**INTRODUCTION**

The appendages of *Drosophila* are derived from imaginal discs, which proliferate during the larval stages and differentiate during the pupal stage to give rise to the adult structures. Segment-specific identity of the appendages is controlled through the action of homeotic selector genes. Homeotic genes are considered to be the genetic switches that govern the choice between alternative developmental programs leading, for example, to leg or antenna development. Antenna and leg are considered to be homologous structures because they can be interconverted through the action of homeotic genes (Gehring, 1966). For example, ectopic expression of Antennapedia (Antp) or other homeotic genes can transform antenna to leg (Gehring et al., 1988; Struhl, 1981a; Yao et al., 1999; Zeng et al., 1993). By contrast, the antenna appears to develop without any input from homeotic genes (Kaufman et al., 1990). Indeed, mutants that lack the entire HOM-C complex in the proximal regions of the flower beetle *tribolium* produce embryos in which all segments bear antenna-like appendages (Beeman, 1987). How is antennal identity specified? The *homothorax* (*hth*) and *Distal-less* (*Dll*) genes have been shown to play a role in antenna development. *Dll* encodes a homeodomain protein expressed in distal leg and antenna that is required for development of distal structures in both appendages (Cohen et al., 1989; Diaz-Benjumea et al., 1994; Gorflinkel et al., 1997). Hypomorphic mutations that reduce *Dll* activity cause transformation of antenna into leg (Cohen and Jürgens, 1989c; Cohen and Jürgens, 1989b). *hth* encodes a TALE-type homeodomain protein expressed in the proximal parts of all imaginal discs. Hth protein promotes nuclear localization of the PBC-class homeodomain protein Extradenticle (Kurant et al., 1998; Pai et al., 1998; Rieckhoff et al., 1997). In leg and wing discs, Hth is expressed and required only in proximal structures (Azpiazu and Morata, 2000; Casares and Mann, 1998; Casares and Mann, 2000; Wu and Cohen, 1999). A similar role has been proposed for the vertebrate Hth protein MEIS in limb development (Mercader et al., 1999). In contrast to the situation in the leg, *Hth* and *Dll* expression overlap extensively in the antenna. This overlap has been linked to antenna identity based on the observation that co-expression of *Hth* and *Dll* can induce formation of antennal structures in the proximal regions of the wing and leg discs (Casares and Mann, 1998; Dong et al., 2000). The *spineless* (*ss*) gene has been implicated in control of antennal identity. *ss* mutants cause transformation of distal antenna to leg (Struhl, 1982). *ss* encodes a bHLH PAS domain transcription factor, homologous to the mammalian dioxin

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**SUMMARY**

Legs and antennae are considered to be homologous appendages. The fundamental patterning mechanisms that organize spatial pattern are conserved, yet appendages with very different morphology develop. A genetic hierarchy for specification of antennal identity has been partly elucidated. We report identification of a novel family of genes with roles in antennal development. The *distal antenna (dan)* and *distal antenna-related (danr)* genes encode novel nuclear proteins that are expressed in the presumptive distal antenna, but not in the leg imaginal disc. Ectopic expression of *dan* or *danr* causes partial transformation of distal leg structure toward antennal identity. Mutants that remove *dan* and *danr* activity cause partial transformation of antenna toward leg identity. Therefore we suggest that *dan* and *danr* contribute to differentiation of antenna-specific characteristics. Antenna-specific expression of *dan* and *danr* depends on a regulatory hierarchy involving *homothorax* and *Distal-less*, as well as *cut* and *spineless*. We propose that *dan* and *danr* are effector genes that act downstream of these genes to control differentiation of distal antennal structures.

Key words: Patterns formation, *Drosophila*, Antennae, Homothorax, Distal-less, Antennapedia, Spineless

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**distal antenna and distal antenna related encode nuclear proteins containing pipsqueak motifs involved in antenna development in *Drosophila***

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receptor (Duncan et al., 1998). ss is expressed transiently in distal domains of the leg and antenna discs during the second larval instar, but is maintained only in the distal antenna. This difference is ss regulation depends on the activity of Hth and Dll (Dong et al., 2002). Ectopic expression of ss in the leg can cause transformation towards antenna, indicating that ss expression in an important determinant of antenna identity (Duncan et al., 1998). The cut gene is also expressed differentially in leg and antenna (Dong et al., 2002), and when misexpressed in distal antenna has been shown to cause misexpression of Antennapedia and concomitant transformation towards leg (Johnston et al., 1998).

Although several genes have been implicated in antenna development on the basis of mutant phenotypes and differential expression (Duncan et al., 1998; Dong et al., 2002), our understanding of the control of antennal identity remains incomplete. We report the identification of two genes, distal antenna (dan) and distal antenna related (danr) that play roles in antennal development. dan and danr encode nuclear proteins that are expressed in the distal antenna imaginal disc, but not in leg. We present evidence that Hth and Dll control Dan and Danr expression through regulation of ss and cut. Loss of Dan and Danr function causes defects in antenna development and partial transformation of distal antennal structures towards leg. Ectopic expression of these genes in the leg disc causes transformation of distal leg to antenna. These findings implicate Dan and Danr as downstream effectors of ss in the specification of distal elements of the antenna.

MATERIALS AND METHODS

Drosophila stocks

Strains used are described in the following references: Act5C>CD2>Gal4 (Pignoni and Zipursky, 1997), e145, UAS-cut (Johnston et al., 1998), UAS-Dll (Wu and Cohen, 1999), DlgGAL4 (Gorfinikel et al., 1997), hthC1 (Rieckhof et al., 1997), UAS-hth12 (Pai et al., 1998), dppGAL4 (Morimura et al., 1996), ss5144-4 and UAS-ss (Duncan et al., 1998). For others, see http://www.flybase.bio.indiana.edu.82. The dan ORF was PCR amplified from EST LD02250 using the primers 5'-AACATATGAAA-CATCCGACATGGG and 5'-CTTTGAGCTCTTGCGACGGCGACT and cloned as a Norl-Xhol fragment into pUAST (Brand and Perrimon, 1993). The dan ORF lacks introns and was PCR amplified from genomic DNA using the primers: 5'-AGAAA-TGAATCTGAGATACTCTCCGGCTAC and 5'-TATAATCCGAGTTGGCTACTGTTGGCGTC and cloned as an EcoRI-Xhol fragment into pUAST.

Isolation of mutants

For danex33 and dan,danex56. EPg-35635 and EPg-J3-220 were recombined onto the same chromosome. Flies containing the recombinant chromosome were identified by their darker eyes, resulting from the presence of mini-w+ genes from each EPg-element. They were obtained at a rate of one in approximately 200,000 screened. Standard methods were used to obtain flies from which both EPg-elements were excised, and whose eyes were therefore white. Fragments containing the breakpoints of the EPg-element excisions were obtained by PCR and sequenced. Sequence of primers for EPg 35635 breakpoints were GATTCGCCAACCACAAGTGCACCC and ACTATGACTACAACTACA. Sequence of primers for EPg J3-220 breakpoints were ATTAGGCTCGTCTCTGTTGCA and TAAAGTCGACGTCCAGAA. Some of the double excisions removed the entire sequence between the two EPg-elements; breakpoints for these were obtained using a forward primer upstream of EPg-35635 and a reverse primer downstream of EPg J3-220. Sequence of primers for large deletion breakpoints were GATTCGCCAACCACAAGTGCACCC and TCTTGTGTCACCGATTCTCTCA. The resulting products were sequenced along with the rest. The danex33 single mutant is missing 314 bp of genomic DNA, including the EPg 35635 insertion point and extending 126 bp into the dan ORF. Sequences surrounding the EPg J3-220 excision point in danex33 mutant DNA are completely wild type.

An EMS screen was carried out to isolate dan mutants. Male flies carrying EPg-J3-220 were mutagenized with 25 mM EMS and crossed to SdGAL4 females. The EMS induced revertant danw3 was isolated by virtue of having normal wings, despite having overexpressed dan. danw3 DNA was cloned from homozygous mutant larvae by PCR using the following primer pairs:

1A, TCTGTGTCACCAAATCTTCAA; 1B, TCTTGTGTCACCAAATCTTCAA;
2A, ATGCCAAGTGTGTCACGTA; 2B, CGTTCTTGTGTCACGTA;
3A, GTTCCAAGCAGCTGACACAG; 3B, CATCGAGATCGTTCTGGT;
4A, TTTGTGTCACCGACTGGA; and 4B, ATAGGGTCACGACCTAGGAGA.

Sequence analysis identified a single nucleotide alteration.

Genetic mosaics

Genotypes of larvae for ectopic expression clones:

eyFlp; FRT82B danex35/FRT82B M+ armw2/z
HSFlp f56a; FRT82B danex35/FRT82B f

Genotypes of larvae for dan danr mutant clones:

HsFlp f56a; FRT82B dan danex56/FRT82B M+ armw2/z f
HsFlp f56a; FRT82B dan danrex35/FRT82B f

eyFlp; FRT82B dan danrex56/FRT82B M+ armw2/z

hh mutant clones:

HsFlp1; FRT82B hthC1/FRT82B UbGF

Dill mutant clones:

HsFlp1; FRT42 DiSIAl/FRT42 Ub GF

cut mutant clones:

cut145 FRT18/FRT18 armw2/z; HSFlp

Genotypes of larvae for ectopic expression clones:

ss, yw Act5C>CD2>Gal4/HSFlp1; UAS-ss; UAS-EGFP

cut, yw Act5C>CD2>Gal4/HSFlp1; UAS-cut; UAS-EGFP

Antibodies

The Dan ORF was PCR amplified (primers AACATATGAAA-ATCCGACATGGG and CTTTGAGCTCTTGCGACGGCCT and cloned into pET23a (Novagen) using

EcoRI and

SacI and

purified by Ni2+ affinity chromatography under denaturing conditions. Terminally HIS-tagged fusion proteins were expressed in bacteria and

EcoRI fragment into SympUAST for Gal4

and cloned as an

sy1.2 kb fragment of the

hth ORF was PCR amplified (primers AAACA TA TGAAC-

TA GCIGC TCG) and cloned into pET23a using

A TCCGCA TGGGC and CTTTGAGCTCTTGCGACGGCGACT)

The Dan ORF was PCR amplified (primers AAACA TA TGAAC-

AAACTCATGGGATACTCTCCGGCTAC and 5'-TATAATCCGAGTTGGCTACTGTTGGCGTC and cloned as an

EcoRI-Xhol fragment into pUAST.

HSFlp f 36a; FRT82B dan danr ex56 /FRT82B M arml acZ

HsFlp f 56a; FRT82B dan danr ex56 /FRT82B M arml acZ

HsFlp f 56a; FRT82B dan danr ex56 /FRT82B M arml acZ

HsFlp f 56a; FRT82B dan danr ex56 /FRT82B M arml acZ

HsFlp f 56a; FRT82B dan danr ex56 /FRT82B M arml acZ

HsFlp f 56a; FRT82B dan danr ex56 /FRT82B M arml acZ

HsFlp1; FRT82B hthC1/FRT82B UbGF

Dill mutant clones:

HsFlp1; FRT42 DiSIAl/FRT42 Ub GF

cut mutant clones:

cut145 FRT18/FRT18 armw2/z; HSFlp

Genotypes of larvae for ectopic expression clones:

ss, yw Act5C>CD2>Gal4/HSFlp1; UAS-ss; UAS-EGFP

cut, yw Act5C>CD2>Gal4/HSFlp1; UAS-cut; UAS-EGFP

The Dan ORF was PCR amplified (primers AACATATGAAA-ATCCGACATGGG and CTTTGAGCTCTTGCGACGGCCT and cloned into pET23a (Novagen) using

EcoRI and

SacI and

purified by Ni2+ affinity chromatography under denaturing conditions. Rats and mice were immunized using RIBI adjuvant at 3-week intervals. Antisera were evaluated by immunostaining imaginal discs. Mouse anti-Antp is described elsewhere (Condé et al., 1991). Mouse anti-Cut (Blochlinger et al., 1990), Rat anti-Hth (Kurant et al., 1998), rat anti-Dll (Wu and Cohen, 2000) and mouse monoclonal anti-

Dill (Duncan et al., 1998) were also used.

RNA interference

Dan

A 1.2 kb fragment of the dan coding sequence was PCR amplified and cloned as an

EcoRI fragment into SympUAST for Gal4 dependent expression of double stranded RNA (Giordano et al., 2002).
RESULTS

Ectopic expression of dan and danr induces leg to antenna transformation
distal antenna (dan) and distal antenna related (danr) were identified in a large-scale modular misexpression screen of ~8500 EPg elements (Mata et al., 2000), as insertions that caused abnormalities in wing development when expressed under sdGal4 control and small rough eyes when expressed under eyGal4 control (not shown). Expression of dan using EPg J3-220, under control of DllGal4 caused transformation of distal leg structures toward distal antennal identity (Fig. 1A,B). The claws, found on the tip of the fifth tarsal segment, were transformed into the distal-most part of the antenna, the arista (compare with the antenna in Fig. 1E.). In addition, there was some overall morphological abnormality of the tarsal region. Expression of danr from EPg35635 using DllGal4 caused loss of the claws but did not produce an overt transformation to arista (not shown).

EPg J3-220 and EPg 35635 lie approximately 45 kb apart on chromosome 3R, 263 bp and 39 bp upstream of the predicted genes, CG11849 and CG13651 (Fig. 2A). To verify that the predicted genes tagged by the EP-element insertions were responsible for the observed overexpression phenotypes, we generated UAS-dan and UAS-danr transgenic strains. Six independent UAS-dan transformants and five independent UAS-danr transformants were tested and found to be lethal when expressed with the DllGal4 driver. However when expressed using dppGal4, UAS-dan and UAS-danr showed distal leg to antenna transformation (Fig. 1C,D). Molecular markers of antennal identity were also examined in the imaginal discs. The zinc-finger protein Spalt is expressed in antenna, but not in leg discs (Wagner-Bernholz et al., 1991). Ectopic expression of dan can induce limited expression of Spalt in the leg disc (not shown), consistent with the observed transformation toward antennal identity. These observations suggested a role for dan and danr in specification of the identity of distal antennal structures.

Dan and Danr encode proteins containing pipsqueak motifs
The predicted proteins encoded by dan and danr are similar, showing 25% identity overall (Fig. 2B). This similarity extended through the entire sequence. In addition, Dan has a C-terminal extension of more than 200 amino acids. The most conserved region is a 64 amino acid sequence beginning with the N terminus, where Dan and Danr share 92% identity. Within this region, both Dan and Danr contain the newly identified ‘pipsqueak’ motif (Siegmund et al., 2002), a helix-turn-helix structure that is likely to be involved in DNA binding (Fig. 2C). Outside the pipsqueak motif, the Dan and Danr proteins contain no regions of significant sequence similarity to other known proteins but show short blocks of strong similarity to each other (Fig. 2B) (F. Ciccarelli and P. Bork, personal communication).

Dan and Danr expression in the distal antenna imaginal disc
Antibodies were raised to the predicted Dan and Danr proteins. Both are nuclear proteins, expressed in the eye-antenna disc (Fig. 3A,B). Double labeling with anti-Dan and anti-Danr showed that the two proteins are co-expressed in the antenna (Fig. 3B). Both proteins are also expressed in the brain and the eye region of the eye-antenna disc (not shown, Fig. 3A). Antibody labeling of other imaginal discs showed limited Dan expression in small groups of cells in leg (Fig. 3C) and wing (not shown). These appear in the location of prominent sense organ progenitors at relatively late stages of disc development. Danr was not detected in other discs.

To define the domain of Dan and Danr expression in the antenna, a series of double labeling experiments were performed with antibodies to other antennal proteins. Homothorax (Hth) is expressed in the presumptive head capsule and in antennal segments A1 to A3 (Casares and Mann, 1998). Hth overlaps with the proximal part of the Dan domain (Fig. 3D). Distal-less (Dll) is expressed in a distal domain comprising A2, A3 and the arista (Diaz-Benjumeda et al., 1994). Dll overlaps the Dan domain, but extends further proximally. Cut is expressed in the proximal part of the antenna (Johnston et al., 1998), in a domain that does not overlap expression of Dan (Fig. 3F). In optical cross-section there appears to be one row of cells with low expression of Dan at the interface between these domains. The domain of Dan/Danr expression appears to coincide with the domain in which expression of ss

Fig. 1. Expression of Dan and Danr induces leg to antenna transformation. (A) Cuticle preparation showing wild-type tarsal segments and claws. (B) Scanning EM of the distal leg of a DllGal4 EPg3-220 fly. Note the absence of claws and presence of two aristae (arrow). Note that each claw produced an arista when Dan was expressed. In contrast to the leg, the antenna disc is not separated into A and P compartments by a lineage restriction until larval stages. The distalmost elements of A and P origin probably merge in antenna to make a single arista, which they cannot do in leg. (C,D) Distal legs from dppGal4 UAS-dan and dppGal4 UAS-danr animals. One claw was replaced by an arista in each case (arrows). (E) Wild-type antenna.
transcript has been reported (Duncan et al., 1998). Antibody to Spinless protein is not available, precluding a more precise comparison.

The relationship between Dan, Hth and Dll expression suggests that the Dan domain corresponds to segment A3 and the arista and that Cut is expressed in A1 and A2 as well as the head capsule. Thus, in addition to the broadly overlapping domains of Hth and Dll, the antenna is subdivided into adjacent and perhaps mutually exclusive proximal and distal domains reflected by ss, Cut and Dan/Danr expression (Fig. 3G). Although we favor the view that the reciprocal expression of Dan/Danr and Cut is likely to define the border between domains of Hth and Dll, the antenna is subdivided into adjacent and perhaps mutually exclusive proximal and distal domains.

**Regulation of Dan by hth and Dll**

The overlap between Hth and Dll has been proposed to define antennal identity, because co-expression of the two proteins in ectopic loci can induce formation of ectopic arista structures in other discs (Casares and Mann, 1998; Dong et al., 2000). To ask whether Hth and Dll have a role in defining the non-overlapping expression domains of Cut and Dan/Danr, we examined clones of cells lacking hth or Dll activity in the antenna. Dan expression was lost in cells mutant for hth in the region where the two expression domains overlap (Fig. 4A). This suggests that Hth activity is required for Dan expression. Likewise, clones of cells lacking Dll activity lost Dan expression in the distal region of the disc (Fig. 4B).

Fig. 2. EPg J3-220 and EPg 35635 target the dan and danner genes, which encode related pipsqueak-motif-containing proteins. (A) (Top) the genomic region containing the dan and danner genes. The insertion points of EPg J3-220 and EPg 35635 (green triangles) lie ~45 kb apart. Predicted genes in the region are shown in blue. (Middle) Red arrowheads indicate the positions of the primer pairs used for genomic PCR to determine the breakpoints of excision mutations; broken red lines between each primer pair indicate the positions of the primer pairs used for genomic PCR to determine the breakpoints of excision mutations. (Bottom) Boxes show the extent of the genomic region deleted in the dan and danner alleles. (B) Alignment of the Dan and Danr sequences showing regions of similarity. Note that the two proteins are most similar in the N terminus. The amino acid change in dan is indicated in red above the alignment. (C) Alignment of pipsqueak motifs from Dan, Daller, Drosophila Pipsqueak, and the transposases Drosophila Pogo and human CENP-B. Asterisks below the alignment indicate identical residues; colons and periods indicate conserved residues (ClustalW). The arrow indicates the residue mutated in dan.
Distal antennal identity genes

antenna cells express Dll but not all express Hth. Our observations point to a non-autonomous effect of Hth on Dan expression, which may explain how Hth can be required for sustained expression of Dan in distal cells where Hth is not expressed.

Regulation of Dan by \textit{ss} and \textit{cut}

\textit{ss} is expressed in the distal antenna in a domain similar to that of Dan/Danr (Duncan et al., 1998). \textit{ss} mutants cause transformation of distal antenna to tarsus, suggesting a role in antennal identity (Burgess and Duncan, 1990; Struhl, 1982). To ask whether \textit{ss} regulates Dan and Danr, we examined antenna discs from \textit{spineless aristapedia} (\textit{ss}a) mutants. \textit{ss}a alleles appear to reduce \textit{ss} activity specifically in the antenna. Dan expression was lost from the distal part of \textit{ss}a mutant antennal discs (Fig. 5A). Loss of Dan expression in \textit{ss}a mutant antenna discs coincided with ectopic expression of Antennapedia (Fig. 5B). We note that these expression domains appear to be non-overlapping. Ectopic expression of Antennapedia has been shown previously to be sufficient to cause transformation of antenna to leg (Gibson and Gehring, 1988). The observation of ectopic Antp in the distal part of the third antennal segment (within the Dan domain) may explain the homeotic transformation of distal A3 and arista in the \textit{ss} mutant. We next asked whether \textit{ss} was sufficient to induce Dan and Danr expression in the leg disc. Ectopic expression of \textit{ss} in randomly positioned clones of cells caused expression of Dan and Danr in the wing and leg discs (Fig. 5C and not shown). These observations suggest that \textit{ss} defines the domain of Dan and Danr expression.

Next, we examined the relationship between \textit{ss} and \textit{cut}. Ectopic expression of \textit{ss} using \textit{ptc}Gal4 caused ectopic expression of Dan and repression of Cut expression (Fig. 5D). Repression of Cut was stronger on one side of the disc (arrow versus arrowhead) and was associated with antenna duplication. Dan was repressed in the region indicated by the arrow in multiple optical sections, indicating that this is not an artifact of the abnormal folding of the disc. Ectopic expression of Dan or Danr had no effect on Cut expression (not shown), suggesting that \textit{ss} may act directly to regulate Cut. The ability of \textit{ss} to repress Cut, contrasts with the observation by Dong et al. (Dong et al., 2002) that cut expression is normal in \textit{ss} mutants. It

![Fig. 3. Dan and Danr expression. (A) Eye-antenna disc showing Dan protein expression. (B) Dan (green) and Danr (red) expression in the antenna region of an eye-antenna disc. (C) Dan expression in the leg disc. A small group of cells in the location of sense organ precursors are labeled late in development. (D) Dan and Hth (red) expression in the antenna. (E) Dan and Dll (blue) expression in the antenna. (F) Dan and Cut (purple) in the antenna. An optical cross-section across the region indicated by the arrow is shown below. (G) Schematic representation of the expression domains of Hth, Dll, Cut, Dan/Danr and the inferred domain of after (SS) expression with reference to antenna segments.]

![Fig. 4. Regulation of Dan by Hth and Dll. (A) Dan expression in hthc1 mutant clones. (Boxed region) The three channels are shown separately at right. The clone was marked by the absence of GFP (green) and Hth protein (red). (B) Dan expression in DllSA1 mutant clones. The three channels are shown separately on the right of the region indicated by an arrowhead. The clone was marked by the absence of GFP (green) and Dll protein (red). Other Dll mutant clones sorted out towards the edge of the Dan domain (arrows). (C) Ectopic expression of Dan (blue) in the distal leg when Hth was misexpressed (Hth expression was marked by co-expression of GFP. Hth-expressing cells sort out from the distal region by this stage (see Wu and Cohen, 1999). Dan expression shown alone in the inset. (D) Ectopic expression (arrow) of Dan (green) in the proximal leg [overlapping Hth (red)], when Dll was misexpressed (Dll not shown).]
is possible that there are redundant mechanisms for Cut repression, one of which is mediated by ss.

To ask whether Cut regulates Dan and Danr expression we examined clones of cells lacking cut activity, generated using the null allele cut^145 and the FLP-FRT system. cut^145 mutant clones did not cause ectopic expression of Dan in proximal regions of the antenna disc (arrow, Fig. 5E), but did show limited expansion of the Dan domain in the region where Dll is expressed (inset, Fig. 5E). We observed comparable effects on Danr expression in cut mutant clones (not shown). Ectopic expression of Cut in the Dan domain using Act>CD2>Gal4 caused repression of Dan (not shown).

Taken together these results suggest that distal expression of ss limits the expression domain of Cut to the proximal antenna. ss is required for Dan and Danr expression in distal antenna. At present it is not possible to determine whether expression of Dan and Danr in response to ss is mediated directly or indirectly by repression of Cut. However, we favor the view that it is direct because removal of Cut did not cause extensive ectopic expression of Dan.

dan and danr are required for distal antennal identity

ss mutants lead to ectopic expression of Antennapedia and concomitant loss of Dan/Danr expression (Fig. 5) and cause a strong phenotypic transformation of distal A3 and arista to tarsus (Fig. 6A,B). To determine whether morphological transformation depends on loss of Dan/Danr, we made use of Gal4 to direct Dan expression in the ss mutant discs. As shown in Fig. 6C, ptcGal4 directed expression of Dan caused strong suppression of the arista-to-tarsus transformation in the ss mutant antenna. ptcGal4 is expressed in a stripe of cells adjacent to the AP boundary in the antenna region of the disc. Dan expression did not repress ectopic expression of Antp in the ptcGal4 stripe of the mutant discs (Fig. 6D). This suggests that Dan can direct antennal differentiation in the presence of Antp, and overcome the ability of Antp to cause transformation to tarsus. Remarkably, this transformation can affect the entire distal arista, even though ectopic Dan is expressed in only a subset of Antp-expressing cells. These observations suggest that Dan plays an important role in specification of antennal identity.

To assess the roles of dan and danr in antenna development in more detail, deletions that remove one or both genes (Fig. 5).
the distal leg and suggest partial transformation of antenna towards leg in the mutant tissue. A similar transformation of the basal cylinder was observed in mosaic antennae derived from ey-FLP danr\textsuperscript{ex35}/Minute heterozygous animals (Fig. 7E). The transformation associated with dan danr\textsuperscript{ex56} homozygous clones was similar, but slightly stronger (Fig. 7F).

Although many dan danr double mutant excisions were recovered, none was singly mutant for dan alone. To generate a dan mutant we performed a screen for EMS-induced revertants of the dan gain-of-function phenotype in the wing. One allele was recovered. Sequence analysis revealed a change of amino acid residue 45 from glutamate to lysine. This alteration lies in the conserved pipsqueak domain and affects a residue thought to be important for DNA binding of a related protein (Fig. 2C). When expressed in the leg disc under control of Dll\textsuperscript{Gal4}, dan\textsuperscript{ems3} caused loss of the claws, but did not cause transformation to arista, suggesting a weaker gain of function phenotype than the wild-type protein (not shown). Thus, dan\textsuperscript{ems3} appears to be a hypomorphic allele that reduces but does not eliminate Dan activity. dan\textsuperscript{ems3} homozygotes were viable and showed a mild antenna defect, including an occasional ectopic bristle in the third antennal segment (Fig. 8A). A stronger ectopic bristle phenotype was obtained when Dan activity was reduced using a Gal4 inducible construct that directs expression of a double-stranded dan RNA (Fig. 8B; Dll\textsuperscript{Gal4}; sympUAST-dan). To verify that the inducible RNAi caused reduction of Dan protein levels, sympUAST-dan was expressed in the antenna disc using dpp\textsuperscript{Gal4}. Dan protein levels were reduced in the RNAi-expressing cells, but Dan levels were unaffected (Fig. 8C).

Together, these observations suggest that both Dan and Danr contribute to specification of antennal identity. danr single mutants produce a partial transformation of arista to tarsus. A similar, but slightly stronger phenotype results when both dan and danr are deleted. Reduced dan activity in the dan\textsuperscript{ems3} mutants or reduced Dan expression caused by RNA interference produced a milder version of the same phenotype.

An additional line of evidence to indicate that both genes contribute to distal antenna identity comes from examining genetic interactions with spineless\textsuperscript{aristapedia}. As noted above, ss\textsuperscript{a} mutants lost Dan/Danr expression and expressed Antennapedia ectopically in the antenna disc (Fig. 5D,F). Restoring Dan expression was able to partially suppress the transformation to antenna, implicating Dan as an effector of ss\textsuperscript{a} function. We therefore examined the consequences of removing one copy each of Dan and Danr in a ss mutant background. The spineless\textsuperscript{114.4} allele shows a mild transformation of the basal capsule of the arista when heterozygous, suggesting that the reduced level of ss activity in this allele is not sufficient to support normal development (Fig. 9A). Removing one copy of danr (using the danr\textsuperscript{ex35} deletion in this background caused a modest increase in the size of the basal capsule and in the number of ectopic bristles (Fig. 9B). The dan danr\textsuperscript{ex56} deletion caused a stronger phenotype, with the basal capsule adopting a two-segmented appearance and the tarsal spineless\textsuperscript{1177} structure with multiple bracted bristles and obvious tarsal morphology (Fig. 9C). Flies heterozygous for the dan danr\textsuperscript{ex56} deletion are morphologically normal. Thus, reduction of both Dan and Danr gene dose led to a more severe phenotype under conditions where ss activity was limiting. Even more extreme arista transformation phenotypes were observed when one

**Fig. 7.** dan danr mutant phenotypes. (A) Wild-type antenna. (B,C) Antennae from danr\textsuperscript{ex35} and dan danr\textsuperscript{ex56} homozygous mutants. Arrowheads indicate reduced third antennal segments with ectopic bristles. Arrow indicates ectopic bristles on the enlarged basal capsule of the arista. (D) Wild-type arista: basal capsule. (E) Basal capsule of the arista from an ey-FLP; FRT82B danr\textsuperscript{ex35}/FRT82B M\textsuperscript{+} arm\textsuperscript{bic} animal. Note the two-segmented appearance and the bracted bristles (arrow). (F) Basal capsule from an ey-FLP; FRT82B dan danr\textsuperscript{ex56}/FRT82B M arm\textsuperscript{bic} animal. Note the clear segmentation of the basal capsule and the bracted bristles (arrow). (G,H) Antenna region of eye-antenna discs carrying danr\textsuperscript{ex35} or dan danr\textsuperscript{ex56} homozygous mutant clones (arrows). Clones were labeled by the absence of \(\beta\)-gal protein (blue). Danr protein is shown in red; Dan protein in green. (G) Note upregulation of Dan in the Danr mutant clone. (H) Dan and Danr were both absent.
copy of ss was removed in animals homozygous for the dan

danr ex56 deletion (Fig. 9G).

We also observed genetic interactions with Dll. Double
heterozygotes for danr and Dll or dan danr and Dll showed
ectopic tarsal bristles in the basal capsule of the arista (Fig.
9D-F; in this case the phenotypes were similar in strength). We
note that the ss/dan danr double heterozygotes produced a
more complete transformation phenotype than the dan danr
homozygous mutants. This raises the possibility that there may
be additional genes acting downstream from ss to specify
antennal identity.

DISCUSSION

Non-autonomous effects of Hth and Dan/Danr in
distal antenna

Insect antennae develop in the absence of input from HOM-C
genes (Beeman, 1987; Stuart et al., 1991). In the anterior head of
Drosophila where Antennapedia-complex and Bithorax
complex genes are not expressed, expression of hth and Dll
overlaps and promotes antenna development (Casares and
Mann, 1998; Dong et al., 2000; Dong et al., 2002). One
consequence of overlapping expression of Dll and Hth is sustained expression of ss in
the distal antenna. ss is expressed in the leg and antenna discs in second instar, but its
expression is not maintained in the leg (Duncan et al., 1998). ss is expressed within
the Dll domain in distal antenna, but does not overlap Hth in the arista (Fig. 10).

Loss of Hth activity has been shown to
cause transformation of arista to tarsus
(Casares and Mann, 1998), presumably
because of loss of ss. It has been suggested
that uniform expression of Hth in second
and early third instar antennae might be
responsible for its role in specification of
distal antenna identity. However, our results
indicate that Hth can have a non-
autonomous effect on the expression of Dan
in the antenna. As described previously, Hth-expressing cells
sort out from the distal part of the leg (Wu and Cohen, 1999).
Nonetheless they were able to induce Dan expression in cells
that remained integrated in the distal leg. This observation is
best explained by a non-autonomous induction of Dan in
response to a signal from Hth-expressing cells. Responsiveness
to this signal apparently requires Dll, which limits it to the
distal region. These effects are presumably mediated by
regulation of ss, which is required for Dan and Danr
expression. These observations provide an explanation for the
apparently non-autonomous role of Hth together with Dll in
the distal antenna.

ss is also required to induce Dan and Danr and to repress
Antp expression. Repression of Antennapedia may be
mediated in part by repression of Cut (Johnston et al., 1998).
Our findings implicate Dan and Danr as downstream effectors
of ss that promote development of distal antennal structures.
Remarkably, we find that expression of Dan or Danr under
Gal4 control can restore antenna development and prevent
transformation of antenna to leg in the ss mutant, even when
Antp is present. A striking feature of these results is that there
appears to be non-autonomous activity. Transformation was
blocked in cells expressing Dan and Danr, as well as in

Fig. 8. dan mutant phenotype. (A) Antenna from a danr ems3 homozygous mutant. Note the
single ectopic bristle in the third segment (arrow). (B) Antenna from a fly expressing dan
double stranded RNA under DllGal4 control. Arrows indicate ectopic bristles. (C) Eye-
antenna disc from a larva expressing dan double stranded RNA under dppGal4 control
(green). Dan protein (red) was reduced. Danr protein (blue) was unaffected. Dan and Danr
expression in the boxed area is shown separately on the right.

Fig. 9. Genetic interaction between
ss, Dll, dan and danr. (A) Basal
capsule of spineless114.4
heterozygous arista shows a mild
defect (compare with Fig. 7D).
(B) spineless114.4/danex53 double
heterozygote. (C) spineless114.4/dan
danr ex56 double heterozygote. Note
the two-segmented appearance and
bracted bristles. (D) DllSA1/+ heterozygote. The basal capsule
appears nearly normal. (E) DllSA1/+ dan
danr ex57/+ double heterozygote.
(F) DllSA1/+ dan danr ex56/+ double
heterozygote. Stout ectopic bristles
were formed on the basal capsule.
(G) spineless114.4 dan danr ex57/dan
danr ex56 antenna. Note the more
extensive transformation of arista to
tarsus.
Fig. 10. Genetic regulatory hierarchy in the antenna. Summary of the regulatory relationships controlling antenna identity. *spineless* plays a central role in specification of distal antenna by repression of Antp and induction of Dan and Danr expression. Dan and Danr can direct distal antenna development when misexpressed in distal leg, and can override the effects of ectopic Antp in the antenna.

nearby cells that did not express these proteins. The identity of the genes responsible for these non-autonomous effects in antenna specification remains to be determined. In view of recent reports of non-autonomous effects of vein/EGFR signaling in development of distal leg pattern (Campbell, 2002; Galindo et al., 2002), it will be of interest to learn if there is a link to this pathway in the non-autonomous effects of Dan and Danr.

Dan/Danr pipsqueak motif

What are the molecular functions of Dan and Danr? The ‘pipsqueak motifs’ found near the N terminus of Dan and Danr are most closely related to those in the ‘transposase group’ of pipsqueak motif proteins, which includes the Pogo transposase and human centromere protein B (CENP-B) (Siegmund and Lehmann, 2002). The pipsqueak motifs of both Pogo and CENP-B are DNA-binding domains. NMR-spectroscopy of the CENP-B pipsqueak motif demonstrates that it has a helix-turn-helix structure (Iwahara et al., 1998; Wang et al., 1999). Interestingly, the glutamate residue that has been transformed to a lysine in the pipsqueak motif of the *dan* 

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