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Control of the *spineless* antennal enhancer: Direct repression of antennal target genes by Antennapedia

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Summary

It is currently thought that antennal target genes are activated in Drosophila by the combined action of *Distal-less*, *homothorax*, and *extradenticle*, and that the Hox gene *Antennapedia* prevents activation of antennal genes in the leg by repressing *homothorax*. To test these ideas, we analyze a 62 bp enhancer from the antennal gene *spineless* that is specific for the third antennal segment. This enhancer is activated by a tripartite complex of Distal-less, Homothorax, and Extradenticle. Surprisingly, Antennapedia represses the enhancer directly, at least in part by competing with Distal-less for binding. We show that Antennapedia is required in the leg only within a proximal ring that coexpresses Distal-less, Homothorax and Extradenticle. We conclude that the function of Antennapedia in the leg is not to repress *homothorax*, as has been suggested, but to directly repress *spineless* and other antennal genes that would otherwise be activated within this ring.

Introduction

Mutations of several genes in Drosophila cause transformations of antenna toward second leg. The best known of these mutations are dominant gain-of-function alleles of the Hox gene *Antennapedia* (*Antp*), which can cause the antenna to develop as a complete leg. Struhl (1981; 1982a) showed that loss-of-function alleles of *Antp* have the opposite effect, causing transformation of leg structures to antenna, but have no effect on development of the antenna itself. He proposed that *Antp* is normally expressed in the legs but not the antenna, and that its function is to repress the activation of antenna-specific genes in the leg. The gain-of-function alleles were suggested to cause ectopic expression of Antp in the antenna. Molecular studies confirmed that *Antp* is expressed as inferred by Struhl (Frischer et al. 1986). However, until recently, the identities of the "antennal genes" controlled by *Antp* remained uncertain, as it was not known how antennal identity is specified.

We now know that the identity of most of the antenna is specified by the combined action of homeodomain transcription factors encoded by the *homothorax* (*hth*) and *Distal-less* (*Dll*) genes (Casares and Mann 1998; Dong et al. 2000). These genes are coexpressed extensively in the antenna, whereas in the leg they are coexpressed in only a narrow proximal ring of cells. Several antennal genes have been shown to be activated independently by combined Hth and Dll expression (Dong et al. 2002). One of the most important of these targets is *spineless* (*ss*), which encodes a bHLH transcription factor homologous to the mammalian dioxin receptor

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(Duncan et al. 1998). The expression patterns of *Dll*, *hth* and *ss* in the antennal imaginal disc, and an adult antenna are shown in Fig. 1A.

Hth is required for normal identity of the entire antenna, and is expressed throughout the antennal disc in the first and second larval instars. *hth*$^{-}$ mitotic recombination clones induced at these times transform the entire antenna to a leg-like appendage (Casares and Mann 1998). Subsequently, Hth expression is lost in the most distal portion of the disc, the primordium of the arista, whose development becomes independent of *hth* (Emmons et al. 2007). Hth is also expressed in the most proximal segments of the leg, where it is required for normal growth and proper formation of segment boundaries (Abu-Shaar and Mann 1998; Wu and Cohen 1999; Casares and Mann 2001). Hth functions as a heterodimer with the homeodomain protein Extradenticle (Exd) (Rieckhof et al. 1997; Pai et al. 1998; Kurant et al. 1998), which is also required for antennal specification and proximal leg development (González-Crespo and Morata 1995). In addition to these roles, Hth and Exd serve as important cofactors that increase the binding specificity of the Hox proteins (for review see Mann et al. 2009).

Dll is required for the development of distal structures in all of the ventral appendages (Cohen et al. 1989). In the antenna, *Dll* is expressed in the primordia of the second (A2), and third (A3) antennal segments and the arista, and this entire expression domain is deleted in *Dll*$^{-}$ mutants (Cohen and Jürgens 1989). However, weak alleles of *Dll* cause transformations of antenna toward leg (Sunkel and Whittle 1987; Dong et al. 2000), suggesting that *Dll* has a role in specifying antennal identity that is distinct from its general role of specifying distal limb structures. Dong et al. (2000) proposed that Dll acts in concert with Hth (and presumably also Exd) to define antennal identity. This proposal is supported by the effects of *hth*$^{-}$ and *Dll*$^{-}$ alleles on the expression of antenna-specific target genes and by the effects of combined ectopic expression of Hth and Dll (Duncan et al. 1998; Dong et al. 2000; 2002; Emmons et al. 2007).

Many of the identity functions of Hth and Dll in the distal antenna are executed by the target gene *ss* (Dong et al. 2002; Emmons et al. 2007), which is expressed in the primordia of A3 and the arista. In *ss*$^{-}$ mutants, A3 lacks all olfactory sensilla, and the arista is transformed to distal leg (Struhl 1982b; Duncan et al. 1998). In previous work (Emmons et al. 2007), we identified the antennal enhancer from *ss* and showed that its expression depends upon Dll and Hth, and that it is repressed by ectopically expressed Antp. The enhancer is also repressed in A2 by the homeodomain protein Cut (Blochlinger et al. 1988).

In this report, we address two major unresolved questions. First, how are inputs from Dll, Hth, and Exd integrated at antennal target genes? To date, no antennal enhancers have been characterized at the molecular level, so the mechanism of action of these factors has remained uncertain. Second, how does Antp repress antennal identity in the leg? Based on the finding that *Antp*$^{-}$ clones in the leg sometimes show ectopic distal expression of Hth, Casares and Mann (1998) proposed that the primary function of Antp is to repress *hth* in the distal leg, which then prevents activation of antennal target genes. Although this view is widely accepted, it has not been subject to direct test.

To address these questions, we focused our attention on the antennal enhancer of *ss*. We identify a 62 bp subregion of this enhancer that drives expression specifically in A3. Like the full antennal enhancer, the A3 enhancer requires Dll, Hth, and Exd for expression. All three of these factors interact directly with the enhancer. The binding of Dll shows strong cooperativity with Hth and Exd, indicating that these proteins bind as a complex. This Dll/Hth/Exd tripartite binding suggests that Dll behaves much like a Hox protein in specifying antennal identity. Surprisingly, we find that Antp also interacts directly with the A3 enhancer. Antp binds cooperatively with Hth and Exd, and represses the enhancer at least in part by competing with Dll for binding.

Our finding that Antp interacts directly with the A3 enhancer led us to reexamine the role of *Antp* in leg development. We find that the A3 enhancer is sometimes activated within *Antp*$^-$ clones in the leg, consistent with the transformation to antenna that such clones can cause. However, this activation occurs only within a narrow ring of cells in the proximal leg that coexpresses Dll, Hth, and Exd (Wu and Cohen 1999). Subsequently, some of the *Antp*$^-$ cells in which the A3 enhancer has been activated begin to express Ss, Cut, and other antennal markers, indicating a transformation to antenna. Importantly, we find that expression of Hth and Dll in the proximal ring is unaffected in *Antp*$^-$ clones, indicating that Antp does not block antennal development in the leg by repressing *hth*, as has been thought. Rather, we conclude that the main, and perhaps sole, function of Antp in the leg imaginal disc is the direct repression of antennal genes that would otherwise be activated by the combined expression of Dll, Hth, and Exd in the proximal ring.

Results

Dissection of the *ss522* antennal enhancer

In a previous report (Emmons et al. 2007), we showed that the antennal expression pattern of *ss* is reproduced by *lacZ* reporters containing a 522 bp fragment from the *ss* 5' region. This fragment contains five conserved (41-90% identity) domains (Stark et al. 2007), each of which was deleted and tested for effect on expression *in vivo*. Expression in the arista and the third antennal segment (A3) prove to be under separate control; expression in the arista requires domains 1, 3 and 5, whereas expression in A3 is lost only when domain 4 is deleted (Fig. 1B). Moreover, reporters containing domain 4 alone show expression in A3 and nowhere else in imaginal discs. Thus, domain 4 is both necessary and sufficient for A3-specific expression. Domain 4 (*D4*) is 62 bp in length and is highly conserved, being invariant at 50/62 base pairs in the 12 Drosophila species sequenced (Fig. 1C).

We first established the boundaries of *D4*/*lacZ* reporter expression relative to Homothorax (expressed in A3 and more proximally) and Cut (expressed in A2 and more proximally) in mature third instar antennal discs. As shown in Fig. 2A-B, the distal boundary of *D4*/*lacZ* expression coincides with the distal limit of Hth expression, and the proximal boundary largely coincides with the distal boundary of Cut expression. *D4*/*lacZ* is therefore expressed throughout A3. *D4*/*lacZ* expression often overlaps Cut expression slightly, indicating that the reporter may also be expressed in a few cells in distal A2.

Trans regulation of *D4*

D4/*lacZ* expression is lost in clones homozygous for null alleles of *Dll*, *hth*, and *exd* (Fig. 2C-E), indicating that Dll, Hth, and Exd are all required for expression. We also examined clones expressing either or both Hth and Dll proteins ectopically (data not shown). Clones expressing Hth show activation of *D4*/*lacZ* in the aristal region of the antenna and the distal part of the leg, regions where Dll is expressed. Similarly, clones expressing Dll activate the reporter in the proximal antenna and wing, regions where Hth is expressed. Clones expressing both Hth and Dll activate *D4*/*lacZ* expression in most locations. A notable exception is the proximal region of the leg discs (see below).

We also find that *D4*/*lacZ* is repressed within antennal clones ectopically expressing Antp (Fig. 2F). Since ectopic Antp is known to repress *hth* in the antenna (Casares and Mann 1998), we tested whether repression of *hth* accounts for the loss of *D4*/*lacZ* expression within Antp-expressing clones by examining antennal clones that express both Antp and Hth ectopically. Surprisingly, *D4*/*lacZ* is fully repressed within such clones (Fig. 2G), just as in clones that express Antp alone, indicating that repression of *D4*/*lacZ* by ectopic Antp is not due to the loss of Hth.

Dll, Hth, Exd, and Antp all interact directly with *D4*

Gel-shift and footprinting studies demonstrate that all four regulators defined above bind *D4* directly. *In vitro* translated Dll and Antp both produce prominent gel retardation bands in gel-shift assays (Fig. 3A). These retardation bands are supershifted by anti-Dll or anti-Antp antibodies, indicating that both Dll and Antp are present in their respective retardation complexes (Fig. 3B). Although Hth and Exd do not produce retardation bands on their own in our assays, when mixed they bind cooperatively to produce a prominent retardation complex (Fig. 3A). Anti-Hth and anti-Exd do not supershift this retardation band, but instead dramatically reduce its intensity, suggesting that these antibodies interfere with the heterodimerization or DNA binding of these proteins (Fig. 3C).

When Dll is mixed with Hth and Exd, strong cooperative binding to *D4* is seen (Fig. 3D); the band corresponding to binding of a single molecule of Dll is replaced by an intense band located higher in the gel than the Hth + Exd band. This upper band is supershifted by anti Dll (Fig. 3E), indicating that it contains Dll. These observations indicate that when all three proteins are present, almost all Dll is bound to probe that is also bound by Hth and Exd. This striking cooperativity implies protein-protein interactions between Dll and Hth and/or Exd. Dll carrying a change of asn51 to ala in the homeodomain, which eliminates DNA binding in other homeodomain proteins (Ades and Sauer 1995), fails to bind *D4* on its own or in combination with Hth and Exd (Fig. 3G). Thus, DNA binding of Dll appears to be required for its interaction with Hth and Exd on *D4*. Antp also binds cooperatively with Hth and Exd (Fig. 3D,F)), although we have not tested whether the ability of Antp to bind DNA is essential for this interaction.

Binding sites for Hth/Exd, Dll, and Antp were defined by footprinting (data not shown) and testing mutant oligonucleotides in gel-shift assays. The sites defined are summarized in Fig. 4. Hth and Exd bind to directly adjacent consensus binding sites (Chang et al. 1997), and mutations in these sites block cooperative binding of these factors (Fig. 4B). Dll binds three sites in *D4*. To characterize these sites, *D4* was subdivided into three oligonucleotides (bp 1-21, 19-41, and 39-62), each containing a single Dll binding site. The Dll binding sites present in oligonucleotides 1-21 and 39-62 are designated Dlla and Dllb, respectively (Fig. 4E). Mutations in these sites almost completely eliminate binding by Dll (Fig. 4A). The central 19-41 oligonucleotide, which contains the Hth/Exd site, also binds Dll. Mutation of the Exd site blocks this binding, indicating that Dll and Exd bind overlapping or identical sites (Fig. 4A). Dll produces three distinct retardation bands when bound to full-length *D4*; we interpret these bands as having one, two, or all three binding sites occupied by Dll. Antp binds only one site in *D4*, which overlaps or coincides with the Dlla site (Fig. 4C). Mutation of this site blocks all binding of Antp.

The finding that Antp binds Dlla raises the possibility that it represses *D4* by competing with Dll for binding. To test this possibility, the ability of combined Dll and Antp to gel-shift the 1-21 oligonucleotide was examined. To achieve robust binding, both Dll and Antp were purified from *in vitro* translation reactions by oligonucleotide selection (Ozyhar et al. 1992). As shown in Fig. 4D, under conditions in which the majority of the 1-21 probe is shifted by either Dll or Antp alone, no additional slower mobility band is seen when these proteins are mixed. This result indicates that Antp and Dll do compete for binding to Dlla.

Finally, to assess the importance of the Hth/Exd, Dlla, and Dllb binding sites *in vivo*, *D4*/*lacZ* reporters carrying mutations in each site were reintroduced into flies. Mutation of the Hth or Exd half sites eliminated enhancer activity in all P-element transformants recovered (10 for the Hth site mutation, and 8 for the Exd site mutation). To assess the importance of the Dlla and Dllb sites, position effects were minimized by using ϕC31-mediated transformation (Bischof et al. 2007) to target integration of mutant derivatives to the same site. Mutations in Dlla cause a dramatic reduction in expression, whereas mutations in Dllb have little or no effect

(Fig. 4F). The double mutant Dlla Dllb is expressed to about the same level as the Dlla mutant. These observations indicate that the Dlla site is of key importance for activation by Dll. Significantly, Dlla is the site at which Antp competes with Dll for binding.

Although not central to this report, we find that *D4* is also regulated by *cut*. Antennal expression of *D4*/*lacZ* is expanded proximally in *cut*$^{-}$ clones, and repressed within clones ectopically expressing Cut (Suppl. Fig. 1). These observations indicate that the proximal limit of *D4*/*lacZ* expression is set, at least in part, by Cut. Gel shift assays indicate that Cut binds to *D4* at two sites simultaneously. These sites overlap the Dlla and Exd sites, and both are required for binding (Suppl. Fig. 1). Binding to these sites is likely mediated by different DNA binding domains within the Cut protein (see Nepveu 2001).

Antp represses *D4* in the proximal leg

Our finding that Antp interacts directly with *D4* was unexpected. What is the relevance of this finding to normal development? The answer turns out to be that the key, and perhaps sole, function of *Antp* during leg development is the repression of *ss* and other antennal target genes within a narrow proximal ring that coexpresses Dll, Hth, and Exd. This ring is shown in Fig. 5A. It is 5-7 cells wide, and is defined by Dll expression; Hth is expressed in the ring as well as more proximally. The function of the ring is not known with certainty. It appears in the early third instar, and overlaps the joint between the trochanter and the femur (Wu and Cohen 2000) (leg segments are shown in Fig. 5B). Although Antp is expressed throughout the leg primordium early in development (Casares and Mann 1998), during larval life its expression becomes limited to a broad proximal domain (Fig. 5C). Within this domain, Antp is most strongly expressed within the proximal ring.

In analyzing *Antp*$^{-}$ clones in second leg discs, we noted that there are two basic types: clones that are well integrated into the disc epithelium and whose borders are interdigitated with their wild-type neighbors, and clones that are rounded up and have smooth borders. Rounded-up clones appear to have reduced affinity for their neighboring cells, and their borders often coincide with novel folds in the disc. Interdigitated *Antp*$^{-}$ clones occur in all regions of the leg disc and appear to develop completely normally. In contrast, rounded-up clones almost always show some connection to the proximal ring, and express *ss* or other antennal markers, indicating they are transformed to antenna.

We first consider *Antp*$^{-}$ clones of the interdigitated type. Such clones can be induced at any time during larval development, and even very large interdigitated clones are well integrated into the disc (Fig. 5D). Clones of this type appear to develop completely normally, as most *Antp*$^{-}$ clones produce normal cuticular structures in adult second legs (Fig. 5E,F). However, when interdigitated *Antp*$^{-}$ clones overlap the proximal ring of Dll, Hth, and Exd expression, *D4*/*lacZ* becomes activated in *Antp*$^{-}$ cells of the ring (Fig. 5D). Importantly, expression of Dll and Hth is unaffected in such clones (Fig. 5G,H). A few cells at the proximal edge of the ring do not activate *D4*/*lacZ* expression. The reason is not known, but both *teashirt* and *dachshund* are differentially expressed within the ring (Wu and Cohen 2000), and may play a role in activating or repressing *D4*. Although *D4*/*lacZ* is activated in the proximal ring in *Antp*$^{-}$ clones of the interdigitated type, Ss itself is not expressed, indicating that such clones are not transformed to antenna (not shown).

Rounded-up *Antp*$^{-}$ clones present a more complex picture. Such clones almost always express *D4*/*lacZ*, and usually extend distally from the ring of Dll, Hth and Exd coexpression. Rounded-up clones express Hth (Fig. 5J), Dll, and usually also Ss (Fig. 5I), indicating they are transformed to antenna. Often clones contain both rounded-up and interdigitated regions; in such cases the rounded-up portion is almost always associated with the ring (Fig. 5J). To determine the origin of rounded-up clones, we examined *Antp*$^{-}$ clones in late larval discs that

were induced at progressively earlier times in development. *D4*/*lacZ*-expressing clones 0-24 hrs of age are almost exclusively of the interdigitated type, with *D4*/*lacZ* expression occurring only within the proximal ring (Fig. 6A). *D4*/*lacZ*-expressing clones 24-48 hrs old show some rounding up, causing distortion of the ring (Fig. 6B). By 48-72 hrs, rounding up of *D4*/*lacZ*-expressing clones is more pronounced (Fig. 6C). Moreover, most clones of this age extend distally from the proximal ring. This distal extension can become very pronounced, with some clones bridging the region between the ring and the distal expression domain of Dll (Fig. 7AB), which includes the tibial and tarsal portions of the disc. Occasionally, rounded up *D4*/*lacZ* expressing clones are found that are entirely distal and not connected to the proximal ring. The presence of intermediates in which distal extensions are connected to the proximal ring by a narrow isthmus (Fig. 7C) suggest that many or all of these strictly distal clones originate within the proximal ring. Of 106 *D4*/*lacZ* expressing clones scored from the 48-72 hr age group, 54 were of the interdigitated type, and 52 contained rounded-up regions. Of the rounded-up clones, only four lacked a connection to the proximal ring. At all times, clones not expressing *D4*/*lacZ* are of the interdigitated type and are well integrated into the disc.

Frequently, a subset of the cells in rounded-up clones expresses Cut, a marker for the A1 and A2 segments of the antenna (Fig. 7D). Cut-expressing and *D4*/*lacZ* expressing regions in such clones usually occupy distinct, although often overlapping, territories. Cut expression is usually not seen in *Antp*$^{-}$ clones of the interdigitated type, although sometimes Cut is weakly expressed in a few cells at the proximal edge of the ring in such clones. The emergence of Cut-expressing cells within transformed clones indicates that such clones can become organized internally to include distinct proximal (Cut-expressing) and distal (*D4*/*lacZ*-expressing) territories.

The overall picture that emerges is that *Antp*$^{-}$ clones in the second leg that lie proximal or distal to the ring of Dll, Hth, and Exd expression develop normally. However, antennal identity is triggered within clones that overlap this ring. Transformed clones then round up, become internally reorganized to include distinct proximal and distal territories, and appear to migrate or extend distally. The *D4*/*lacZ* reporter was of key importance in working out these events, as it allowed visualization of steps prior to the overt antennal transformation of *Antp*$^{-}$ clones.

Although Antp is expressed in a proximal ring in all three legs, *Antp*$^{-}$ clones show transformations to antenna only in the second leg (Struhl 1981, 1982a, Abbott and Kaufman 1986). A likely explanation is that antennal genes are repressed in the first and third legs by the Hox proteins Scr and Ubx, respectively, as well as by Antp (Struhl 1982a). Consistent with this possibility, we find that, like Antp, both Scr and Ubx repress *D4*/*lacZ* in the antenna when ectopically expressed on their own or in combination with Hth. In addition, both proteins bind *D4* cooperatively with Hth and Exd (Suppl. Fig. 2).

Discussion

In this report we study the regulation of an enhancer from the antennal gene *ss* that drives expression specifically in the third antennal segment (A3). Our work provides the first look at how the homeodomain proteins Dll, Hth, and Exd function in the antenna to activate antennal target genes. We find that these proteins form a trimeric Dll/Hth/Exd complex on the enhancer, suggesting that Dll acts much like a Hox protein in antennal specification. Our work also reveals how the Hox protein Antp functions in the leg to repress antennal development. The conventional view has been that the primary function of Antp is to repress *hth* in the distal leg, which then prevents the activation of all downstream antennal genes. However, we find that Antp represses the *ss* A3 enhancer directly. This repression is essential within a proximal ring in the leg that coexpresses the antennal gene activators Dll, Hth, and Exd. We show that Antp competes with Dll for binding to the enhancer, and that this competition is part of a molecular switch that allows the *ss* A3 element to be activated in the antenna, but represses its activation

in the leg (Fig. 7E). Our results suggest that repression of antenna-specific genes in the proximal ring is the sole function of Antp in the leg imaginal disc.

At 62 bp, the *ss* A3 enhancer (called *D4*) is one of the smallest enhancers to be identified in Drosophila, and yet it is quite strong; only a single copy is required to drive robust expression of *lacZ* reporters. The enhancer is also very specific, driving expression in A3 and nowhere else in imaginal discs. Dong et al. (2000) proposed that antennal identity in Drosophila is determined by the combined action of Dll, Hth, and Exd. Consistent with this proposal, we find that all three of these factors are required for *D4* expression. Although these activators are coexpressed in both A2 and A3, *D4/lacZ* expression is restricted to A3 by Cut, which represses the enhancer in A2. Like *ss* itself (Duncan et al. 1998), *D4/lacZ* is also repressed by ectopically expressed Antp.

Surprisingly, Dll, Hth, Exd, Cut, and Antp all act directly upon *D4*. The activators Hth and Exd bind with strong cooperativity to directly adjacent sites. Their joint binding site matches the optimum site for *in vitro* binding of the mammalian homologs of Hth and Exd (Meis and Prep) (Chang et al. 1997), consistent with the robust activity of the enhancer *in vivo*. Mutation of either of these sites abolishes activity of the enhancer. The coactivator Dll binds three sites in *D4*; one of these sites (Dlla) is required for almost all activity of the enhancer. Dll shows strong cooperativity with Hth and Exd for binding to *D4*, indicating that Dll interacts physically with these proteins. This interaction requires DNA binding, as Dll protein containing a missense change that blocks DNA binding (a change of asn51 to ala in the homeodomain) shows no ability to associate with *D4*-bound Hth and Exd. A curious feature of the cooperativity seen in our binding studies is that although Hth and Exd increase the affinity of Dll for *D4*, Dll appears to have little effect on the affinity of Hth and Exd for the enhancer (see Fig. 3). Since Hth and Exd already bind cooperatively with one another, it may be that additional cooperative interactions with Dll have little effect. Alternatively, it may be that Hth and Exd interact with Dll only after binding DNA. If so, Hth and Exd would be expected to increase Dll binding to *D4*, but Dll would have little effect on the binding of Hth and Exd, as observed. Panganiban and Rubenstein (2002) have reported detecting interactions between Dll and Hth in the absence of DNA in immunoprecipitation experiments. However, we have been unable to repeat these observations (data not shown). Moreover, our finding that the asn51 mutant of Dll fails to associate with *D4*-bound Hth and Exd argues strongly against such interactions.

The repressor Cut also acts directly upon *D4*. Binding of Cut requires two sites, one overlapping Dlla and the other overlapping the joint Hth/Exd site. These binding sites suggest that *D4* is controlled by Cut in much the same way that a structurally similar Abdominal-A (Abd-A) regulated enhancer from the *rhomboid* gene is controlled by the repressor Senseless (Sens) (Li-Kroeger et al. 2008). In the *rhomboid* enhancer, adjacent Hth and Exd sites are also present, and these create a binding site for Sens. Activity of the *rhomboid* enhancer is controlled by a competition between binding of the Sens repressor and binding of the activators Abd-A, Hth, and Exd. It seems likely that *D4* is controlled similarly, with the repressor Cut competing for binding with the activators Dll, Hth, and Exd. It will be of interest to determine whether enhancers similar to *D4* are used more widely to control Cut targets involved in its role as an external sense organ determinant.

A key finding in our work is that Antp represses *D4* by direct interaction. We show that Antp binds a single site in *D4*, which overlaps or is identical to the Dlla binding site. Like Dll, Antp binds cooperatively with Hth and Exd. Using purified proteins, we show that binding of Dll and Antp to the Dlla site is mutually exclusive. This indicates that Antp represses the enhancer at least in part by competing with Dll for binding. Similar competition may occur at other enhancers; when Antp expression is driven artificially in the distal leg, variable deletions of the tarsal segments occur (Emerald and Cohen 2004). These defects might arise because Antp

competes with Dll for binding to its target genes in the distal leg. In most other contexts examined, Antp is an activator of transcription (Capovilla et al. 2001; Winslow et al. 1989; Reuter and Scott 1990); why it fails to activate *D4* is not clear. The similar behavior of Dll and Antp in binding to *D4* supports the idea that Dll behaves like a Hox protein in activating *D4*.

Although our initial focus was on the antenna, the finding that Antp interacts directly with *D4* led us to examine *D4* regulation in the leg, where Antp is normally expressed. We find that in second leg imaginal discs, *Antp* is required only in a proximal ring of cells that coexpresses Dll and Hth. This ring appears in the early third instar, and is of uncertain function. Large *Antp*$^{-}$ clones in T2 leg discs that do not enter this ring appear to develop completely normally, regardless of whether they are located distal or proximal to the ring. However, clones that overlap the ring show activation of *D4*/*lacZ* within the ring cells. Importantly, such clones have no effect on the expression of Dll or Hth within the ring. By examining *Antp*$^{-}$ clones of increasing age the following sequence of events is inferred. First, *D4*/*lacZ* is activated in cells of the ring that are included within *Antp*$^{-}$ clones. Second, many such clones begin expressing the antennal markers Ss and Cut, indicating a transformation to antenna, and round up as if they have lost affinity for neighboring cells. Third, such clones appear to extend and move distally in the disc.

The events we describe for *Antp*$^{-}$ clones in the leg make sense of several previously enigmatic observations. Struhl (1981; 1982a) noted that many *Antp*$^{-}$ clones in the leg do not transform to antenna and appear to develop normally. Our finding that only clones that overlap the proximal ring undergo transformation accounts for this observation. Struhl also found that *Antp*$^{-}$ clones that do contain transformations usually show apparent nonautonomy in that not all cells in the clone are transformed to antenna. Our results account for this observation as well, since within an *Antp*$^{-}$ leg clone only those cells located in the proximal ring undergo transformation to antenna; cells located elsewhere in the clone retain normal leg identity. Most importantly, our observations provide an explanation for why *ss* is controlled directly by *Antp*. We find that *Antp*$^{-}$ clones have no effect on *hth* or *Dll* expression in the proximal ring. Therefore, *Antp* must function in the ring at the target gene level to repress antennal genes that would otherwise be activated by combined Hth and Dll (and Exd). Since several such targets are known (Dong et al. 2002), it seems likely that several, perhaps many, antennal genes in addition to *ss* are repressed directly by *Antp*.

The findings of McKay et al. (2009) challenge our inference that transformed *Antp*$^{-}$ clones extend or migrate distally in the leg. These authors show that distal migration of cells from the *hth*-expressing domain of the leg does not occur during normal development. However, coexpression of Dll and Hth in leg discs normally occurs only within the proximal ring, whereas such coexpression in the antenna extends far more distally, including all of A2 and A3. Therefore, *Antp*$^{-}$ cells from the proximal ring that transform to antenna likely assume a more distal identity as well as an altered segmental identity, perhaps allowing them to migrate more distally. Alternatively, it is possible that the transformed clones we interpret as having migrated distally were in fact generated early in leg development, when Hth expression overlaps Dll expression more distally in the leg primordium (McKay et al. 2009). We favor the first possibility because almost all transformed clones retain a clear, although sometimes tenuous, connection to the ring.

We confirm the finding of Casares and Mann (1998) that transformed *Antp*$^{-}$ clones in the leg often show ectopic *hth* expression in distal locations. If *hth* is not directly controlled by Antp in the leg, as we suggest, then why is *hth* ectopically expressed within such clones? A likely explanation is that downstream antennal genes that have become activated in such clones feed back to activate *hth*. This interpretation is strongly supported by the finding that ectopic expression of the antennal genes *ss*, *dan*, or *danr* in the distal leg causes ectopic activation of

hth (Suzanne et al. 2003). Thus, the distal expression of *hth* seen in *Antp*$^-$ leg clones is likely a consequence rather than a cause of the transformation to antenna. Whether repression of *hth* in the antenna by ectopic Antp is also indirect is not clear. *Dll* is also expressed ectopically in transformed *Antp*$^-$ leg clones, suggesting that it is also subject to feedback activation by downstream antennal genes.

The function of the proximal Dll- and Hth-expressing ring in the proximal leg is not well understood. The ring is highly conserved among the insects (Angelini and Kaufman 2005), and may serve as a boundary between the proximal and distal portions of the legs (Wu and Cohen 1999; McKay et al. 2009). In the context of our work, a striking feature of the ring is that it contains a microcosm of gene expression domains corresponding to the three major antennal segments. Thus, proceeding from proximal to distal through the ring, cells express *hth* alone, *hth* + *Dll*, and *hth* + *Dll* + strong *dachshund* (Wu and Cohen 2000). These expression combinations are characteristic of the A1, A2, and A3 antennal segments, respectively. Looked at in this way, the ring would appear to resemble a repressed antennal primordium within the leg.

It has been known for almost thirty years that *Antp* is required in the leg to repress antennal identity. However, an understanding of how this repression occurs has been lacking. Our results indicate that Antp functions within the proximal ring to directly repress antennal genes that would otherwise be activated by combined expression of Dll, Hth, and Exd. This appears to be the only function of *Antp* in the leg, at least during the third instar larval stage. Our results are entirely consistent with the ideas of Struhl (1981; 1982a), who argued that second leg is the “ground state” ventral appendage (the limb type that develops in the absence of identity specification) and that the role of *Antp* in the leg is to preserve this ground state by repressing the activation of “head-determining” genes.

Experimental Procedures

Antibody Staining

Antibody stainings were performed as described previously (Kankel et al. 2004). Primary antibodies used were mouse anti Dll (Duncan et al. 1998), rabbit anti Dll (gift of Grace Boekhoff-Falk), rabbit anti Hth (gift of A. Salzberg), mouse anti Ubx and mouse anti Exd (gifts of R. White), guinea pig anti Ss (gift of Michael Kim), mouse anti Cut, mouse anti Scr, and mouse anti Antp (all from the Developmental Studies Hybridoma Bank), mouse anti β-galactosidase (Promega), and rabbit anti β-galactosidase (Cappel). Secondary antibodies used were Cy3 donkey anti rabbit, Cy3 donkey anti mouse, Cy3 donkey anti guinea pig, Cy5 donkey anti rabbit, Cy5 donkey anti mouse (Jackson), and FITC goat anti rabbit (Cappel). Images were captured on a Nikon A1 scanning confocal microscope.

Gel shift assays

Unless otherwise noted, all chemicals were from Sigma-Aldrich. The sequences of the oligonucleotides used as probes are in Figure 5. Oligonucleotides were labeled with α ^{32}P dCTP (Perkin Elmer) using the Klenow fragment of DNA pol I (New England Biolabs). 10 to 50 ng of annealed oligonucleotide was used per reaction. Unincorporated label was removed with P6DG spin columns (Biorad) and amounts were normalized using DE81 filters (Whatman).

All proteins were produced by *in vitro* translation using the TnT T7 Rabbit Reticulocyte Lysate kit (Promega). 1 μg of circular plasmid DNA was used per reaction and 5% of the translation was incubated with ^{35}S methionine to assay translation efficiency. The unlabelled translated protein was used without further purification. 1 to 10 μl of *in vitro* translation reaction product were used per reaction. Total protein was kept constant among samples in an experiment by

addition of control luciferase translations. Luciferase translations were also used in control lanes to assess non-specific binding of proteins in the lysate. Poly (dI·dC) was used to reduce nonspecific binding. For super-shift experiments, 1 μl of antibody (1:10 dilution in PBS) was added for the final 5 minutes of incubation prior to gel loading.

The plasmids used for *in vitro* translation were as follows: Dll, Hth, and Exd constructs contain the full-length coding regions of the respective genes cloned into pT7βplink (Dalton and Treisman 1992), a generous gift of G. Boekhoff-Falk. The Dll coding sequence used includes an additional 20 codons relative to the standard sequence due to alternate splicing between exons 2 and 3. Antp, Scr and Ubx constructs contain the full length coding regions cloned into pTnT (Promega). The Cut construct includes nucleotides 2632-5434 (numbering as in Blochlinger et al. 1988) of the *cut* cDNA, which includes the coding sequences for all three Cut domains and the homeodomain, cloned into pTnT.

For competition assays, *in vitro* translation products were purified from the lysate and concentrated using oligonucleotide selection as described by Ozyhar et al. 1992. Based on Coomassie staining of pre- and post-purification lysate, greater than 90% of nonspecific lysate protein was removed by this protocol. Copper phenanthroline footprinting of shifted bands excised from gels was as described by Sigman et al. (1991).

Generation of *lacZ* reporter lines

Deletion derivatives of the *ss522* sequence were generated by recombinant PCR, verified by sequencing, and subcloned into either *pCaSpeR-hs43-βgal* (Thummel and Pirrotta, 1992) or *placZattB* (Bischof et al. 2007). *pCaSper-hs43-βgal* constructs were transformed into *y* w^{67c23} flies by standard methods, and a minimum of 5 separate insertions per construct assayed. *placZattB* constructs were transformed as described by Bischof et al. (2007). For *placZattB*, all integrations were at a site at 22A. X-Gal staining was as described by Emmons et al. (2007).

Mitotic recombination clones: A *D4*/*lacZ* reporter line containing a dimer of the *D4* sequence inserted at the 22A site (line *81d42*), was used in almost all experiments. In a few early experiments, a P-element *lacZ* reporter containing a multimer of *D4* was used and gave similar results. Clones were generated by the FLP-FRT method using the following chromosomes: exd^{1} *FRT18E*, cut^{145} *FRT18E, FRT82B* $hth^{64\text{-}1}$, *FRT82B* $Antp^{25}$, and *FRT42D* Dll^{SA1}. In all cases, mitotic recombination clones were identified in discs by the loss of the *Ubi-GFP* marker. *hs-FLP122* and *hs-FLP38* were used as sources of recombinase. Crosses were made in plastic vials, and cultures were immersed for 30 minutes (*hs-FLP122*) or 1 hr (*hs-FLP38*) in a water bath at 37° to induce recombinase expression.

Ectopic expression clones: Males carrying appropriate *UAS* constructs were crossed to *y w hs-FLP12*/*y w hs-FLP12*; *D4lacZ 81d42*/*D4lacZ 81d42*; Act>y^{+}>Gal4 UAS-GFP/*TM6B, Tb* females or to *y w hs-FLP12*/*y w hs-FLP12*; Act>y^{+}>Gal4 UAS-GFP/*TM6B, Tb* females. Clones were induced by immersion at 37° for 8 minutes. The *UAS* lines used were *UAS-Cut* (provided by Helen McNeil), *UAS-Hth* (line 12; provided by Henry Sun), *UAS-Antp* (provided by T. Kaufman), and *UAS-Scr*, *UASUbx*, and *UAS-Dll* (all from the Bloomington Stock Center).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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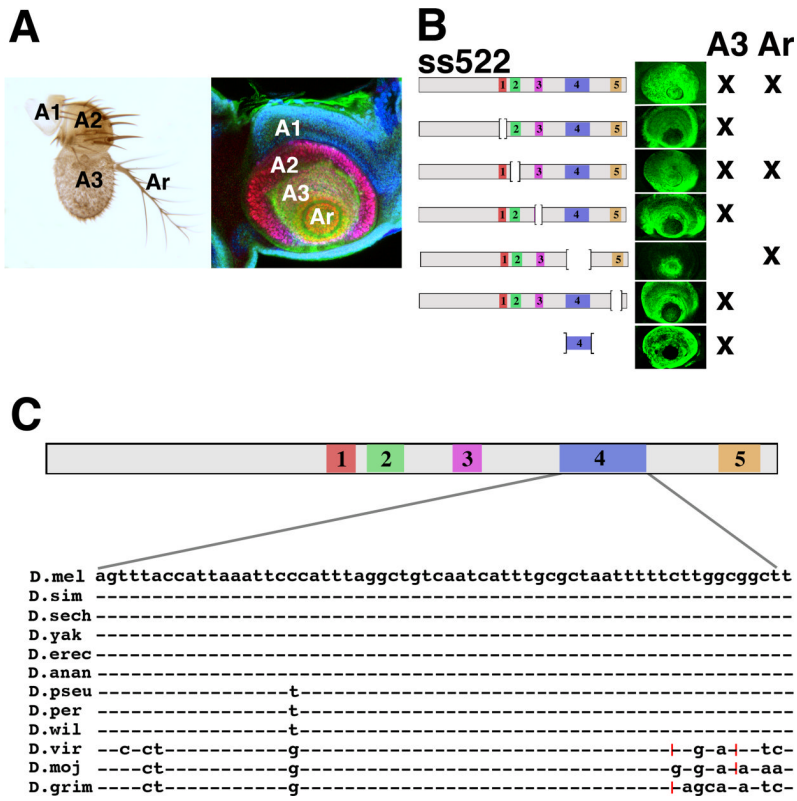

Figure 1.

(A) Left: A wild-type adult antenna. The first (A1), second (A2), and third (A3) antennal segments and the arista (Ar) are indicated. Right: A mature antennal disc stained for Hth (blue), Dll (red), and the *ss* reporter *B6.9*/*lacZ* (Emmons et al. 2007) (green). Hth is expressed in the primordia of A1, A2, and A3; Dll is expressed in A2, A3, and the arista; and *ss* is expressed in A3 and the arista. (B) Five conserved domains within *ss522* and their deletion derivatives are indicated. The antennal expression each drives *in vivo* is shown to the right. (C) Conservation of the sequence of domain 4 in 12 Drosophila species; dashes indicate identity, red hatch marks indicate 3 bp insertions relative to the *D. melanogaster* sequence.

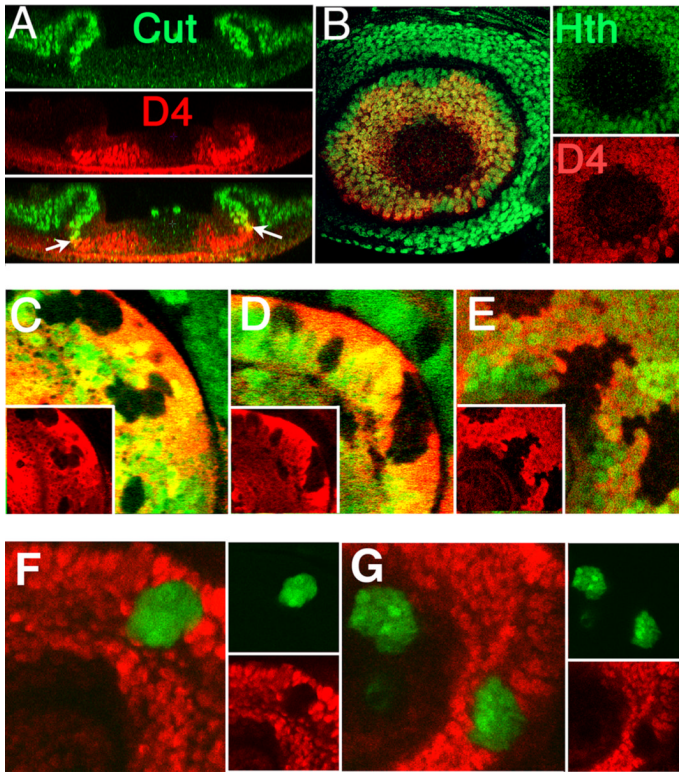

Figure 2.
(A) Cross section of an antennal disc showing expression of Cut and *D4*/*lacZ*. The distal boundary of Cut expression and the proximal boundary of *D4*/*lacZ* expression closely match, although a few cells at the interface often express both (arrows). (B) Antennal disc stained for expression of Hth and *D4*/*lacZ*. The distal boundaries of expression coincide precisely. (C - E) Expression of *D4*/*lacZ* (red) is lost within clones mutant for *Dll* (C), *hth* (D), or *exd* (E). All clones are marked by the loss of GFP (green). (F,G) Clones expressing either Antp alone (F) or both Antp and Hth (G) (green) fully repress *D4*/*lacZ* (red). Expression of Hth was confirmed by antibody staining (not shown).

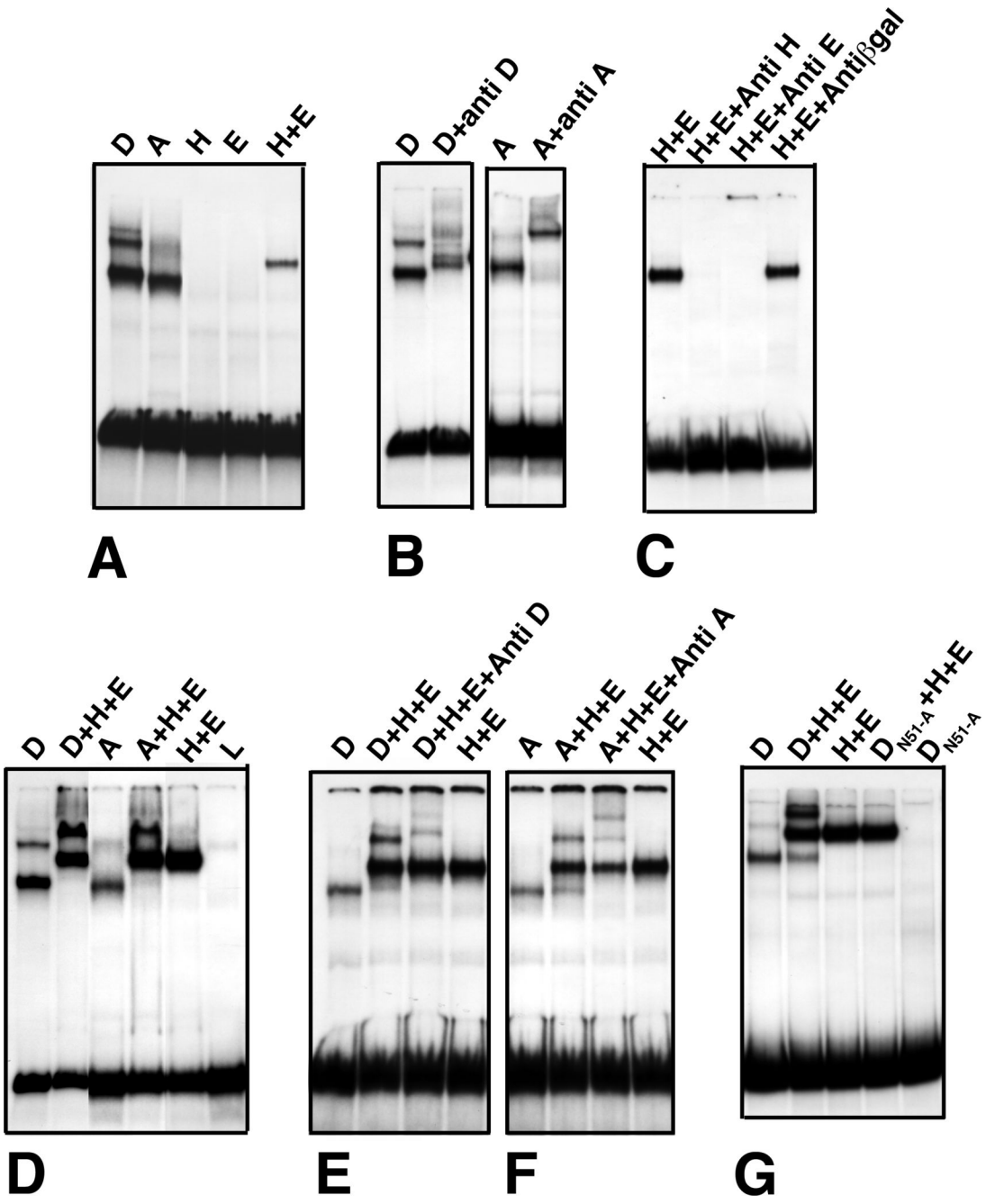

Figure 3.

(A) Binding of Dll (D), Antp (A), Hth (H), and Exd (E) to *D4*. Dll produces three retardation bands, whereas Antp produces a single major retardation band. Hth and Exd produce no shift on their own, but generate a prominent retardation band when mixed. (B) Anti-Dll and anti-Antp supershift the respective retardation complexes. (C) Anti-Hth and anti-Exd antibodies block production of the Hth+Exd retardation band. (D) When combined with Hth and Exd, both Dll and Antp produce slowly migrating bands, but show very little of the singly bound species produced by Dll or Antp on their own. L = lysate control. (E-F) Antibodies to Dll (E) and Antp (F) supershift the slow moving bands formed when these proteins are mixed with Hth and Exd. (G) Dll protein in which asn51 of the homeodomain has been changed to ala does

not bind *D4* on its own or when mixed with Hth and Exd. *In vitro* translation of the mutant protein was confirmed by ^{35}S-methionine labeling (not shown).

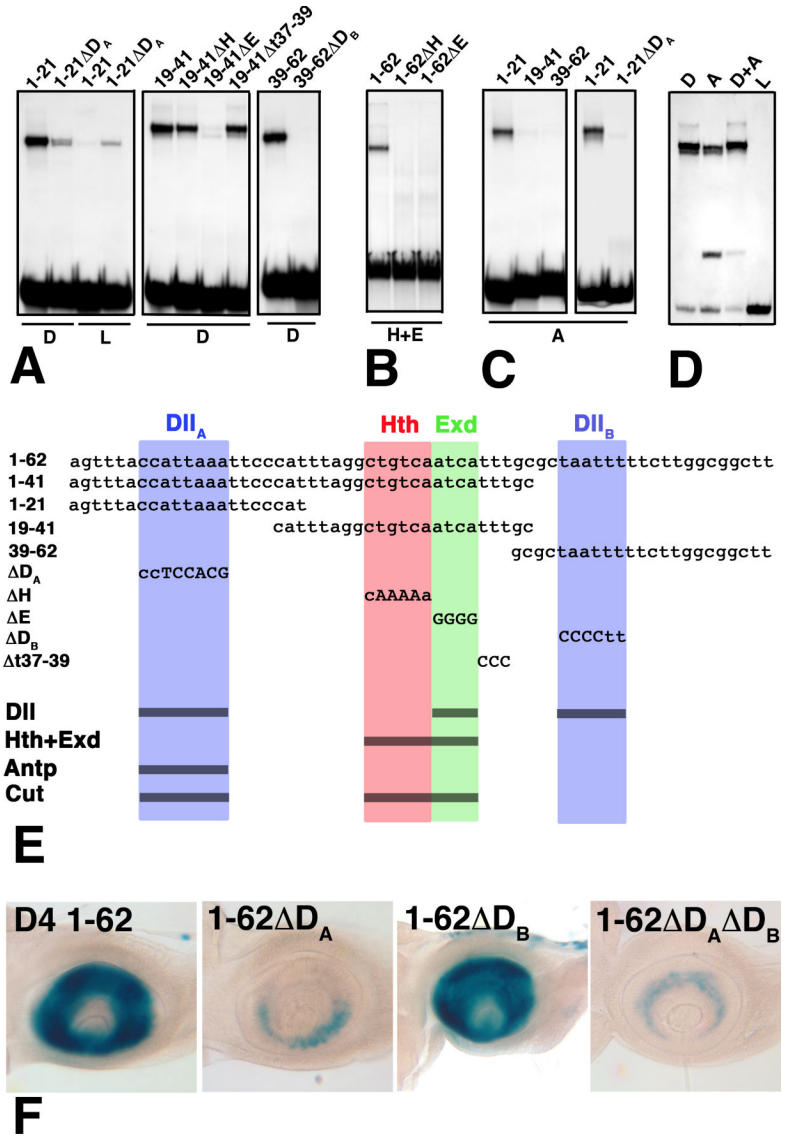

Figure 4.

Gel-shift assays of mutant and wild-type derivatives of *D4*. The sequences of full length *D4*, four fragments, and five clustered site mutations are shown in (E). Abbreviations as in Fig. 3. (A) Dll binds three sites in *D4*, one in each of the subfragments 1-21, 19-41, and 39-62. Binding to these fragments is almost completely eliminated by the ΔD_A, ΔE, and ΔD_B mutants, respectively. (B) Cooperative binding of Hth and Exd is eliminated in both the ΔH and ΔE mutants, indicating that Hth and Exd bind adjacent consensus sites. (C) Antp binds only the 1-21 fragment, and this binding is lost in the ΔD_A mutant. Lysate (L) control lanes were blank (not shown) for all but the 1-21ΔD_A probe. (D) Purified Dll (D) and Antp (A) compete for binding to the 1-21 probe. The faster migrating band in the Antp lanes is likely due to the binding of a breakdown product generated during purification. (E) Summary of the DNA sequences tested in (A-D). (F) Effects of the ΔD_A and ΔD_B mutations on antennal expression *in vivo*.

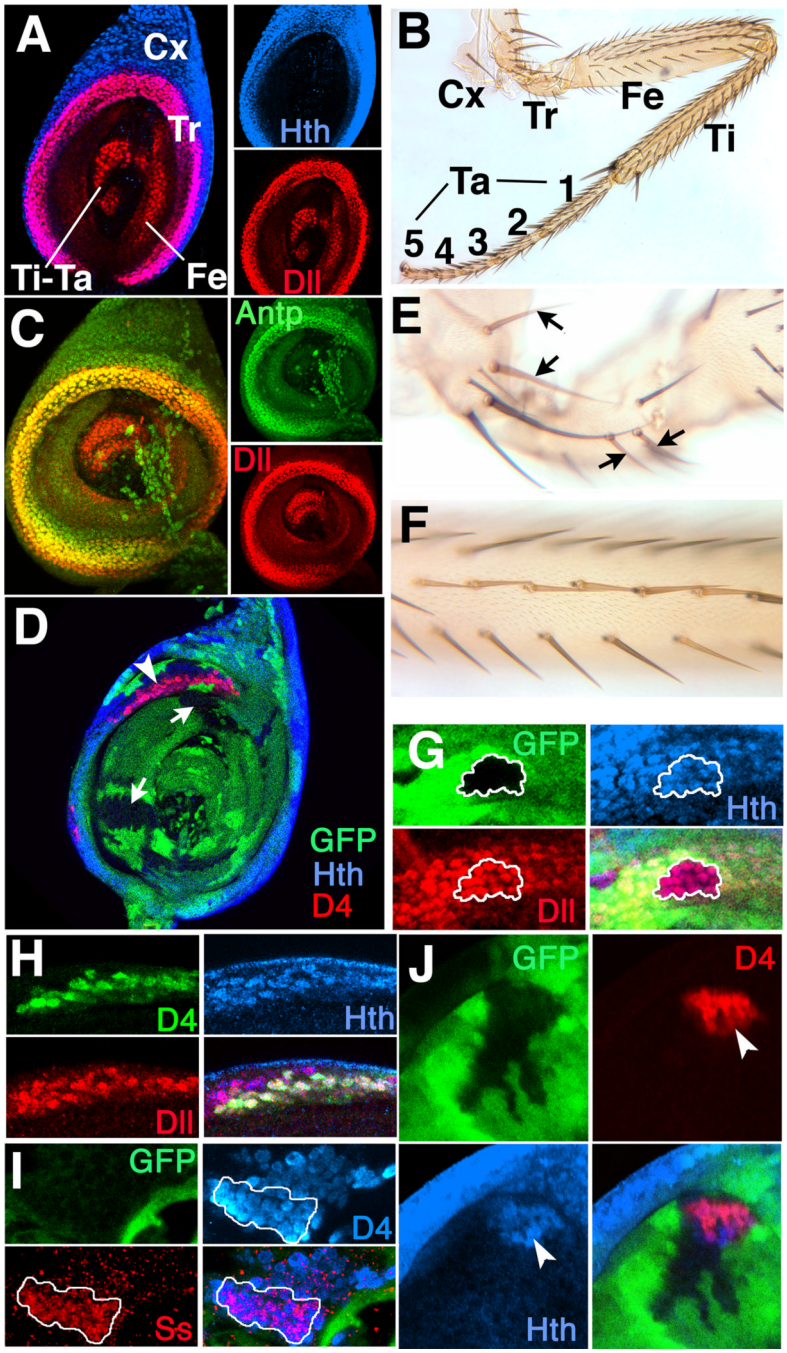

Figure 5.

Antp represses *D4*/*lacZ* in the proximal Dll+Hth ring of the second leg imaginal disc. (A) A second leg disc stained for Hth and Dll. Hth is expressed in a broad proximal domain, whereas Dll is expressed in a 5-7 cell wide proximal ring whose distal border coincides with the distal limit of Hth. Dll is also expressed in the central (distal) region of the disc, which is only partly in the plane of focus. Cx=coxa, Tr=trochanter, Fe=femur, Ti=tibia, and Ta=tarsus. (B) An adult second leg. Abbreviations as in (A). (C) A second leg disc stained for Dll and Antp. Antp is expressed in a broad proximal region, and is upregulated within the Dll ring. (D) *Antp*$^{-}$ clones, marked by the loss of GFP, in a second leg disc. *D4*/*lacZ* is activated in *Antp*$^{-}$ clones where they overlap the Hth + Dll ring (arrowhead). *Antp*$^{-}$ clones that do not overlap the ring (arrows)

show no activation of *D4*/*lacZ*, have interdigitated borders, and appear to develop normally. (E) *Antp*$^{-}$ clones in the coxa and trochanter marked by yellow bristles (arrows) produce normal cuticular structures. (F) An *Antp*$^{-}$ clone in the femur marked by yellow bristles produces normal structures. (G) An *Antp*$^{-}$ clone (outlined in white) in the proximal ring has no effect on expression of Hth or Dll. (H) All cells expressing *D4*/*lacZ* within an *Antp*$^{-}$ leg clone also express both Hth and Dll. Clones are not marked in this disc to allow direct comparison of Dll, Hth, and *D4*/*lacZ*. (I) A partially rounded up *Antp*$^{-}$ clone showing expression of Ss within part (white outline) of the *D4*/*lacZ*-expressing region. (J) An *Antp*$^{-}$ clone marked by the absence of GFP in a second leg disc showing activation of *D4*/*lacZ* and ectopic expression of Hth. Note that *D4*/*lacZ* and Hth are expressed in a rounded-up portion of the clone (arrowheads), which is presumably transformed to antenna, whereas the remainder of the clone is interdigitated. Although not visible in this focal plane, the region of the clone expressing *D4*/*lacZ* and Hth retains a connection to the proximal Hth + Dll ring.

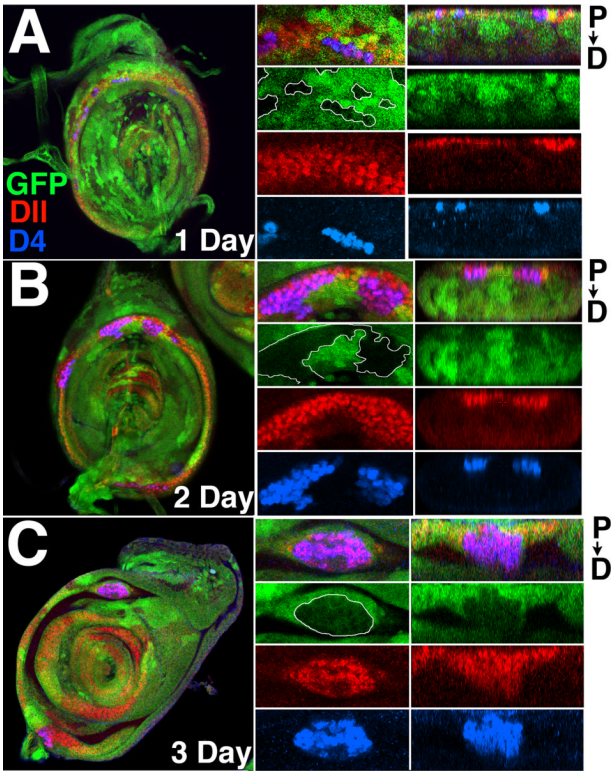

Figure 6.

D4/*lacZ* activation in *Antp*$^{-}$ clones of increasing age in second leg discs. *Antp*$^{-}$ clones are marked by the loss of GFP (green), and all discs are stained for Dll (red) and *D4*/*lacZ* (blue). Left-hand panels show merged images of the entire disc. The central panels show an enlarged region, with the merged image at top. The right hand panels show cross sections at the same level as the central panels. Distal extension of clones from the ring is seen as downward extension in these cross sections. (A) 1 day old *Antp*$^{-}$ clones. Note in central panels that *Antp*$^{-}$ clones activate *D4*/*lacZ* in the distal part of the Dll ring, but not in the proximal portion. No distal extension of the *D4*/*lacZ*-expressing clones has taken place. (B) By day two, *D4*/*lacZ*-expressing clones are beginning to round up and distort the ring (central panels). Slight distal extension of these clones has occurred (right panels). (C) By day three, rounding up of *D4*/*lacZ*-expressing clones is advanced (middle panels), and significant distal extension is seen (right panels).

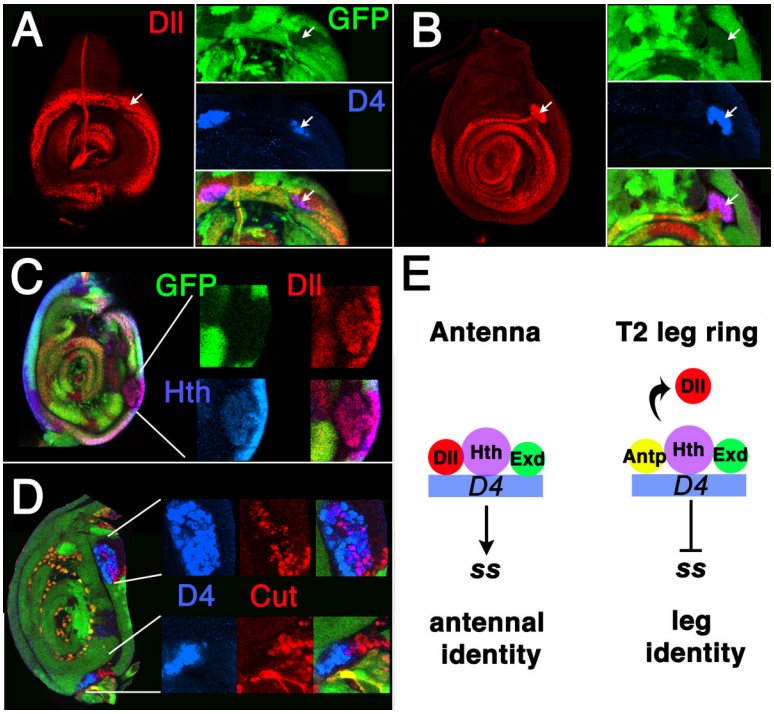

Figure 7.

In all panels, *Antp*$^{-}$ clones are marked by the loss of GFP (green). (A-B) A *D4*/*lacZ*-expressing *Antp*$^{-}$ clone in a second leg disc that extends from the proximal ring to the central domain of Dll expression. (A) Proximal focal plane, showing the Dll ring. Note activation of *D4*/*lacZ* in a clone overlapping the ring (arrows). (B) Distal focal plane, showing that the same transformed clone (arrows) connects to the distal domain of Dll expression. (C) A transformed *Antp*$^{-}$ clone in the second leg stained for Hth and Dll expression. Part of the clone has rounded up, but remains connected to the ring by a narrow isthmus. In some transformed clones, the connection is much narrower and thread-like. (D) Second leg disc containing *Antp*$^{-}$ clones stained for Cut and *D4*/*lacZ* expression. Note two rounded-up clones in which both Cut and *D4*/*lacZ* are expressed. Although Cut and *D4*/*lacZ* are coexpressed in many cells in the upper clone, Cut is expressed adjacent to a *D4*/*lacZ* expressing region in the bottom clone. (E) Model summarizing the control of *D4* in the antenna and leg. Left: In the antenna, *D4* is activated by binding of a Dll/Hth/Exd complex. Right: In the proximal ring of the leg, Antp displaces Dll and prevents activation of the enhancer.