Coexpression of the homeobox genes Distal-less and homothorax determines Drosophila antennal identity

P. D. Si Dong, Jessie Chu and Grace Panganiban*

Department of Anatomy, University of Wisconsin, Madison, WI 53706, USA

*Author for correspondence (e-mail: gepangan@facstaff.wisc.edu)

Accepted 5 November, published on WWW 20 December 1999

SUMMARY

The Distal-less gene is known for its role in proximodistal patterning of Drosophila limbs. However, Distal-less has a second critical function during Drosophila limb development, that of distinguishing the antenna from the leg. The antenna-specific activity of Distal-less is genetically separable from the proximodistal patterning function in that certain Distal-less allelic combinations exhibit antenna-to-leg transformations without proximodistal truncations. Here, we show that Distal-less acts in parallel with homothorax, a previously identified antennal selector gene, to induce antennal differentiation. While mutations in either Distal-less or homothorax cause antenna-to-leg transformations, neither gene is required for the others expression, and both genes are required for antennal expression of spalt. Coexpression of Distal-less and homothorax activates ectopic spalt expression and can induce the formation of ectopic antennae at novel locations in the body, including the head, the legs, the wings and the genital disc derivatives. Ectopic expression of homothorax alone is insufficient to induce antennal differentiation from most limb fields, including that of the wing. Distal-less therefore is required for more than induction of a proximodistal axis upon which homothorax superimposes antennal identity. Based on their genetic and biochemical properties, we propose that Homothorax and Extradenticle may serve as antenna-specific cofactors for Distal-less.

Key words: Distal-less, homothorax, Antennapedia, spalt, Antenna, Leg, Limb, Homeotic transformation

INTRODUCTION

The similar developmental potentials of the Drosophila antenna and leg primordia are evidenced by the number of mutations that result in transformation of one tissue into the other. Genes in which loss-of-function mutations lead to partial antenna-to-leg transformations include homothorax (hth) (Casares and Mann, 1998; Pai et al., 1998), Distal-less (Dll) (Sunkel and Whittle, 1987), and spineless (ss) (Balkaschina, 1929; Struhl, 1982). Complete transformations of the antenna into leg are observed with gain-of-function alleles of the Drosophila trunk Hox gene Antennapedia (Antp) (Gehring, 1986). Transformations of leg into antenna are much less common and have been observed with loss of Antp function (Struhl, 1981). Analysis of the genetic hierarchies among genes required to distinguish the antenna and the leg is likely to provide insights into both limb development and the generation of morphological diversity. Dll and Antp previously were found to repress hth in the developing leg (Casares and Mann, 1998; Gonzalez-Crespo et al., 1998; Abu-Shaar and Mann, 1998), and Dll shown to be required for ss expression in both leg and antenna (Duncan et al., 1998). Here, we analyze the relationship between Dll and hth in the antenna.

Drosophila heterozygous for intermediate and strong loss of function of Dll alleles exhibit truncations of the Drosophila antenna and leg, indicating that Dll plays an essential role in forming the proximodistal (PD) axis in both limb types (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989; this work). Ectopic expression of Dll can induce the formation of new PD axes in various positions of the body (Gorfinkiel et al., 1997). These ectopic limbs take on identities appropriate to their anteroposterior position along the main body axis, e.g. antennal elements on the head and leg elements on the wing (Gorfinkiel et al., 1997). This is consistent with the idea that Dll plays a single role during limb development, that of inducing the formation of PD axes upon which selector genes, such as the Hox genes, superimpose information regulating limb identity. However, in Drosophila carrying only one functional copy of the Dll gene or heterozygous for combinations of hypomorphic Dll alleles, the antenna is partially transformed toward leg (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989; this work). The transformation can occur without any concomitant loss of PD information. This indicates that Dll has a second function during limb development, specifying antennal cell fates. How Dll effects the differentiation of distinct limb types is not immediately apparent since Dll is expressed in similar patterns in the presumptive distal and medial cells of both the antenna and the leg (Cohen, 1990; Diaz-Benjumea et al., 1994). One plausible mechanism would be via interaction with other factors whose expression is limb specific.

hth meets three important criteria for a factor that could cooperate with Dll to regulate antennal identity: (1) it is expressed throughout the antennal primordium for much of development,
(2) it is required for the differentiation of proximal, medial and distal antennal elements, and (3) it is differentially expressed between the antenna and the leg (Casares and Mann, 1998; Pai et al., 1998). In developing legs, hth expression is restricted during embryogenesis to presumptive proximal cells. hth andDll expression therefore do not overlap in the leg throughout most of development (Casares and Mann, 1998). hth encodes a TALE-class homeodomain protein required for the nuclear localization of a PBC-class homeodomain protein encoded by extradenticle (exd) (Pai et al., 1998; Rieckhof et al., 1997; Kurant et al., 1998). Exd is a transcriptional cofactor for a variety of homeodomain proteins, including several Hox gene products (reviewed in Mann and Chan, 1996). Genetic studies indicate that both exd and hth are needed for normal development of the entire antenna and of the proximal leg. Loss of either exd or hth function in the developing antenna causes cell-autonomous transformation of medial and distal antennal structures into medial and distal leg structures (Casares and Mann, 1998; Pai et al., 1998; Gonzalez-Crespo and Morata, 1995). Ectopic expression of Meis-1, a murine homolog of hth, can induce the formation of antennae in the genital disc derivatives (Casares and Mann, 1998). However, ectopic expression of either Meis-1 or hth in the developing leg, wing or head does not lead to antennal differentiation (Casares and Mann, 1998; this work), therefore hth is insufficient to induce antennal development in most contexts.

In this study, we demonstrate that Dll and hth are independently regulated and expressed in partially overlapping domains in the developing antenna. Reducing Dll activity or eliminating hth activity transforms the antenna to leg and results in reduction or loss of spalt (sal) expression. Ectopic expression of Dll in domains of endogenous hth expression or coexpression of Dll and hth using the GAL4/UAS system (Brand and Perrimon, 1993) induces expression of sal, and leads to the differentiation of antennal cuticular structures from the eye, leg, wing and anal/genital primordia. Based on these results, we propose that Dll and hth act at the same level of the genetic hierarchy to coordinately activate the antennal developmental program.

MATERIALS AND METHODS

Immunohistochemistry

Antibody and X-gal stainings were carried out as described (Halder et al., 1998). Antibodies used were: chicken anti-Hth (Casares and Mann, 1998), rabbit anti-Hth (Pai et al., 1998), rabbit anti-Dll (Panganiban et al., 1995), mouse anti-Dll (Vachon et al., 1992), rabbit anti-Hth (Kuhnlein et al., 1998), rabbit anti-Dll (Vachon et al., 1992), and rabbit anti-Sal (Kuhnlein et al., 1998). Secondary antibodies coupled to act>CD2>GAL4 (Pignoni and Zipursky, 1997), (3) w; UAS-Dll/In (2LR) Gla, Gla, Be, Lcp (Konrad Basler), (4) w; UAS-GFP-hth8/TM6B, Tb, Hu (Casares and Mann, 1998), (5) w; FRT82B hthP1 (Pai et al., 1998), (6) w; FRT82B AntpP5C (Gary Struhl), (7) hth-lacz (= hthP7253; Bloomington), and (8) sal-lacz (= salh6562; Bloomington).

Stocks constructed by us for these experiments were: (1) Dll1/Cyo wg-lacZ, (2) Dll3/Cyo wg-lacZ, (3) Dll/Sal1/Cyo wg-lacZ, (4) DllS2A/Cyo wg-lacZ, (5) y, hs-FLPase; FRT82B, 2piM, (6) y; UAS-Dll/In (2LR) Gla, Gla, Be, Lcp; UAS-GFP-hth8/TM6B, Tb, Hu, (7) y, hs-FLPase; UAS-Dll/TM6B, Tb, Hu, (8) y, hs-FLPase; FRT43D, 2piM, (9) w; FRT43D DllS2A, and (10) sal-lacz/Cyo; dpp-GAL4/TM6B, Tb, Hu.

Genetic manipulations

Dll hypomorphic larval imaginal discs were generated by crossing heterozygous Dll mutant animals in which each Dll mutant chromosome was balanced over CyO, wg-lacZ. Mutant animals were identified by the absence of X-gal staining in the larval tails. Ectopic expression of Dll and hth was induced using the GAL4-UAS binary system (Brand and Perrimon, 1993). dpp-GAL4 was used to activate UAS-hth and/or UAS-Dll along the anteroposterior compartment boundary of the developing imaginal discs. Clones of cells ectopically expressing Dll were generated using a modified GAL4/UAS system (Pignoni and Zipursky, 1997) in which y, hs-FLPase; UAS-Dll/TM6B, Tb, Hu flies were crossed to act>CD2>GAL4 and the resulting larvae heatshocked at 37°C for 10 minutes at 72-96 hours after egg laying (AEL) to induce site-specific recombination between the FRT sites, which in turn results in constitutive GAL4 expression in the clones. Dll, hth and Antp null clones were generated using the FLP/FRT system (Xu and Rubin, 1993). Animals of the genotypes: (1) y hs-FLPase; FRT43D, 2piM/FRT43D DllS2A, (2) y, hs-FLPase; FRT82B, 2piM/FRT82B hthP1, and (3) y, hs-FLPase; FRT82B, 2piM/FRT82B AntpP5C were heat shocked at 37°C for 1 hour at 48-72 hours AEL and examined in mid- to late-third instar.

RESULTS

Dll is required for antennal identity

Animals heterozygous for Dll null alleles exhibit partial antenna-
Distal-less and homothorax determine antennal identity to-leg transformations (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989), indicating thatDll levels may be important for antennal determination. Weak hypomorphic combinations ofDll alleles also lead to partial transformation of the third antennal segment (a3) and the arista into leg-like structures (Fig. 1A,B). Intermediate hypomorphic combinations ofDll alleles transform the medial antenna toward leg and exhibit distal truncations (Fig. 1C). Strong combinations ofDll alleles exhibit more severe antennal truncations (Fig. 1D). These same allelic combinations result in progressively more severe truncations of the distal leg (Fig. 1E-H). Notably, the antenna-to-leg transformations are not a property of a specific subset ofDll alleles, but are observed with allDll alleles when assayed in appropriate combinations. For the transformation phenotype to be apparent,Dll PD function must be largely intact. This is likely due to the necessity of having a PD axis for either antennal or leg identity to be manifested. That we observe transformation without limb truncation indicates that the antennal selector function is more sensitive toDll dosage than its PD function. We emphasize that transformation is a loss-of-function phenotype ofDll, not a neomorphic or hypermorphic one. Together, theDll phenotypic

Fig. 2.Dll and hth are not required for each others expression in the larval antenna. (A-C) Dll (blue) and Hth (green) expression overlap (turquoise) in the presumptive second (a2) and weakly in the third (a3) antennal segments of a wild-type late third instar antennal disc. (D-F) Hth expression (green) is normal inDll null clones (arrowheads). Dll is in blue (G-I) Dll expression (blue) is normal in anhth (green) null clone in a3 (arrow). to-leg transformations (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989), indicating thatDll levels may be important for antennal determination. Weak hypomorphic combinations ofDll alleles also lead to partial transformation of the third antennal segment (a3) and the arista into leg-like structures (Fig. 1A,B). Intermediate hypomorphic combinations ofDll alleles transform the medial antenna toward leg and exhibit distal truncations (Fig. 1C). Strong combinations ofDll alleles exhibit more severe antennal truncations (Fig. 1D). These same allelic combinations result in progressively more severe truncations of the distal leg (Fig. 1E-H). Notably, the antenna-to-leg transformations are not a property of a specific subset ofDll alleles, but are observed with allDll alleles when assayed in appropriate combinations. For the transformation phenotype to be apparent,Dll PD function must be largely intact. This is likely due to the necessity of having a PD axis for either antennal or leg identity to be manifested. That we observe transformation without limb truncation indicates that the antennal selector function is more sensitive toDll dosage than its PD function. We emphasize that transformation is a loss-of-function phenotype ofDll, not a neomorphic or hypermorphic one. Together, theDll phenotypic

Fig. 3. Sal expression in the antenna is dependent upon Dll and Hth. (A,B) Dll (blue) and Sal (red) overlap (pink) in the presumptive second (a2) and weakly in the third (a3) antennal segments of a wild-type late third instar antennal disc. The proximal boundaries of both coincide. (C) Hth (green) and Sal (red) overlap (yellow) in presumptive a2 and weakly in a3 of a wild-type antennal disc. The distal boundaries of both coincide. (D,E) In Dll\textsuperscript{D}Dll\textsuperscript{D} antennal discs, Dll (blue) is expressed at reduced levels in a normal pattern, and Sal expression (red) is greatly reduced. Note that Sal expression is normal in the eye. (F) Hth expression is normal in Dll\textsuperscript{D}Dll\textsuperscript{D} antennal discs. (G-I) Sal (red) cannot be detected in a Dll null clone in a3 (arrow). Dll is in blue (J-L) hth null clones (arrows) in the antenna exhibit loss of detectable Hth (green) and Sal (red). The arrowhead indicates an hth null clone positioned on a fold in the disc. Neither Sal nor Hth are detected in the clone, but can be seen as faint staining in the wild-type cells beneath the clone.

Fig. 4. Ectopic Hth in the Dll domain or ectopic Dll in the Hth domain activates sal. (A,B) When Hth (green) is ectopically expressed in the Dll domain of the antenna, sal (red) is activated (arrow). In this case, a dpp-GAL4 driver was used to drive UAS-GFP-hth expression along the anterior-posterior compartment boundary. Ectopic Hth is green because it is tagged with GFP (green fluorescent protein). Endogenous Hth is not visible. Note that sal expression is not activated by ectopic Hth proximally, i.e. outside of the Dll expression domain, and that there is a low level of ectopic sal induced in the area immediately anterior to the ectopic hth stripe. This may be because the dpp pattern is dynamic and dpp-GAL4 drove hth expression from the UAS element in these cells earlier in development. When Dll (blue, C,D) is expressed proximally in the antennal disc using a flip-out GAL4 cassette and a UAS-Dll construct, Sal (red, C,F) is induced in cells expressing Hth (green, C,E). The arrow points to a cluster of cells in which sal is activated. The arrowhead indicates a cluster of cells with higher levels of Dll in which sal is not activated. We sometimes see downregulation of Hth in such clones.
analysis indicates that *Dll* is required for antennal identity, as well as for limb outgrowth.

**Dll and hth are not required for each others expression in the larval antenna**

Both loss-of-function *Dll* alleles and loss-of-function *hth* alleles lead to antenna-to-leg transformations. It therefore was possible that *Dll* might activate *hth* expression in the antenna or vice versa. To test whether this is the case, we examined expression of each gene in animals mutant for the other. At late third instar, *Dll* is expressed in presumptive a2, a3 and arista (Fig. 2A,B), while *Hth* is expressed in presumptive a1, a2 and, weakly, a3 (Fig. 2A,C). Thus the expression of *Dll* and *Hth* overlaps in a2 and a3 (Fig. 2A). In *Dll* hypomorphic antennal discs, both the pattern and level of *Hth* appear normal (Fig. 3F). In *Dll* null clones in a2 or a3, *Hth* levels appear normal (Fig. 2D-F). In *hth* null clones in presumptive a2 and a3, *Dll* levels appear normal (Fig. 2G-I). Thus *Dll* and *Hth* are not required during larval stages for each others expression in the antenna.

**Dll and hth are required together for sal expression in the antenna**

The experiments described above establish that *Dll* and *hth* act in parallel as antennal selectors. An obvious next question was whether mutations in both genes affect the expression of antennal markers in the same way. To test this, we made use of the antennal marker *spalt* (*sal*). *sal* is one of few genes known to be expressed in the antenna but not in the leg (Wagner-Bernholz et al., 1991). Consistent with the idea that *sal* lies genetically downstream of both *Dll* and *hth*, we found that *sal* expression is restricted to the presumptive medial cells of the antenna (a2 and a3), precisely where the domains of *Dll* and *Hth* expression overlap (Fig. 3A-C). To test whether both *Dll* and *Hth* are required for *sal* expression in the antenna, the expression of *sal* was examined in *Dll* hypomorphs and in clones null for either *hth* or *Dll*. In *Dll* hypomorphic combinations exhibiting antenna-to-leg transformations, e.g. *Dll*/*Dll* (Fig. 3D,E) and *Dll*/*Dll* (not shown), *Sal* is significantly reduced (Fig. 3E). In *Dll* null clones, *Sal* is undetectable (Fig. 3G-I). In *hth* null clones, *Sal* is also undetectable (Fig. 3I-L). Together, these results demonstrate that *sal* expression in the antenna requires both *Dll* and *hth* functions.

**Coexpression of Dll and Hth in the antenna activates sal**

The results described above indicate that *Dll* and *Hth* are both necessary for *sal* expression in the antenna. If *Dll* and *Hth* were sufficient to activate *sal*, we would predict that ectopic expression of *Hth* in the *Dll* domain or ectopic expression of *Dll* in the *Hth* domain would induce the expression of *sal*. To test this, *dpp-GAL4* (Morimura et al., 1996) was used to drive expression of a *UAS-GFP-hth* construct (Casares and Mann, 1998) along the anteroposterior compartment boundary of the antennal disc. This resulted in the expression of *Hth* in a subset of the presumptive distal a3 and arista cells that normally express *Dll*, but do not normally express *Hth* during the third instar (Fig. 4A). Under these conditions, *Sal* expression is induced (Fig. 4A,B).

To test the consequences of expressing *Dll* in the *Hth* domain, a combined ‘flip-out’ *GAL4/UAS* system was used. In this case, clones of cells expressing *Dll* were generated in the proximal part of the *Hth* domain where *Dll* is normally not expressed (Fig. 4C-F). Only small clones could be recovered (Fig. 4C,D), suggesting that high levels of *Dll* may be cell lethal. However, cells in these clones frequently express *Sal* (Fig. 4C,F). We therefore conclude that antennal cells both proximal and distal to the normal *Sal* domain are competent to express *Sal* if provided with *Dll* and *Hth*, i.e. within the context of the antennal disc, neither *Dll* nor *Hth* alone can activate *sal*, but that together they are sufficient to do so.

**Ectopic Dll induces sal expression and antennal differentiation where there is endogenous Hth**

To test whether coexpression of *Dll* and *Hth* induces antennal differentiation, we examined the phenotypic consequences of ectopically expressing *Dll* in *Hth* domains in areas outside of the antenna. As previously reported (Gorfinkiel et al., 1997), we found that ectopic *Dll* can induce the differentiation of antennal tissue elsewhere in the head (Fig. 5E-G) and ectopic leg tissue on the wing (not shown). However, we also find that ectopic expression of *Dll* induces the differentiation of antennal structures, primarily arista, in the proximal wing and, less frequently, in the proximal leg (Fig. 6A-D).

The locations where ectopic antennal structures can be induced are correlated with both endogenous *Hth* expression and ectopic *Sal* expression. In the eye-antennal imaginal disc, *Hth* is expressed in the presumptive head capsule and behind the morphogenetic furrow (Fig. 5A,C). Ectopic *Dll* expression in the *Dpp* domain of the eye disc creates a region of overlap of *Dll* with the presumptive head capsule domain of *Hth* expression (Fig. 5A-C). Ectopic *Sal* can be detected in the region of overlap, but not in adjacent cells that express *Dll* but lack endogenous *Hth* (Fig. 5A,D). Using X-gal stainings of *hth-lacZ* pupae, we observe that the sites where differentiation of antennal structures can be induced by ectopic *Dll* express endogenous *hth* (Fig. 6E,F). Together, these results are consistent with *Dll* being able to induce antennal structures and *sal* expression only where *Hth* is present.

**Ectopic Dll and Hth together can induce sal expression and antennal differentiation in the leg, head and genital discs**

*hth* expression is restricted to presumptive proximal leg cells and *Dll* expression is restricted to presumptive distal leg cells during embryogenesis (Casares and Mann, 1998; Goto and Hayashi, 1997). Thus their expression does not overlap for most of leg development. If *Dll* and *Hth* cooperate to induce antennal differentiation, then leg tissue may express *sal* and differentiate antennal structures if provided with *Dll* and *Hth* simultaneously. To test this, *Dll* and *Hth* were ectopically expressed together using the *dpp-GAL4* driver. Under these conditions, *Sal* expression was induced in the medial to distal leg disc (Fig. 7A,B) and ectopic antennal structures could be detected on the adult legs (Fig. 7C,D).

*Dll* and *Hth* expression also do not normally overlap in the eye, the presumptive head capsule, or the genital disc. When *dpp-GAL4* is used to coexpress *Dll* and *Hth* in these tissues, ectopic *Sal* expression (Figs 8A-C, 9A,C,E) and the formation of ectopic antennal cuticular structures result (Figs 7C,D, 8D, 9B,D). X-gal staining of *UAS-Dll*; *UAS-GFP-hth/dpp-GAL4* pupae harboring a *sal-lacZ* enhancer trap, results in specific stainings of the ectopic antennal structures (not shown), indicating that *sal* expression can be correlated directly with antenna formation.
We note that ectopic Dll and Sal are sometimes found where GFP-Hth is very low or not detected (Figs 4A, 5A, 7A, 8A, 9C). This probably reflects our detection methods and not an absence of Hth in these cells. Both Dll and Sal proteins were visualized using antibody reagents that amplified the signals. Hth was visualized by means of the GFP tag in the UAS construct, thus the sensitivity was not as high. The cells in which ectopic Sal can be detected therefore probably contain Hth as well as Dll.

We have compared the frequencies of ectopic antenna formation induced by ectopic coexpression of Dll and Hth in the legs and genitalia with the frequencies of ectopic antenna formation induced by either ectopic Dll or Hth alone. With ectopic Dll alone, we have observed only one recognizable arista on a leg in more than 20 animals, i.e., 120 legs, examined, a frequency of less than 1%. The reason for this low frequency may be due to the fact that there is little overlap of the ectopic Dll with endogenous Hth in the leg disc when UAS-Dll is expressed using the dpp-GAL4 driver. A second possibility is that antennal development is impeded when Antp (or another trunk Hox gene product) is present. As for why ectopic expression of Hth in the Dll domain of the leg does not induce antennal differentiation, when Hth is expressed ectopically there using dpp-GAL4, Dll is repressed (not shown). Thus if both Dll and Hth are required for antennal differentiation, these conditions would not lead to ectopic antennae. Indeed, no recognizable antennal structures were found in the legs of the more than 20 animals examined with ectopic Hth alone (not shown). We also have observed no ectopic aristae in the genital disc derivatives of animals ectopically expressing either Dll or Hth alone. In each case, at least 20 animals were examined. In contrast, with ectopic Dll and Hth together, we found 8 aristae on the legs of 7 animals, or 42 legs, a frequency of 19%. These contrast, with ectopic Dll and Hth together, we found 8 aristae Hth alone. In each case, at least 20 animals were examined. In disc derivatives of animals ectopically expressing either Dll or Hth, we hypothesized that Antp repression of sal indirectly via hth developmental program.

**Antp may repress sal indirectly via hth**

Antp represses hth, thereby restricting hth expression to the proximal region of the leg (Casares and Mann, 1998). Antp also represses sal in the leg (Wagner-Bernholz et al., 1991). Because antennal sal expression is dependent upon both Dll and Hth, we hypothesized that Antp repression of sal might be mediated via Antp repression of hth, which in turn prevents the overlap of Dll and Hth in the distal leg. Consistent with this possibility, Sal is expressed in Antp null clones in the Dll domain where hth is derepressed (Fig. 10A-D). We therefore think it likely that Antp may be repressing sal expression indirectly by preventing hth from being expressed in the Dll domain of the leg. Since both Dll and Hth are required for antennal differentiation, by preventing the coexpression of Dll and Hth, Antp can preclude antennal development.

**DISCUSSION**

**Dll and hth act in parallel as antennal selectors**

Dll is required for the formation of distal elements in all ventral appendages, including the antenna and the leg. hth is required for the formation of proximal elements in the antenna and the leg. For their roles in PD patterning, Dll and hth act independently. However, Dll (Sunkel and Whittle, 1987; Cohen and Jurgen, 1989) and hth (Pai et al., 1998; Rieckhof et al., 1997) also function as selector genes in the antenna. Via both loss- and gain-of-function experiments, we have demonstrated here that they cooperate to regulate antennal differentiation (Fig. 11).

Not only are Dll and hth required in parallel for normal antennal development and antennal sal expression, but coexpression of Dll and Hth activates sal expression and can induce the formation of antennal structures in many different areas in the body, including the head, wings, legs and anal plates. It has been reported that ectopic expression of either Dll or Hth alone can result in the formation of ectopic antennal structures. This includes ectopic expression of Dll in the head capsule (Gorfinkiel et al., 1997) and ectopic Hth or its vertebrate homolog, Meis-1, in the anal plates (Casares and Mann, 1998). Both cases are consistent with the results and model presented here (Fig. 11), in which coexpression of Dll and Hth is required to determine antennal fates. For instance, we find that the ectopic antennal tissue induced by ectopic Dll alone in the head capsule, proximal leg and proximal wing correlates with sites of endogenous hth expression. We also demonstrate that ectopic Dll alone in the presumptive head capsule region of the eye imaginal disc induces sal expression only when Dll overlaps endogenous Hth. Together, these results suggest that ectopic expression of Dll alone can only lead to formation of antennal tissue from cells with endogenous Hth.

If Dll and Hth collaborate to regulate antennal development, then the converse should also be true, i.e. ectopic expression of Hth should induce the differentiation of antennal structures wherever Dll is expressed endogenously. Indeed, in the reported instances of arista induction with ectopic Meis-1 or Hth in the anal plates, the ectopic expression was driven by a Dll-GAL4 line (Casares and Mann, 1998). This produced coincident expression of Dll and Meis-1 or Hth, again consistent with the proposed model (Fig. 11).

However, Hth or Meis-1 alone, when ectopically expressed in Dll domains elsewhere in the body does not lead to ectopic Sal expression (our observations) or to the differentiation of recognizable antennal structures (Casares and Mann, 1998). The explanation for this that we favor is that when Hth is ectopically expressed using the GAL4/UAS system, Dll expression is downregulated in the cells producing Hth. These cells would then have Hth, but insufficient Dll. If both are required for antennal differentiation, antennal differentiation would not be possible. Consistent with this idea, we and others (Gonzalez-Crespo et al., 1998) have observed a decrease in Dll expression in leg cells ectopically expressing Hth.

**Hth and arista formation**

Coexpression of Dll and Hth in the leg, the wing, and the genital discs using the dpp-GAL4 driver frequently leads to the formation of ectopic aristae. However, while Hth is required cell autonomously for arista development, Hth expression is not detected in the presumptive arista of third instar antennal discs, and ectopic expression of either Hth or Meis-1 in the presumptive arista late in development prevents arista formation (P. D. S. D., unpublished results). Since Dll
has functions in PD outgrowth independent of Hth, a plausible explanation is that the ectopic Hth is titrating out the Dll needed for PD outgrowth. If Hth must be lost or reduced to permit arista differentiation, why then does coexpression of Dll and Hth lead to arista development? The explanation that we favor is that it reflects both the ability of Dll to autoregulate and the dynamics of dpp-GAL4 expression. The width of the stripe of dpp expression remains fairly constant as the discs grow (Masucci et al., 1990; Weigmann and Cohen, 1999). As a result, the anteriormost cells that express the dpp-GAL4 driver early, give rise to cells that do not express dpp-GAL4 later in development. Thus some cells in the anterior compartment will express Hth and Dll from the UAS elements only transiently. Because Dll can autoregulate in imaginal discs (Gorfinkiel et al., 1997), transient expression of Dll from the UAS element activates the endogenous Dll gene. By late third instar, portions of the imaginal discs resemble normal presumptive aristal cells in expressing Dll and having expressed, but lost, hth expression. It may be that these cells differentiate as arista.

**Could Dll form a functional complex with Hth and Exd in the antenna?**

Given that Dll and Hth cooperate to regulate antennal differentiation, it is of interest to elucidate the molecular basis...
Distal-less and homothorax determine antennal identity

Coexpression ofDll (blue) and Hth (green) can induceSal expression (red) in the eye (arrowheads). Only the ectopic Hth is visible because it is tagged with GFP. Arrows indicate sites where Dll is expressed, but Hth can no longer be detected. This probably reflectsDll autoregulation. Sal is expressed in these cells, which probably express endogenous Hth. Asterisks indicate sites in which coexpression of Dll and Hth does not induce Sal. (D) Extra antennal tissue induced on the head of an animal in which Hth and Dll were ectopically expressed. As many as three ectopic antennae are frequently observed on each side. Aristae are indicated with arrows. a3-like tissue is indicated with arrowheads. The eye is marked with an astersk.

Ectopic Dll and hth together induce sal expression and antenna formation in the genital disc derivatives. (A) Sal expression (red) in a wild-type male genital disc. (B,D) Aristae induced on female (B) and male (D) genital disc derivatives by ectopically expressing both hth and Dll with dpp-GAL4 and UAS-Dll and UAS-GFP-hth constructs. (C,E) Sal (red) induced in a male genital disc by ectopically expressing both GFP-Hth (green) and Dll (not shown) with dpp-GAL4 and UAS-Dll and UAS-GFP-hth constructs. Note that the ectopic hth and Dll repress endogenous sal expression, and that sal is not activated everywhere Dll and Hth are expressed.

Antp may repress sal via hth. (A-D) Antp null clone in the leg disc in which Dll expression (blue) is normal, and both Hth (green) and Sal (red) are derepressed. Sal is only produced by Hth expressing cells within the Dll domain. The arrow indicates the distalmost portion of the clone.

of this synergy, Exd and its vertebrate counterpart, Pbx, are known cofactors for a variety of homeodomain proteins, including Labial, Engrailed and Ultrabithorax (reviewed in Mann and Chan, 1996). Hth is required for retention of Exd in the nucleus (Pai et al., 1998; Rieckhof et al., 1997; Kurant et al., 1998) and may form part of the functional Exd/Hox complex (Rieckhof et al., 1997). Vertebrate homologs of Hth, the Meis and Prep proteins, have been shown to form trimeric complexes with Hox and Pbx proteins (Berthelsen et al., 1998; Swift et al., 1998; Goudet et al., 1999; Shen et al., 1999). Several lines of evidence now support the idea that Exd and Hth are cofactors for the Dll homeodomain protein in the developing Drosophila antenna. These include: (1) the similar antenna-to-leg transformation phenotypes of Dll, hth and exd mutants, (2) the known physical interactions of Exd and Hth with other homeodomain proteins, (3) the fact that Dll and hth function in parallel to regulate antennal development, and (4)
the fact that ectopically expressing Hth can mimic loss of Dll function in the antenna. Testing whether DII, Hth and Exd interact physically and whether such a complex activates antennal enhancers will be important steps toward understanding limb development and tissue-specific gene regulation.

We appreciate the comments of Reese Bolinger, Sean Carroll, Karen Downs, Kirsten Guss, Georg Halder and Al Laughon on the manuscript. We are grateful to Jen Scholz for technical support, Sean Carroll for access to his confocal microscope, Richard Mann for the chicken Hth antibody and UAS-GFP-Inh Drosophila lines, Konrad Basler for UAS-Dll Drosophila lines, Henry Sun for the FRT-Inh Drosophila line and rabbit Hth antibody, Gary Struhl for the FRT-Antp Drosophila line, Reinhard Schuh for the Sal antibody, and the Bloomington Drosophila Stock Center for many of the Drosophila lines used in these experiments. P. D. S. D. was the recipient of a fellowship from the University of Wisconsin Graduate School. J. C. was supported by NIH Predoctoral Training Grant #T32-HD07477. G. P. is the recipient of an award from the Howard Hughes Medical Institute and the University of Wisconsin Medical School.

REFERENCES


