The *Drosophila* proboscis is specified by two Hox genes, *proboscipedia* and *Sex combs reduced*, via repression of leg and antennal appendage genes

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SUMMARY

The proboscis is one of the most highly modified appendages in *Drosophila melanogaster*. However, the phenotypes of *proboscipedia* (*pb*) mutants, which transform the proboscis into leg or antenna, indicate a basic homology among these limbs. Recent genetic studies have revealed a developmental system for patterning appendages and identified several genes required for limb development. Among these are: extradenticle (*exd*), homothorax (*hth*), dachshund (*dac*), Distal-less (*Dll*) and spalt (*sal*). These limb genes have not been well studied in wild-type mouthparts and their role if any in this appendage is not well understood. Here we demonstrate that the homeotic gene products Proboscipedia (*Pb*) and Sex combs reduced (*Scr*) regulate the limb genes in the labial disc to give rise to a unique type of appendage, the proboscis. *Pb* inhibits *exd*, *dac* and *sal* expression and downregulates *Dll*. This observation explains the ability of *Pb* to inhibit the effects of ectopically expressed trunk Hox genes in the proboscis, to suppress leg identity in the trunk and to transform antenna to maxillary palp. *Scr* suppresses *sal* expression and also downregulates *Dll* in the labial discs; discs mutant for both *pb* and *Scr* give rise to complete antennae, further demonstrating appendage homology. In the labial disc, *Pb* positively regulates transcription of *Scr*, whereas in the embryo, *Scr* positively regulates *pb*. Additionally, our results suggest a revised fate map of the labial disc. We conclude that the proboscis constitutes a genetically distinct type of appendage whose morphogenesis does not require several important components of leg and/or antennal patterning systems, but retains distal segmental homology with these appendages.

Key words: *Drosophila*, Proboscis, Labial disc, Homeotic, *dachshund*, extradenticle, Distal-less, *spalt*, *proboscipedia*, *Sex combs reduced*

INTRODUCTION

A highly conserved set of homeotic (Hox) genes is required for the specification of structures in segments along the main body axis of many animals. In insects, including *Drosophila melanogaster*, these structures include a diverse set of specialized appendages: antennae, mouthparts, wings, legs, analia, etc. Moreover, genes that are involved in leg morphogenesis are also employed in the development of structures as morphologically dissimilar as antennae and wings (Lecuit and Cohen, 1997; Abu-Shaar and Mann, 1998; Casares and Mann, 1998). Although many of these appendages have been studied in detail, the deployment of the same appendage patterning genes in the development of the specialized mouthparts, namely the proboscis, of *Drosophila* is not well understood.

Some of the more dramatic *Drosophila* homeotic mutations cause transformations of the proboscis into other types of appendages (Kaufman et al., 1990). A null mutation in the gene *proboscipedia* (*pb*), which is normally expressed in the labial discs, transforms the proboscis into a pair of tarsi; *pb* hypomorph mutations, such as *pb*Δ, results in the transformation of the proboscis into a pair of aristae; and hypomorphic *Scr* mutations cause a transformation into what has been interpreted as a pair of maxillary palps (Kaufman et al., 1990; A. M. Pattatucci, PhD thesis, Indiana University, 1991; Aplin and Kaufman, 1997). Mutations in both *pb* and *Scr* transform the proboscis into a complete antenna (Percival-Smith et al., 1997). These transformations indicate that the proboscis is a highly derived appendage developmentally related to both antennae and legs.

In order to address the segmental organization and homology of the proboscis vis-a-vis legs and antennae, we have studied wild-type labial discs, as well as the individual and combined effects of *pb* and *Scr* mutations on the distribution of the known appendage and homeotic genes in the labial anlagen. Both *hth* and *exd* are required for proximal leg development and the formation of antennal structures (Casares and Mann, 1998). We found that wild-type labial discs expressed low levels of *Hth* and *Exd*, *pb* mutants displayed levels of *Hth* and *Exd* that were comparable with wild-type legs and antennae, and that ectopic expression of *Pb*
in other imaginal discs suppressed Exd. Furthermore, we provide evidence that Pb is both required and sufficient to suppress dac in all appendages, thereby preventing formation of structures that correspond to the intermediate portions of legs or antennae. In addition, Pb suppresses sal expression; for example, ectopic expression of Pb in the eye-antennal disc suppresses sal and phenotypically transforms the antenna to maxillary palp. However, Teashirt (Tsh), another protein necessary for proximal leg development is not expressed in the eye-antennal disc. For example, ectopic expression of Pb in the eye-antennal disc suppresses sal and phenotypically transforms the antenna to maxillary palp. We conclude from these observations that the proboscis represents a highly modified appendage missing the regulatory program to properly specify the coxa (Cx), trochanter (Tr), femur (Fm) and, at least part of, the tibia (Tb). We argue that this modification is due in large part to the action of Pb and Scr. The distal portion of the appendage, homologous to the leg tarsus (Tar) and antennal arista (Ar), is present but is completely re-patterned, owing to the joint effect of Pb and Scr.

We have also investigated possible regulatory effects of the gnathal homeotic genes on each other. It has been shown that Dfd and Scr positively regulate pb and are required for its correct deployment during embryogenesis (Rusch and Kaufman, 2000). We confirm that Pb and Scr are co-expressed in most of the labial disc, while Dfd is expressed in a small proximal domain separate from the expression domains of Pb and Scr (Mahaffey and Kaufman, 1987; Randazzo et al., 1991). We report that Pb is necessary for Scr expression in the distal but not in the proximal half of the labial disc. We propose a model for the imaginal disc regulation of the gnathal homeotic genes that is strikingly different from the one demonstrated for the same genes in the embryo (Rusch and Kaufman, 2000).

Dill is required for the development of the labellar structures on the distal part of the proboscis, such as pseudotracheae and border hairs (Cohen and Jürgens, 1989). The Dil expression domain for wild-type and mutant discs in the part of the labial disc opposite the peripodial stalk argues for a revision of the existing fate map (Kumar et al., 1979). We suggest that the proximodistal axis of the proboscis coincides with the main anatomical axis of the labial disc. This is supported by the expression domains of Pb and Scr, both of which are required for development of the labellar structures and are expressed chiefly in the distal two-thirds of the labial disc (Mahaffey and Kaufman, 1987; Randazzo et al., 1991; Pattatucci and Kaufman, 1991).

MATERIALS AND METHODS

Fly stocks
Drosophila stocks were maintained at 25°C; all Gal4 crosses were performed at 29°C unless otherwise noted. All of the homeotic mutations singly and in combination were balanced using the TM6B, Tb chromosome. Crosses of mutant/TM6B, Tb with mutant/TM6B, Tb were performed and the progeny raised in uncrowded conditions. Progeny that were homozygous or carried heterozygous combinations of Hox mutations were selected as third instar larvae by their Tb+ phenotype. In some cases, the selected larvae were allowed to develop and the adult phenotype examined; the remainder were dissected and their imaginal discs removed for staining. In addition to the mutant fly lines, imaginal discs and adult progeny examined in this study were derived from crossing P[w+Wh=Gal4-dpp.k1]140C.6 (a dpp-Gal4 driver obtained from M. Hoffman) to a transgenic P[UAS-pb]49.1 stock (Aplin and Kaufman, 1997). We also crossed the same driver to a line carrying a P[UAS-Scr]4E4 transgene. The progeny of this cross will be referred to as dpp->pb and dpp->Scr throughout the rest of the paper. In all cases, virgin females from the Gal4 driver line were crossed to males of the UAS responder line. As controls, a fly line carrying P[ry+;y=BS3.0], a dpp-lacZ reporter, as well as progeny derived from crossing P[w+Wh=Gal4-dpp.k1]140C.6 with a P[UAS-lacZ-NLS] line (T. Jacobsen, PhD thesis, 1999) were carried through the analyses. Homeotic mutant lines and the dpp-lacZ reporter line were obtained from the Bloomington Stock Center.

Immunohistochemistry
Imaginal discs were dissected and fixed as described in Pattatucci and Kaufman (Pattatucci and Kaufman, 1991) with the following modifications: inverted larvae were rocked for 20 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS) with 2.5% dimethylsulphoxide, then washed twice with absolute methanol. These were then stained as follows: after two quick washes in PB1 (1xPBS with 0.1% Triton X-100) and four 10 minute washes in PB1T (PB1 with 0.5% bovine serum albumin), larvae were blocked for 30 minutes with 30 µl normal goat serum in 270 µl of PB1T. Larvae were rocked overnight at room temperature after addition of the appropriate primary antibodies. After overnight incubation, larvae were washed four times for 10 minutes each with PB1T, reblocked with 30 µl of normal goat serum in 470 µl of PB1T for 30 minutes, incubated with secondary antibody for 2 hours and then washed once with PB1T. After this wash, 100% glycerol was added, and the discs were dissected and mounted in Aqua PolyMount. All fluorescent labeling was carried out with Jackson Labs fluorescently labeled secondary antibodies and were viewed using confocal microscopy.

Antibodies
The polyclonal rabbit β-galactosidase antibody was produced and affinity purified in our laboratory by David Miller. The Dac monoclonal antibody, developed in the laboratory of Dr Rubin, was obtained from the Developmental Studies Hybridoma Bank. These and all other antibodies used and their dilutions were as follows:

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RESULTS

The morphology of the labial disc

The labial discs begin their development in the embryo as ventralateral thickenings of the atrium (mouth cavity) that are just anterior to the position of the common salivary duct. These epidermal invaginations grow to produce two round lateral sack-like buds that are attached to the larval mouthparts by a peripodial stalk (Poulsone, 1950; Fig. 1A). For the purposes of the description of the gene expression patterns in this paper, we will refer to the stalk end of the labial disc as proximal and the region opposite to that attachment as distal (Fig. 1B). The labial discs then fuse during metamorphosis to create the proboscis of the adult fly (Fig. 1C). In wild-type labial discs, Pb and Scr are co-expressed in all but the most proximal cells of the disc (Fig. 1D-F). In the figures, all labial discs are viewed laterally. This is in contrast to leg and antennal discs, in which the distal regions of each disc are at the center. The position of the peripodial stalks in the confocal images is marked with a white dot.

Pb is required for suppression of dac, DII and exd in the labial discs

The proboscipedia null mutation pb\(^5\), when homozygous, displays a homeotic transformation of the proboscis into a pair of tarsi (Kaufman et al., 1990). In order to address the molecular nature of this transformation and the role of Pb in mouthpart morphogenesis, we analyzed the expression of genes involved in limb patterning: dac, DII and exd, in both wild-type and mutant labial discs. No Dac is detected in wild-type labial discs (Fig. 2A). However, Dac is strongly expressed in a distinct circular domain in the labial discs from pb\(^5\) mutants (Fig. 2D,E). We detect DII in the wild-type labial discs in a small, well-defined, crescent-shaped domain in a position located directly opposite the peripodial stalk (Fig. 2B). This DII accumulation is weaker than the signal detected in the leg and antennal discs of the same animals (Fig. 2HJ). In pb\(^5\) mutants, however, DII is expressed in the distal part of the labial disc at levels comparable with those seen in the leg and antennal discs. Additionally, its domain is now expanded more proximally towards the peripodial stalk (Fig. 2D,F).

In addition, we looked at the expression of Exd in pb\(^5\) labial discs. Low levels of Exd (Fig. 2C) are detected in wild-type labial discs, whereas detectable levels of nuclear protein are observed in haltere and leg discs from the same animals (Fig. 2K). The labial discs from pb\(^5\) mutants, however, have easily detectable nuclear accumulation of Exd in their proximal half. Fig. 2D,E,G also shows that most of the Exd domain is just proximal to that of Dac with only a few overlapping cells. As a result, triple staining with Exd, Dac and DII antibodies of the pb\(^5\) mutant labial discs reveals a pattern comparable with that seen in the leg discs (compare Fig. 2D with 2H). The salient difference is that leg imaginal discs have a much broader Dac domain, which includes ‘Dac+DII’ and ‘Dac only’ territories, whereas the entire Dac domain in the pb\(^5\) labial discs overlaps the DII domain.

In summary, wild-type labial discs express no Dac, a reduced domain of DII and low levels of Exd. pb\(^5\) null mutant labial discs express Dac, a larger domain of DII and express Exd at levels comparable with those in leg discs, indicating that Pb is required for the negative regulation of dac, DII and exd genes in labial discs.

Pb is sufficient for suppression of dac and exd

Using the GAL4 system (Brand and Perrimon, 1993), we examined whether Pb was sufficient for the negative regulation of dac, DII and exd outside the normal expression domain of pb, namely the leg imaginal discs. We ectopically expressed Pb in a dpp pattern in the leg discs (dpp\(\rightarrow\)pb) and analyzed the expression of the appendage genes (Fig. 3A-H). Most dpp\(\rightarrow\)pb imaginal discs are mis-shapen because of ectopic Pb; the discs have abnormal folding and do not lay flat like wild-type discs. First, we determined that a stripe of β-galactosidase, produced with the dpp: Gal4 driver (dpp\(\rightarrow\)lacZ), partly overlaps with the circular Dac domain in wild-type leg discs (Fig. 3A,B). However, in dpp\(\rightarrow\)pb flies, the Dac domain is disrupted by Pb-expressing cells (Fig. 3C,D). No nuclei accumulate both Pb and Dac are observed. A similar effect of Pb was observed on Exd Fig. 3E,F). In dpp\(\rightarrow\)lacZ animals, β-galactosidase expression broadly overlaps the DII domain (Fig. 3G). When Pb was expressed in the dpp pattern, although the pattern of DII expression was altered, there was clear co-expression of these two proteins (Fig. 3H). So while Pb is sufficient for the repression of dac and exd, it is not sufficient for the repression of DII.

Pb is necessary and sufficient for the regulation of Scr in imaginal discs

In addition to Pb, Scr is expressed in the labial discs. In order to more fully understand the nature of the homeosis associated with pb loss of function, we studied the concomitant expression patterns of Scr and some of the appendage gene products in wild-type and pb mutant labial discs (Fig. 4A-F). In pb\(^5\) mutant labial discs, Scr expression is clearly lowered in the distal portion of the disc (Fig. 4A,F; compare with wild-type Scr expression in Fig. 1F). Scr protein accumulation in the proximal third of pb\(^5\) labial discs is apparently normal. Therefore, Pb is required for normal expression of Scr in the distal but not in the proximal portion of the labial disc. In addition, we determined the relative positions of the Scr, Dac and DII expression domains in pb\(^5\) mutant labial discs (Fig. 4A-F). Scr expression shows little overlap DII or Dac, albeit there appear to be a few co-expressing cells. As noted above, Dac expression completely overlaps the expression domain of DII (Fig. 4B).

Surprisingly, we also found that Pb was capable of activating Scr when expressed ectopically (Fig. 4G-I). In wild-type T3 leg discs, no overlap exists between Dac and Scr (Fig. 4G). As noted above in dpp\(\rightarrow\)pb discs, the Dac ring is disrupted by a stripe of pb-expressing cells. These cells also accumulate Scr and no overlap between the Scr and Dac is observed (Fig. 4H). Additionally, we detected a stripe of Scr expression in the wing discs of dpp\(\rightarrow\)pb flies (Fig. 4I), where Scr is normally not expressed (not shown). Thus, ectopic Pb accumulation is associated with the negative regulation of the limb-patterning genes and the positive regulation of another homeotic locus, Scr. This raises the question as to whether or not Pb negatively influences the limb patterning genes through Scr. To test this possibility, we expressed Scr directly using the dpp GAL4 driver (dpp\(\rightarrow\)Scr). While the legs are morphologically normal in these animals, the antennae are transformed distally to a tarsus-like identity. Thus, the Scr protein is having a demonstrable effect on the development of the antennae. However staining of the eye-antennal discs from these animals,
while showing clear ectopic accumulation of Scr, shows no gap in the Dac ring of expression. There is however a clear repression of \textit{spalt} (\textit{sal}).

\textbf{Molecular analysis of the labial discs from pb\textsuperscript{4} mutants}

Unlike the transformation of the proboscis into a pair of tarsi in pb\textsuperscript{5} null mutants, the proboscis is transformed into a pair of aristae in pb\textsuperscript{4} hypomorphic mutants (Kaufman et al., 1990; Aplin and Kaufman, 1997). Recall that in wild-type labial discs, we observe low levels of Exd/Hth, no Dac and a small domain of Dll at the distal tip of the labial disc. We analyzed the molecular patterning of pb\textsuperscript{4} mutant labial discs and compared them with wild-type antennal disc patterning (Fig. 5A-G). In the wild-type antennal disc, the Hth domain overlaps the domain of Dll in a ring, which surrounds a ‘Dll only’ region (Fig. 5B). Sal, an important antennal marker (Dong et al., 2000), is expressed in a circular domain at the junction of the Dll and Hth expression domains (Fig. 5A,C). In addition, the circular domain of Sal surrounds a crescent-shaped domain of Dac (Fig. 5G). No Sal was detected in the labial discs of wild-type animals (not shown). Scr is expressed at the base of the antennal disc.
but not in the disc proper, revealing little overlap with Sal accumulation in wild-type eye-antennal discs (Fig. 5G). Unlike wild-type labial discs, pb4 labial discs contain distinct domains of Dac, Dll, Hth and Sal expression (Fig 5D-F,H,I), similar to wild-type antennae but in diminished domains. We note that the Dac and Sal domains often form a spot or a short crescent-shaped stripe instead of the ring seen in wild-type eye-antennal discs (compare Fig. 5G with 5I).

We also determined the effect of the hypomorphic allele on the expression of Scr in the labial discs. Similar to pb5 mutants, we see a distinct, albeit weaker, regulatory effect on Scr expression in hypomorphic pb4 mutants. The level of Scr accumulation is diminished most clearly distal to the spot of Dac accumulation (Fig. 5H), the diminished domain coinciding and narrowly overlapping with the distal domain of Sal (Fig. 5H).

**Distribution of Tsh in wild-type and mutant labial discs**

We also studied the distribution of Tsh, a molecule required for development of the maxillae and antennae and the proximal segments of the leg (Bhojwani et al., 1997; Erkner et al., 1999). Using an enhancer trap, tsh expression has been shown to be absent from the proboscis in adults (Bhojwani et al., 1997). We confirmed the lack of Tsh accumulation in the labial discs with both rat and rabbit anti-Tsh antibodies (Table 1). This was in contrast to easily detectable protein levels in leg discs from the same animals (not shown). We also found that no ectopic Tsh expression is seen in pb5 mutant labial discs (not shown). This suggests that the leg-like structures of the pb5 homeotic mutants lack properly specified proximal podomeres.

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**Fig. 3.** The effect of ectopic accumulation of Pb on the expression of dac, Dll and exd. (A) There is a circular Dac (red) domain in wild-type leg discs (T2 is shown). (B) The pattern of accumulation of β-galactosidase in the T3 leg disc of a dpp=>lacZ animal. Note the yellow nuclei expressing both β-gal (green) and Dac (red). (C,D) dpp=>pb T2 and T1 leg discs showing a broken Dac (red) ring. The ring is interrupted by a stripe of Pb-expressing cells (green). (E) dpp=>lacZ is capable of driving lacZ (green) in Exd-expressing cells (red) of the T2 leg disc. (F) Little or no co-expression of Pb (green) and Exd (red) is seen in a T1 leg disc of dpp=>pb flies. (G) β-gal (green) expression overlaps with that of Dll (red) in a dpp-lacZ T3 leg disc. Note multiple yellow nuclei expressing both Dll and β-gal. (H) dpp=>pb showing Pb (green) in a stripe across a T3 leg disc stained with anti-Dll antibody (red). Although the normal expression pattern of Dll is altered, there is co-expression in many cells.

**Fig. 4.** Regulation of Scr by Pb. (A-C) Expression of Dll (green), Dac (red) and Scr (blue) in a pb5 labial disc. (B) The Dac (red) expression domain completely overlaps the expression domain of Dll (green). (C) Scr (blue) and Dac (red) expression in a pb5 labial disc. Note the gap in Scr accumulation that coincides with the band of Dac-positive cells and that there is no detectable Scr accumulation in the distal end of the disc. For comparison with wild-type Scr expression in the labial disc, refer to Fig. 5A. (D-F) Same disc showing individual channels of Dll, Dac and Scr. (G) Wild-type T3 leg disc stained with anti-Scr (green) and anti-Dac (red) antibodies. There is no overlap (yellow) of accumulation. (H) The Dac (red) ring is interrupted by the Scr (green)-expressing cells in a dpp=>pb T3 leg disc. No overlap (yellow) between Dac and Scr is detected. (I) Ectopic expression of Scr (green) in a dpp=>pb wing disc. Again there is no co-expression of Scr and Dac. W, wing disc.
Fig. 5. Distribution of Dac, Dll, Hth, Sal and Scr in pb4 mutant labial discs. (A-C) Wild-type eye-antennal disc stained with Dll (red), Hth (green) and Sal (blue). (B,C) The same disc showing the ring of overlap between Dll and Hth in yellow, and the separate blue channel of Sal alone in the antennal disc. (D-F) A labial disc from a pb4 mutant also stained with Dll (red), Hth (green) and Sal (blue). Note that where the expression of Dll and Hth overlap (yellow, E), Sal is expressed in a small band of cells (F). (G-I) Comparison of Dac, Sal and Scr in a wild-type eye-antennal disc and a pb4 labial disc. (G) In a wild-type eye-antennal disc, a distinct ring of Sal surrounds a crescent-shaped ring of Dac; Scr is not expressed in the antennal disc proper. (H) Scr is expressed in the proximal portion of the pb4 mutant labial disc but is expressed at lower levels at the most distal tip (also see Fig. 5E), coinciding and slightly overlapping with the region of Sal (overlap appears turquoise). (I) Same disc as in (H) showing only Dac and Sal expression. pb4 mutant labial discs express a small band of Sal-expressing cells just proximal to a small region of Dac-expressing cells, similar to antennae. E-A, eye-antennal disc.

Molecular analysis of Scr mutants

Hypomorphs of Scr that survive to adulthood exhibit a transformation of proboscis into what has been interpreted to be a maxillary palp identity (A. M. Pattatucci, PhD thesis, Indiana University, 1991). To understand the changes taking place at the molecular level, we analyzed the expression patterns of Pb and products of several appendage genes: dac, Dll, exd and sal in this class of Scr mutants.

Fig. 6A-C show the pattern of accumulation of Scr and Pb in a labial disc from an Scr+/Scr- hypomorph. Levels of Scr accumulation in these mutants are variable and usually lower than in wild-type labial discs (Fig. 6B). However, there is easily detectable Pb protein in these discs (Fig. 6C). As these Scr lesions are leaky and because Scr has been shown to positively regulate pb in the embryo (Rusch and Kaufman, 2000), we thought it prudent to further investigate the regulatory relationship of the two homeotic genes in imaginal cells. Imaginal discs from dpp->Scr animals were stained for Pb accumulation. No ectopic expression could be detected, despite readily detectable levels of the protein in the labial discs of these animals (not shown). Thus, in contrast to what is observed in the embryo (Rusch and Kaufman, 2000), Scr does not act as a positive regulator of pb in the imaginal discs.

Analysis of Dac, Dll, Exd and Sal in Scr mutant labial discs reveals a complete absence of Dac (not shown) and Sal (Fig. 6F), and only low levels of Exd (Fig. 6E), similar to wild-type labial discs. This is consistent with the posited role of Pb as a determinant of the maxillary palp anlagen (Aplin and...
Fig. 7. Patterns ofDll, Exd, Hth and Sal accumulation in
pb\(^5\)Scr\(^5\)/pb\(^5\)Scr\(^4\) mutants. (A) Scanning electron micrograph
showing a transformation of the proboscis into an antenna in a
pb\(^5\)Scr\(^5\)/pb\(^5\)Scr\(^4\) mutant. Note the absence of claws or pulvillae.
(B) Wild-type labial discs express low levels of Hth (green)
and no Sal (blue). (C) pb\(^5\)Scr\(^5\)/pb\(^5\)Scr\(^4\) mutant labial discs
express domains of Dll (red), Exd (green) and Sal (blue) that
resemble those in the antennae. (For reference, wild-type labial
disc expression patterns of Dll and Exd are shown in Fig.
2B,C.) (D,E) In the labial discs of pb\(^1\)Scr\(^4\)/pb\(^5\)Scr\(^4\) mutants, Sal
expression (blue) is localized to cells expressing both Dll (red)
and Hth (green, shown with arrows in D).

Kaufman, 1997), and the inferred phenotype of Scr mutant
mouthparts. The wild-type maxillary anlagen in eye-antennal
discs expresses little Exd and no Dac (not shown), which
correlates well with these results. Surprisingly, the labial discs
of Scr\(^6\)/Scr\(^5\) mutants show a slightly expanded domain of Dll
(Fig. 6D,G). In summary, these results demonstrate that Pb
alone is sufficient to repress dac and exd but that Scr is also a
negative regulator of Dll in the labial discs. Thus Pb may be
having its negative effect on Dll in the labial discs indirectly
through Scr. This regulatory relationship will be elaborated on
in the Discussion.

**Molecular analysis of the labial discs from
pb\(^1\)Scr\(^4\)/pb\(^5\)Scr\(^5\) mutants**

We also analyzed the labial discs of pb\(^1\)Scr\(^4\)/pb\(^5\)Scr\(^5\) double
mutants, where Scr function is impaired and Pb is absent. As
Scr\(^6\)/Scr\(^5\) is hypomorphic with variable but detectable levels
of Scr accumulation, we observed several animals for their
mutant proboscis phenotypes. These phenotypes varied from
thick and shortened aristae with claws and pulvillae to
apparently complete antennae with aristae, third and second
antennal segments bearing characteristic bristles (Fig. 7A).
This latter observation is consistent with the results of a
clonal analysis in which Scr\(^{pb}\) cells produced antennal
structures (Percival-Smith et al., 1997). In accordance with
the observed phenotypic variation, we found variable
patterns of accumulation of Dac, Dll, Hth and Sal. Whereas
no Dac (Fig. 2A), a small domain of Dll (Fig. 2B), low
expression of Exd (Fig. 2C) and Hth, and no Sal (Fig. 7B)
are expressed in wild-type labial discs, we observed
expression patterns of these appendage genes that were very
similar to those seen in either wild-type antennae (Dac not
shown; compare Fig. 5A,G with Fig. 7C,D) or in wild-type
legs (presumably owing to leaky expression of Scr). These
antennal expression patterns (and importantly the presence
of Sal) are consistent with the genetic make-up of the wild-
type antennal disc, which does not exhibit any HOM-C gene
expression in the antennal disc proper (Yao et al., 1999). As
in pb\(^4\) mutant labial discs, which display a transformation to
aristae, there is clear Sal expression where the domains of
Hth and Dll overlap (Fig. 7D,E). However, unlike pb\(^4\) mutant
discs, these pb Scr double mutant labial discs are more
similar in shape and size to wild-type antennal discs, and the
domain of Sal is more circular and not at the distal tip as in
pb\(^4\) mutant labial discs (compare Fig. 7D,E with Fig. 5H).

**Regulation of sal by Pb and Scr**

In order to further understand the nature of the pb\(^4\) aristal
transformation versus the tarsal transformation in pb\(^5\) mutants,
we examined eye-antennal discs of dpp\(\rightarrow\)Scr and dpp\(\rightarrow\)pb
animals for Sal. In wild-type eye-antennal discs there is clear
overlap between the normal dpp\(-\)lacZ pattern and the ring of
Sal (Fig. 8A). However, in dpp\(\rightarrow\)Scr antennal discs, whose
adult phenotype is a transformation to tarsal identity, there is
clear repression of sal where Scr accumulates in a dpp pattern
(Fig. 8B-D). A similar effect is seen in dpp\(\rightarrow\)pb eye-antennal
discs, in which the antennae are transformed toward a
maxillary palp identity (Aplin and Kaufman, 1997): there is no
overlap of Pb- and Sal-expressing nuclei and there is a clear
disruption of the ring of Sal (Fig. 8E-G). As we have observed
activation of Scr by ectopic Pb in imaginal discs, including the
antennal anlagen, this repression could be due to Scr induction
and not a direct effect of Pb. This interpretation is supported
by the fact that the residual Scr expression seen in pb\(^2\) mutant
labial discs is apparently sufficient to repress sal expression
and when both pb and Scr are inactivated Sal accumulation
observed in the labial discs (Fig. 7C,D). It must be noted
however, that the pattern of sal expression in pb\(^4\) mutant labial
discs appears to contradict this interpretation. As is the case for
the pb null allele there is residual Scr expression in the pb\(^4\)
mutant discs (Fig. 5H). Nevertheless, in the hypomorphic
phenotype we do observe Sal accumulation (Fig. 5F), consistent
with the observed transformation of the distal proboscis to
arista. Thus, it would appear that the truncated protein
produced by the pb\(^4\) allele retains an activity not seen in cells
that completely lack Pb. A rationalization of this result will be
offered in the Discussion.

**DISCUSSION**

**Homeotic specification of the proboscis**

The mouthparts of Drosophila melanogaster are some of the
most highly derived appendages in insects, their appearance
having little in common with antennae and legs. In addition to having specialized structures for feeding, there is little obvious segmentation. However, mutations in pb display antennal and tarsal phenotypes that imply an underlying homology among these appendages (Johnston and Schubiger, 1996). Moreover, pb and Scr work together to specify proboscis-specific structures: pb4 hypomorphs show a transformation of mouthparts to aristae (Kaufman, 1978); pb5 nulls transform to tarsus (Kaufman, 1978); and Scr4/Scr5 mutants transform to a maxillary palp-like identity (Pattatucci et al., 1991). Simultaneous mutation of both genes transforms the proboscis to a complete antennal identity (Percival-Smith et al., 1997). This work represents a detailed analysis of a group of gene products required for the development of legs and antennae, and how their expression patterns are governed by a regulatory program controlled by both pb and Scr (Fig. 9). Furthermore, these data suggest that the proboscis is an appendage whose morphological characteristics are dictated by pb, that identifies multiple targets of Pb and uncovers some unexpected regulatory paradigms in the labial discs.

In wild-type labial discs, we did not detect accumulation of Dac, a protein expressed in the intermediate portions of legs and antennae (Fig. 9; Mardon et al., 1994). This observation suggests that the proboscis lacks segments homologous to the femur and tibia of walking legs. The inhibition of Dac is due to the activity of Pb, which we have also shown to be sufficient for the suppression of Dac. As Pb is a homeodomain transcription factor, it is possible that it acts as a direct

Fig. 8. Ectopic expression of Pb or Scr represses Sal expression in the antennae. (A) dpp-lacZ reporter stained for β-gal (green) and Sal (red). Note the region of overlap between the reporter and Sal. (B–D) There is no overlap between Scr-expressing cells and the broken ring of Sal in a dpp=>Scr eye-antennal disc. (E–G) Similarly, there is no overlap between Pb-expressing cells and Sal expression in dpp=>pb eye-antennal discs. (B,E) Merged images; (C,D,F,G) separated channels.

Fig. 9. A summary diagram showing the expression domains in wild-type leg, wild-type antenna, wild-type labial discs and mutant labial discs of the appendage patterning genes analyzed in this study. Wild-type leg and antenna with their respective imaginal disc expression patterns are at the top for reference. The wild-type appendage of the labial disc, the proboscis and various mutant phenotypes are diagramed below and to the right. The genotype of the individual mutants is given above each appendage. The corresponding patterns of protein accumulation in the normal and mutant discs are shown to the right of the appendage phenotypes. Dark blue indicates relatively high levels of Hth/Exd protein accumulation and pale blue indicates low levels. The proboscis (asterisk) is displayed laterally to represent the same plane of view as the labial disc in this figure, and half is lightened to represent a single disc derivative.
regulator of the dac gene; however, additional experimentation will be required to establish this point. It is relevant to mention here that Dac is not expressed in the wild-type maxillary palp anlagen or in the labial discs of Scr mutants, which adopt a more maxillary identity and still express the Pb protein. Therefore, we propose that the maxillary palps, like the labial mouthparts, have lost their intermediate podomers and this is at least partially due to the repression of dac by Pb. The ring domain of Dac expression is narrow in pb5 labial discs relative to the rings in the wild-type leg disc. Moreover, it appears that in the transformed labial disc, all of the Dac-positive cells co-express Dil; i.e. there are no ‘Dac-only’ cells similar to those seen in the leg discs. This observation correlates with the ability of pb5 mutant labial discs to develop tarsus (including the first tarsal segment), owing to the presence of Scr, but not the more proximal podomers, the tibia and femur. These latter segments are thought to be derived from the ‘Dac-only’ domain of the normal leg disc (Mardon et al., 1994). Labial discs mutant for both pb and Scr develop an almost fully articulated set of antennae, which implies that Scr, unlike Antp, is incapable of specifying more than tarsus (distal appendage) identity. This limited capability of Scr is further supported by the observation that animals ectopically expressing Scr in the antennal anlage display an aristato-tarsus transformation only, as opposed to ectopically expressing Antp, which displays a more complete antenna-to-leg transformation (Gibson and Gehring, 1988; Gibson et al., 1990; Zeng et al., 1993; Yao et al., 1999). Finally, it is possible that some as yet unidentified genes are responsible for the suppression or activation of the ‘Dac-only’ domain, just as Pb is necessary for preventing the formation of the ‘Dac+Dil’ domain.

In addition to Dac, pb mutants display levels of Exd and Hth comparable with antennae and legs (Fig. 9); both are expressed at low levels in wild-type labial discs (Fig. 2; Fig. 7B). Exd is an important co-factor of many Hox genes and its absence can alter homeotic gene product function and specificity (Pinsonneault et al., 1997; Ryoo and Mann, 1999). The fact that pb suppresses the expression of Hth and Exd also implies that the three homeotic genes expressed in the labial disc (Dfd, Scr and pb) are functioning largely in the absence of the Exd co-factor and that this may contribute to the uniqueness of regulatory interactions in the labial appendage.

Dil, a protein needed for the development of most of the distal structures in both legs and antennae, is normally expressed at low levels (relative to antennal and leg discs) in a restricted distal domain in the labial disc (Fig. 9). Analysis of pb and Scr mutants demonstrates that Dil is expressed in the distal half of the labial disc at levels comparable with legs and antennae. Both pb and Scr are required for the development of the distiproboscis, a structure dependent at least in part on the function of Dil (Mahaffey and Kaufman, 1987; Cohen and Jürgens, 1989; Randazzo et al., 1991). As Pb is required for activation of Scr in the distal half of the labial disc and the expression domains of Scr and Dil are reciprocal in pb5 mutants, it is likely that Scr is the principal negative regulator of Dil expression in the labial disc.

We propose that the proboscis constitutes an appendage type that is distinct from both antenna and thoracic leg, owing at least in part to the repression in the labial discs by pb of several of the genes normally involved in patterning legs and antennae (Fig. 10). As a result, the fly proboscis appears to lack elements serially homologous to the proximal and intermediate leg segments. We support the hypothesis that the labellum, the distalmost part of the proboscis that bears the pseudotracheae, is homologous to the leg tarsus. However, our analysis demonstrates that the labellum is thoroughly modified and it is likely that other, as yet unidentified, genes are also specifically involved in the morphogenesis of this unique appendage and are controlled by both Scr and pb. Additionally, there may be loci not influenced by but similar to pb and Scr; for example, genes not normally expressed in the leg but involved in the suppression in the labial discs of limb segmental anlagen.

Regulatory relationship between Pb and Scr in the imaginal discs

The regulatory interaction between the homeotic genes pb and Scr in the imaginal discs is distinct from the interaction seen in embryos (Fig. 10; Rusch and Kaufman, 2000). During embryogenesis in Drosophila and other insects, Pb is expressed in the maxillary and labial lobes where it overlaps with both Dfd and Scr (Rogers and Kaufman, 1997; Peterson et al., 1999; Rusch and Kaufman, 2000), and this expression domain is established in part through positive regulation by Dfd and Scr in the maxillary and labial segments, respectively (Rusch and Kaufman, 2000). Moreover, Scr positively regulates pb in the red flour beetle Tribolium castaneum (Denell et al., 1996; S. Brown, personal communication). We found that Pb expression in the adult derivatives of the labial segment does not depend on Scr or Dfd (Fig. 10; not shown). Dfd is expressed in a domain that is mutually exclusive from the expression domain of Pb in the labial disc; therefore, Dfd is an unlikely candidate for direct positive regulation of pb in this tissue. Moreover, rather than activating pb, Scr is positively regulated by Pb in the imaginal labial discs. Note that although Pb controls Scr in the distal half of the labial disc, Pb has very little effect on Scr expression in the more proximal part of the labial disc (Fig. 10). Clearly, two distinct genetic/developmental territories exist within an individual labial disc.
Arista versus tarsus identity in pb mutant labial discs

One of the more interesting phenotypes observed is the aristal transformation in pb<sup>5</sup> hypomorphs. What makes this interesting is that Scr, whose function normally is to specify tarsal identity, is present throughout the pb<sup>5</sup> mutant labial discs except for the distal tip. Additionally, we observe that when Scr is ectopically expressed in the antennal disc using the Gal4 system, sal, a gene important for antennal/aristal development is repressed cell autonomously (Fig. 8). In addition, Scr can repress antennal identity and promote tarsal identity via repression of hth (Yao et al., 1999). In light of the fact that Scr is expressed in the proximal region of pb<sup>5</sup> null labial discs, represses sal and specifies tarsus, one might predict that the more robust expression pattern of Scr in pb<sup>4</sup> labial discs should be sufficient to repress Sal accumulation in these mutant discs as well. However, in pb<sup>4</sup> mutant labial discs, at the point of decreased Scr expression, co-expression with Sal is observed. While it is possible that in the hypomorphic genotype, Scr levels fall below a threshold level in some cells (such that Scr cannot repress sal), we favor an alternative hypothesis. The products of several hypomorphic alleles of pb, including pb<sup>4</sup>, are partially functional, truncated peptides that still contain the DNA-binding homeodomain (Cribbs et al., 1992). A closer look at the pb<sup>4</sup> adult phenotypes reveals that the proboscis of these mutants, in addition to having aristae, have fewer pseudotracheal rows compared with wild type, but do form these labial-specific structures (Cribbs et al., 1992). Moreover, antibody raised to epitopes encoded by the second exon of pb shows wild-type accumulation of the truncated pb<sup>4</sup> encoded peptide (not shown). Therefore, the truncated Pb protein must still be sufficiently functional for specification of mouthpart components such as pseudotracheae, despite the concomitant expression of Scr. Additionally, while full-length Pb protein can suppress sal expression in the antennal disc (Fig. 8), it is evident that these truncated, partially functional proteins do not prevent Sal accumulation in the labial discs. We conclude therefore that the presence of the partially functional, truncated peptide is in some way interfering with the activity of Scr in the cells in which both proteins are accumulated. Determining the validity of this ‘interference’ model will, of course, require further investigation. It should also be noted that the pb<sup>4</sup> encoded peptide is deficient in its ability to repress dac expression in the labial discs, as easily detected levels of Dac are found in these mutant discs.

Finally, there are many appendage genes that are involved in antenna and leg patterning, but they have not been studied here. It is possible that yet another factor or co-factor is needed to activate tarsal identity, which the compromised Pb protein is still capable of repressing.

Distinct genetic environments in different imaginal discs

Pb suppresses dac gene expression cell autonomously in the labial discs and can suppress its expression in the antennal or leg anlagen; however, other regulatory relationships appear to be unique to the labial discs. An example of this is the regulation of Dil by either Pb or Scr. Mutants show clear derepression of Dil to resemble Dil accumulation levels and pattern in leg and antennal discs, yet ectopic expression of either Pb or Scr shows that these proteins do not repress Dil in other imaginal discs. Thus, it would appear that other, as yet unidentified, factors are probably involved in the development and specification of the different imaginal discs. The fact that each disc type represents a different ontogenic environment is not an entirely surprising or unanticipated finding.

Fate map of the labial disc

The original fate map of the labial disc, based on surgical fragmentation and implantation experiments, indicated that the future distalmost part of the proboscis, which gives rise to the labellum and is surrounded by the border hairs, lay in the proximoventral region of the labial disc (Fig. 11; Kumar et al., 1979). Thus, the proximodistal axis of the adult proboscis on the published labial disc fate map is pointed towards the peripodial stalk (Fig. 11B). Analysis of Dil mutants has shown that, in part, the pseudotracheae of the labellum and the border hairs depend on the function of this gene (Cohen and Jürgens, 1989). We have shown in this analysis that Dil expression is localized to a well-defined domain opposite to and not near the peripodial stalk. This expression pattern suggests that the proximodistal axis of the proboscis points away from the stalk and coincides with the main anatomical axis of the balloon-shaped labial discs (Fig. 11B). Nevertheless, the map of the labial disc and the position of Dil accumulation become coincident, if (while the relative positions of the fate mapped structures are maintained) the map is simply rotated 180° (Fig. 11B). The expression pattern of Pb in wild-type labial discs supports this interpretation. Loss of Pb
function is relative to the proximodistal axis of the proboscis, as mutants have a stronger effect on more distal structures (Bouhe et al., 1998). Notably, the highest expression levels of Pb are observed in the area of the labial disc opposite the peripodial stalk (these results).

The inferences derived from wild-type labial discs are supported by the molecular analysis of the homeotic mutants. In pb null and pb Scr double mutant labial discs, the main growth associated with strong Dil and Dac expression is directed away from the stalk, not towards it. This finding suggests that the proximodistal axis is preserved in the mutant labial discs (Fig. 11B). Additionally, the pattern of expression of all of the appendage regulating genes in the transformed labial discs indicates a proximodistal axis that runs from the proximal peripodial stalk to the anatomically distal end of the disc (Fig. 11B).

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REFERENCES


Gibson, G., Schier, A., LeMotte, P. and Gehring, W. J. (1990). The specificity of Sex combs reduced and Antennapedia are defined by a distinct portion of each protein that includes the homeodomain. Cell 62, 1087-1103.


