody) and anti-h/anti-en to determine the position of the h stripes relative to the bristle rows (Fig. 5). The en protein is expressed in the posterior compartment of all imaginal discs (Kornberg, 1985). Tarsal bristle rows 1-3 are located in the posterior compartment of the leg with the compartment boundary running between rows 1 and 8 (Held, 1979b; Lawrence et al., 1979); thus, sc rows 1, 2 and 3 should be contained within the en domain of expression. We observe that this is the case and that row 4 lies adjacent to the dorsal boundary of en expression (Figs 5C, 6). h stripe 1 runs through the center of the en domain, and h stripe 2 lies 3-4 cell rows away from the dorsal boundary of en expression, leaving enough room for ac/sc stripe 4 in the non-expressing cells in between (Fig. 5F). This result shows that h stripe 2 falls between ac/sc stripes 4 and 5, and since h is expressed between every other pair of ac/sc stripes, the other h stripes must be expressed between sc stripes 8 and 1, 2 and 3, and 6 and 7 (summarized in Fig. 6), which is consistent with the phenotypic effects we observe in h mutants (Fig. 3B).

**DISCUSSION**

Patterning of leg bristle rows is effected largely through the functions of h and ac

The data presented in this work suggest the following model for the generation of the periodic pattern of bristles in the adult legs. Beginning early in pupal leg development, h is expressed in 4 longitudinal stripes. In the tarsal segments, ac has the potential to be expressed in 4 broad stripes but its expression is repressed within the h stripes and is confined to 8 narrow stripes, each pair of which straddles a h stripe. The narrow stripes of ac expression would define the limits within which bristles may be produced. This mechanism appears to be evolutionarily conserved as the expression of h (Gates and Carroll, unpublished data) and sc (J. Gates, personal communication) in other Drosophila seem to be identical to that in D. melanogaster. The notion that bristle rows on the legs are preceded by stripes of competent cells was proposed by Lawrence et al. (1979), who found that certain bristles can be embraced by clones from either the anterior or posterior leg compartment. The bristle row at this boundary (row 1) would thus have to originate from a band of cells at least 2 cell diameters in width. Interestingly, the thoracic microchaetes may also arise from rows of competent cells as ac expression in narrow stripes of cells within the notal region of the wing disc has been observed at approximately 6 hours APF (Skearth and Carroll, unpublished data).

The role we propose for ac in the leg is consistent with proneural gene function in other adult tissues, such as the notum where the pattern of the ac proneural clusters, from which 1 or 2 bristles are selected as SMCs, prefigures the final pattern of macrochaete SMCs. By analogy, the stripes of ac expression in the tarsal legs could be composed of fused proneural clusters, each of which produces one microchaete SMC. However, the variability of bristle number and position within each row (Held, 1979a) suggests an alternative explanation: each stripe may repre-
sent a coherent ‘proneural field’ (Cubas and Modollel, 1992) within which few (if any) SMC sites are predetermined (Held, 1990, 1991). Our finding that \textit{ac} is expressed in stripes 3-4 cells wide is consistent with such a model.

**Phenotypic effects of expansion of the proneural fields**

Since in the mutant pairs of \textit{ac} stripes, or proneural fields, are expanded producing an area 3 times wider than either of the original \textit{ac} stripes, one might expect a severe disorganization of the bristle rows coupled with a 50% increase in bristle number. We observe, however, that although the bristles are arranged in a zig-zag pattern, the bristle rows are still discernable and there is only a 10% increase in bristle number. One possible explanation for this discrepancy is that even though the proneural field has been expanded, there is still a bias toward forming bristles in the ‘wild-type’ regions. Consistent with this hypothesis, Rodriguez et al. (1990) have shown, by ubiquitously expressing \textit{sc} in imaginal discs from \textit{sc} null larvae, that cells at or near the sites where notal macrochaetes normally form preferentially produce S0s as compared to cells from other regions of the disc. The bias toward SO formation in the ‘wild-type’ regions of the leg is not complete because there are ectopic bristles and some disorganization in the rows is observed in the mutant suggesting that bristles are selected at a low frequency from the would-be interstripe region. This bias may be due to the existence of a bristle promoting factor in the ‘wild-type’ regions or to the presence of a factor that opposes \textit{ac} function in the would-be interstripe region. An example of a factor that opposes proneural function is found in the wing. Expression of \textit{sc} alone is not sufficient to promote bristle formation in all areas of the notal region of the wing disc. The \textit{emc} gene appears to counteract \textit{sc} expression and function in certain regions of the wing disc (Skeath and Carroll, 1991; Cubas et al., 1991; Cubas and Modollel, 1992). \textit{emc} or an analogous gene could function to oppose \textit{ac} function in the leg \textit{ac} interstripe regions.

An alternative explanation is based on the possibility that although in the wild-type situation, where the bristle rows are only 3-4 cells wide, the lateral inhibitory effect exerted by the SMC would not necessarily have to be very far-reaching, it is possible that the SMC may be capable of exerting an inhibitory influence over several cell diameters. Thus, although in the mutant, the proneural field is significantly wider, it may only be possible to produce ectopic bristles if a cell from each extreme position of the field is selected to become an SMC. In this case a more central cell may be distant enough to escape an inhibitory effect from either of these cells and vice-versa. If, however, two somewhat more central cells were chosen, cells on either side of them might not be far enough away to escape a lateral inhibitory effect. If there were a bias toward selection of somewhat more central cells, formation of many ectopic bristles or severe disruptions of the bristle rows would not be expected.

Another possible dimension of the bristle patterning problem and interpretation of the phenotype is related to the observation that the single cells that are selected within the proneural stripes could theoretically deviate by as much as 2 cell diameters in their relative lateral positions, thus requiring an alignment step to produce the very ordered pattern of bristles in the adult. In this regard, a suggestion by Lawrence et al. (1979) is illuminating. Because they assumed that the A/P compartment boundary is straight, they were also faced with the problem of how initially misaligned cells (on either side of the boundary) could become aligned into a row, and they proposed (see their Fig. 5) that the SMCs could become aligned by using the boundary itself. That is, the SMCs could move left or right until they reached the boundary and then stop moving. The A/P boundary coincides with the interface between \textit{ac} stripe 1 and \textit{h} stripe 4. Hence, it is possible to extend this line of reasoning to suppose that all such interfaces between \textit{h} and \textit{ac} stripes provide reference lines along which SMCs can become precisely aligned. Directional movements of bristles relative to bract cells have indeed been implicated for all basitarsal bristle rows by a map of their cell lineage (Held, 1979b). In the absence of \textit{h} function, the movements would be futile, and accurate alignment would not be possible. Thus, frequent misalignments should occur, which is indeed what we observe as a key feature of the \textit{cosh\textsc{null}/h\textsc{null}} phenotype.

**Relationship between \textit{h} leg function and its function in other tissues**

The pattern of bristle rows appears to be determined in part by positional cues specified via the function of \textit{h}. One might expect that a similar mechanism may be utilized in the development of ordered spatial arrays of other epidermal structures. This appears to be the case for the generation of the periodic segmented pattern of the \textit{Drosophila} embryo. In the embryo \textit{h} functions as one of the pair-rule genes that divide the embryo into its segmental repeats (Nüsslein-Volhard, 1980; Jurgens et al., 1984). The pair-rule genes are all expressed in periodic patterns in the embryo, and \textit{h} is one of the three primary pair-rule genes that are required for the periodic expression of the other pair-rule genes. For, example, the \textit{fushi tarazu} (\textit{fz}) gene is expressed in seven stripes in the embryo, and \textit{h} expression is largely out of register with \textit{fz} expression (Hafen et al., 1984; Carroll and Scott, 1985) and acts as a negative regulator of \textit{fz} expression in these regions (Carroll et al., 1986; Howard and Ingham, 1986). This function of \textit{h} in establishing the periodicity of \textit{fz} stripes in the embryo is strikingly similar to its function in patterning the \textit{ac} stripes of expression in pupal legs. Thus, \textit{h} appears to be performing a very similar function in two different primordia, the embryo and leg discs. The outcome of these processes is a periodic pattern of denticles and naked cuticle in the embryo epidermis and the alternation of bristle row with bristleless cuticle in the legs. Interestingly, in both cases, \textit{h} obeys a ‘pair-rule’: there are always half as many \textit{h} stripes as the number of final pattern elements (segment or rows respectively).

The phenotype of \textit{h} loss of function in the legs has an obvious similarity to that in other adult structures, such as the wing, i.e., the formation of ectopic bristles. Genetic analysis suggests that \textit{h} also represses \textit{ac} expression in the developing wing (Botas et al., 1982; Moscoso del Prado and Garcia-Bellido, 1984a,b). In spite of these similarities,
analysis at the molecular level indicates that the regulation of ac by h in these two imaginal discs is quite distinct. h is expressed along the presumptive veins in the pupal wing (Carroll and Whyte, 1989). In viable h mutants ac is expressed ectopically along the wing veins, which causes the formation of ectopic bristles, but this occurs 2 hours after the normal expression and function of ac in the region (Skeath and Carroll, 1991; Blair et al., 1992). This suggests that h does not play a major role in the initial patterning of wing disc SOs. Consistent with this, the initial SMC pattern is normal in h<sup>1</sup> (Blair et al., 1992), a hypomorphic mutation, and in cosl:h<sup>null</sup>/h<sup>null</sup> wings only one extra SMC is seen early (S. Blair, personal communication). The observation in mutant pupal leg discs that the expansion of ac expression occurs by 6 hours APF, coincident with the time when the ac stripes are first seen, suggests, on the other hand, that h is directly involved in the initial patterning of the ac stripes in legs.

Regulation of h and ac gene expression in the pupal leg
The 6-hour pupal leg expression of ac in narrow longitudinal stripes appears to be the product of a relatively simple mechanism. In the absence of h function, the positional cues present in pupal leg discs are apparently sufficient to pattern ac expression into wide stripes, each of which spans a region that in wild-type legs would encompass two wild-type ac stripes and an interstripe. In wild-type leg discs, however, the presence of h function represses ac within h-expressing cells, resulting in 8 narrow stripes of ac expression. Repression of ac expression by h may occur through direct transcriptional regulation by h. h is a member of the basic helix-loop-helix (bHLH) family of transcription factors (Rushlow et al., 1989). The basic domain of these proteins is involved in DNA binding and the HLH domain in protein-protein interactions (Murre et al., 1989a,b; Davis et al., 1990). Thus, h may directly repress
transcription of ac by binding to its promoter sequences. On the other hand, h may indirectly repress ac expression by interfering with an activator of ac. The HLH proteins can form homo and heterodimers (Murre et al., 1989a,b; Davis et al., 1990) and heterodimerization between HLH proteins can interfere with DNA binding ability (Benezra et al., 1990). h may interfere with DNA binding of an as yet unidentified activator of ac or with ac itself, which is also a bHLH protein (Villares and Cabrera, 1987).

The regulation of the 4 h stripes is likely to be more complex than that of ac. The h stripes are present from the beginning of pupal development; in fact, they can be traced back to the larval leg disc. Possible candidate regulators of h are the segment polarity genes, which are expressed early during imaginal disc development in a position-specific, but not periodic, pattern (reviewed by Whittle, 1990; Wilkins and Gubb, 1991). Indeed, a number of segment polarity mutations cause defects in defined regions of the leg, such as deletions of specific bristle rows (Held, 1993). These genes may function in concert to draw the h boundaries of expression in the pupal leg, similar to the way in which the gap genes establish the boundaries of pair-rule gene expression in the embryo (reviewed by Carroll, 1990).

h specifies the boundaries of only half of the ac stripes. The question of how the other boundaries of ac expression are determined remains open. One possibility is that another gene, in a manner analogous to h, is expressed within the regions that give rise to the other ac interstripes. Alternatively, it is also possible that the boundaries of the other ac stripes might be determined in a manner analogous to that suggested above for h. For example, the segment polarity gene en could participate in drawing some of the boundaries. We showed that ac stripes 1-3 are expressed within the en domain, while ac stripe 4 is positioned adjacent to the dorsal border of en expression. We have also observed (not shown) that h stripe 4 abuts the ventral border of en expression. This suggests a possible mechanism for the production of one of the ac and h boundaries of expression. Other segment polarity genes could function to establish the other boundaries of ac and h expression.

We gratefully acknowledge Jim Skeath and Nadine Brown for technical advice, for providing photographs for figures and for many thoughtful discussions. We thank David Ish-Horowicz and laboratory members for the kind gift of several cosh lines. We also thank Seth Blair, Grace Panganiban and Jim Skeath for their critical review of the manuscript. We are grateful to Leanne Olds for much help in photography and artwork. Scanning electron microscopy was performed at the Texas Tech University EM Facility. T. Orenic is supported by an NIH Postdoctoral Fellowship (GM-13899-01A1); work in the Carroll Laboratory is supported by the National Science Foundation, The Shaw Scholars’ Program of the Milwaukee Foundation and the Howard Hughes Medical Institute, and work in the Held laboratory is supported by Grant 003644-044 (to L. I. H.) from the Texas Advanced Research Program.

REFERENCES


REFERENCES


Ghysen, A. and Dambly-Chaudiere, C. (1979). Determination of sensory bristles and
Hafen, E., Kuroiwa, A. and Gehring, W. J. (1979). Genetic analysis of the
20
(1991). The choice of cell fate in the
(1979b). A high-resolution morphogenetic map of the second-
Held, L. I., Jr (1979a). Pattern as a function of cell number and cell size on the


(Accepted 19 February 1993)