Control of distal antennal identity and tarsal development in *Drosophila* by *spineless–aristapedia*, a homolog of the mammalian dioxin receptor

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We report the molecular characterization of the *spineless* (*ss*) gene of *Drosophila*, and present evidence that it plays a central role in defining the distal regions of both the antenna and leg. *ss* encodes the closest known homolog of the mammalian dioxin receptor, a transcription factor of the bHLH–PAS family. Loss-of-function alleles of *ss* cause three major phenotypes: transformation of distal antenna to leg, deletion of distal leg (tarsal) structures, and reduction in size of most bristles. Consistent with these phenotypes, *ss* is expressed in the distal portion of the antennal imaginal disc, the tarsal region of each leg disc, and in bristle precursor cells. Ectopic expression of *ss* causes transformation of the maxillary palp and distal leg to distal antenna, and induces formation of an ectopic antenna in the rostral membrane. These effects indicate that *ss* plays a primary role in specifying distal antennal identity. In the tarsus, *ss* is expressed only early, and is required for later expression of the tarsal gene *bric à brac* (*bab*). Ectopic expression causes the deletion of medial leg structures, suggesting that *ss* plays an instructive role in the establishment of the tarsal primordium. In both the antenna and leg, *ss* expression is shown to depend on *Distal-less* (*Dll*), a master regulator of ventral appendage formation. The antennal transformation and tarsal deletions caused by *ss* loss-of-function mutations are probably atavistic, suggesting that *ss* played a central role in the evolution of distal structures in arthropod limbs.

[*Key Words:* Homeotic gene; dioxin receptor; aryl hydrocarbon receptor; PAS domain; antennal specification; leg development; *spineless-aristapedia*]

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The identities of body segments in the trunk and posterior head of *Drosophila* are controlled by the homeotic genes of the Antennapedia (ANT-C) and bithorax (BX-C) complexes (for reviews, see Lewis 1978; Duncan 1987; Kaufman et al. 1990). Although the ANT-C and BX-C genes specify most aspects of body segment specialization, at least three appendage structures develop independently of these genes. First, Carroll et al. (1995) have shown that wing formation in the second thoracic segment (T2) requires no input from the ANT-C and BX-C genes. Instead, these genes function to repress wing development in all segments but T2. Second, the antenna receives no input from the ANT-C and BX-C genes, as these are not expressed within the antennal segment (Kaufman et al. 1990; Jürgens and Hartenstein 1993). Third, formation of the distal part (the tarsus) of the T2 leg appears not to require ANT-C or BX-C genes, as normal second leg tarsi are produced by clones homozygous for null alleles of *Scr, Antp,* or *Ubx* (Struhl 1981, 1982b; Abbott and Kaufman 1986), the ANT-C and BX-C genes that specify segment identities in the thorax. These genes do, however, play an important role in more proximal regions of the second leg, and in both proximal and distal regions of the first and third legs (Struhl 1982b).

What genes are responsible for specifying development of the wing, antenna, and T2 tarsus? For the wing, a key gene appears to be *vestigial* (*vg*). *vg* encodes a novel nuclear protein that is expressed in the pouch region of the wing imaginal disc (Williams et al. 1991). Ectopic expression of *vg* causes the development of wing tissue in other discs, indicating that *vg* is a primary determinant of wing development (Kim et al. 1996). However, no genes have been identified that play similar roles for the antenna or T2 tarsus. In this report, we present evidence that the *spineless* (*ss*) gene plays a key role in specifying the development of both structures. Loss-of-function alleles of *ss* cause transformation of the distal part of the antenna (arista) to distal second leg, and deletion of most of the tarsal region of each of the legs (Lindsley and Zimm 1992). In addition, these alleles cause a severe reduction in size of most bristles. Almost all *ss* alleles

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cause the antennal homeotic transformation, and are known as *spineless–aristapedia* (*ss*^{a}) alleles. Most alleles affect bristle size to some degree, but only very strong alleles also cause tarsal deletions (Struhl 1982a). Although *ss* has been known for many years, there has been little interest in the gene because its pleiotropy has been taken to suggest that it plays some general, perhaps metabolic, role (see, e.g., Raff and Kaufman 1983).

In this report we describe the molecular characterization of *ss*. The results are not consistent with a general metabolic function. We find that *ss* encodes the closest known homolog of the mammalian dioxin receptor, a bHLH–PAS domain transcription factor. *ss* is not expressed uniformly, but shows distinct expression patterns that correspond to its three major functions. *ss* transcripts accumulate in the distal portion of the antennal disc, in the tarsal regions of the leg discs, and in bristle precursor cells. *ss* is also expressed in the embryo in the antennal segment, the thoracic imaginal primordia, and the peripheral nervous system. Ectopic expression of *ss* causes transformation of the maxillary palp and distal leg to distal antenna, and induces development of an ectopic antenna in the rostral membrane, which normally has no appendage. These effects argue that *ss* is a primary determinant of antennal identity. In the tarsus, *ss* is expressed only early in development and is required for later expression of the tarsal gene *bric à brac* (*bab*) (Godt et al. 1993). This suggests that *ss* functions in the establishment of the tarsal primordium. Ectopic expression of *ss* causes the deletion of medial leg structures, perhaps because these are incorrectly specified as tarsus. *ss* expression in both the antenna and leg is shown to be dependent upon *Distal-less* (*Dll*), a master regulator of ventral appendage development (Cohen and Jürgens 1989; Gorfinkiel et al. 1997). We argue that *ss* has played an important role in the evolution of both the antenna and the distal leg.

Results

Cloning of the ss *gene*

The *ss* locus was tagged by a P-element insertion. The insertion allele, now lost, caused a weak *ss*^{a} phenotype when homozygous, carried a P-element at the *ss* locus (polytene bands 89C1,2; Lewis 1963), and was revertable to *ss*$^+$ by mobilization of the P element. Following isolation of P-element containing clones from the locus, phage clones covering ~30 kb to either side of the P-element insertion were recovered by chromosome walking. A map of the cloned DNA is presented in Figure 1A.

To clarify the genetic organization of *ss*, a screen for new X-ray-induced alleles was conducted. The 25 mutants recovered are summarized in Table 1. Eleven of these, as well as six pre-existing rearrangements, were

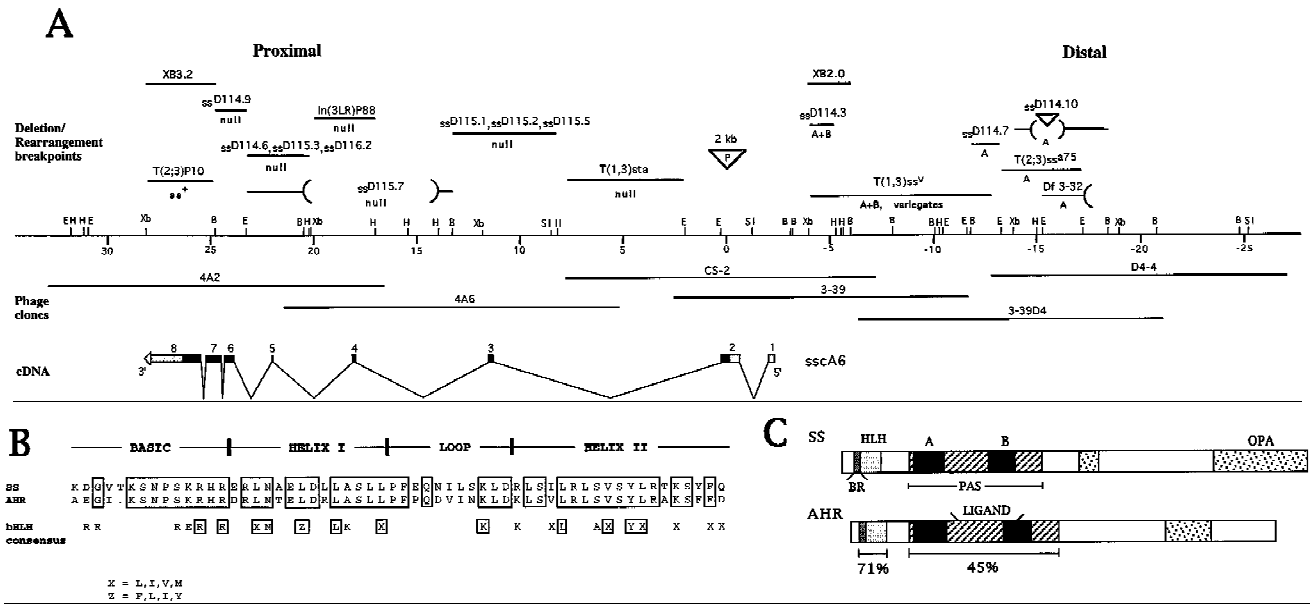

Figure 1. Molecular analysis of the *ss* locus. (*A*) Map of the cloned DNA. Coordinates are in kb; position 0 is the insertion site of the P-element tag used to clone the locus. (B) *Bam*HI; (E) *Eco*RI; (H) *Hin*dIII; (Sl) *Sal*I; (Xb) *Xba*I. Overlapping phage clones are indicated below the restriction map; phage 3-39 is the P-element containing clone that initiated the walk. The positions of 17 breakpoint mutants are noted *above* the map; breakpoints and their uncertainties are indicated by lines, deletions by parentheses, and insertions by inverted triangles. The phenotypes of these mutants are noted as null, antennal transformations (A) or antennal transformations coupled with bristle defects (A+B). XB3.2 and XB2.0 indicate genomic fragments that detect transcripts when used to probe Northern blots. Coding sequences and untranslated regions within the exons of the sscA6 cDNA are indicated by black and shaded boxes, respectively. (*B*) Amino acid identity of ss to AHR in the bHLH domain. Boxed amino acids are identical between the two proteins, and identities of both ss and AHR to the bHLH consensus is noted below. (*C*) Structure of the ss and AHR proteins drawn to scale. Motifs are noted on the ss protein schematic. (BR) Basic region; (HLH) helix–loop–helix; (A, B, PAS) A and B repeats of the PAS domain; (OPA) region of opa or CAX repeats. The position of the ligand binding domain of AHR is also noted.

localized by Southern blotting and in situ hybridization to mutant polytene chromosomes. A proximal limit to the locus at +25 to +28 kb (0 being the site of P-element insertion) is provided by the breakpoint of *Tp(3;2)P10*, which does not affect *ss* function. All ten of the null mutations mapped are broken within the region from about +28 through +2. As described below, these alleles all interrupt the *ss* transcribed region. Six of the alleles mapped lie in the upstream region from −4 to −18. These mutants all cause strong homeotic transformation of the distal antenna (arista) to tarsus, but do not cause distal deletions in the legs or transformed antennae. Effects on bristle size vary with position; the most proximal of the mutants in the upstream group ($ss^{D114.3}$) causes a strong reduction in bristle size, the *T(1;3)*ss^{V} allele variegates for bristle size, and the four more distal mutants in this group cause a weak reduction in bristle size. The distal limit of *ss* is not known.

Northern blotting revealed two transcribed regions distal to the *Tp(3;2)P10* breakpoint. A probe from the more proximal of these (from +25 to +28 kb) detects a smear of RNAs ranging from 0.7 to 6.5 kb that has several embedded bands, the largest of which is 5.4 kb (not shown). The second transcribed region lies more distally, at −4 to −6 kb. This region hybridizes to a major transcript of 1.2 kb and minor transcripts of 2.2 and 3.3 kb. These are detected from the second larval instar through adulthood (not shown).

To assess which transcripts from *ss* are most important functionally, genomic regions were used as probes for in situ hybridization to transcripts in imaginal discs. The proximal region of *ss* gave specific signal in the three regions expected for *ss:* the antenna, legs, and bristle precursor cells. This, and other evidence described below, indicates that transcripts from this region are the major functional products of *ss*. In contrast, the distal transcribed region gave no specific signal in imaginal discs or embryos, and cDNAs from this region do not contain appreciable ORFs (data not shown). The significance of these transcripts is unknown. They are transcribed from a probable regulatory region of *ss* (see below), and may therefore be similar to the *bxd* and *iab* region transcripts of the bithorax complex (Lipshitz et al. 1987; Cumberledge et al. 1990).

Isolation of ss *cDNAs*

To isolate cDNAs for the proximal transcribed region of

Table 1. *New X-ray-induced* ss *alleles*

Allele	Cytology	Hemizygous phenotype[a]	Breakpoints[b]
$ss^{D114.1}$	Df(3R)89A-B;89E present	—	—
$ss^{D114.2}$	normal	null	N.D.
$ss^{D114.3}$	T(1;3)6C;89C1-2	A, B	–4/–5.5
$ss^{D114.4}$	Df(3R)89B;89C	—	—
$ss^{D114.5}$	T(2,3)55C;80AB, normal at 89C	moderate A, moderate B, variable L	N.D.
$ss^{D114.6}$	Tp(3;het);89C;90B	null	+21/+23
$ss^{D114.7}$	89C/heterochromatin	moderate A, moderate B	–12/–13
$ss^{D114.8}$	normal	null	N.D.
$ss^{D114.9}$	In(3R)89B9-14;C1-2	null	+24/+25
$ss^{D114.10}$	normal	moderate A	–14/–17, internal deletion of at least 1 kb + insertion
$ss^{D114.12}$	Complex T(Y;3). Probable new order: Y/84D-89CD/99F-90A/84D-61 + Y/90A-89CD/100A-100F	A, moderate B	N.D.
$ss^{D115.1}$	In(3R)88CD;89C1-2	null	+8/+13.5
$ss^{D115.2}$	normal	null	+8/+13.5
$ss^{D115.3}$	normal	null	+21/+23
$ss^{D115.5}$	T(2;3)43A;89C1-2	null	+8/+13.5
$ss^{D115.6}$	normal	null	N.D.
$ss^{D115.7}$	normal	null	internal deletion; proximal break at +21/+23, distal break at +13/+14
$ss^{D115.8}$	Df(3R)89B18-22;89D3-5	—	—
$ss^{D115.12}$	Tp(3;3)71A;89E;90B + Df(3R)89B20-22;89E	—	—
$ss^{D116.1}$	In(3R)81;89C1,2	null	N.D.
$ss^{D116.2}$	T(Y;3). New order: Y/89C-84E/89C-100 + 61-84E/Y	null	+21/+23
$ss^{D116.4}$	normal	A, B	N.D.
$ss^{D116.5}$	normal	A, B	N.D.
$ss^{D116.7}$	normal	null	N.D.
$ss^{D116.8}$	In(3R)81;99E. 89C normal	null	N.D.

[a]Phenotype when heterozygous with *Df(3R)*$ss^{D114.4}$. (A) Antennal transformation; (B) reduction in bristle size; (L) tarsal deletions.
[b]As determined by Southern blotting. Coordinates indicate altered restriction fragments. (N.D.) No changes detected.

ss, a genomic fragment from +25 to +28 was used to screen an eye–antennal imaginal disc library (kindly provided by Dr. G. Rubin, University of California, Berkeley). Approximately 1.5×10^6 plaques were screened, and 10 distinct cDNAs obtained. The largest of these, which is 5.2 kb in length, was chosen for sequence analysis. This cDNA, designated sscA6, is probably near full length, as it is only 200 bp shorter than the largest band seen on Northerns. Exons in this cDNA are spread over a 30-kb genomic region that encompasses all the *ss* null breakpoints mapped, indicating that it represents a functional transcript of the locus.

The ss *protein*

Sequencing of *ss*cA6 revealed a single ORF that encodes a protein of 884 amino acids (Fig. 2). This ORF has good *Drosophila* codon bias throughout. Although the sequence surrounding the putative initiating methionine does not match the *Drosophila* consensus well (Cavener 1987), homology considerations (see below) suggest this codon is the initiator. The 3′ portion of the ORF is rich in opa (CAX) repeats, which encode primarily glutamine or histidine. These repeats extend beyond the ORF for an additional 600–700 nucleotides. Curiously, the 3′ end of *ss*cA6 lies to the left of the *Tp(3;2)P10* breakpoint, which is ss^+, implying either that the 3′ end of the *ss* transcribed region is dispensable, or that sequences able to substitute for the *ss* 3′ end are introduced by this rearrangement. As indicated in Figure 2, an additional cDNA of 2.8 kb (sscA5) was sequenced. The protein it encodes is three residues shorter than that encoded by sscA6. This difference likely results from alternate splicing.

The putative *ss* protein shows extensive similarity to the human and murine aryl hydrocarbon receptor (AHR, also known as the dioxin receptor), as well as lower similarity to the human aryl hydrocarbon nuclear translocator, hypoxia-inducible factor 1, and endothelial PAS-1 (Tian et al. 1997) and the *Drosophila* proteins single-minded (sim), period, and trachealess (trh) (for review, see Schmidt and Bradfield 1996). These proteins comprise a recently recognized family distinguished by the PAS domain, which can mediate protein–protein interactions (Huang et al. 1993). Most members of the family also contain a bHLH domain. *ss* is most similar to AHR; as can be seen in Figure 1, there is 71% identity between the two proteins in the bHLH region, 45% identity in the PAS domain, and 41% identity overall. The organization of domains within the two proteins is also closely similar. *ss* and AHR share almost twice as much sequence identity as either shares with their next closest relative, sim.

Sequencing of exons reveals that all five of the *ss* splice sites within the bHLH and PAS coding sequences are conserved in *Ahr* (Fig. 2). These splice sites lie within conserved codons in the two genes, and separate precisely the same nucleotide positions within these codons (Schmidt et al. 1993). *Ahr* has three additional splice sites in the PAS domain coding sequence that are not shared with *ss*. Three of the five splice junctions of *ss* are also shared with *sim*.

Transcript distributions in imaginal discs and the ss *mutant phenotype*

In situ hybridization of *ss* probes to imaginal discs reveals distinct phases of transcript accumulation that correspond to the leg, antennal, and bristle functions of *ss*.

The leg In leg discs, *ss* staining is first seen in the late second instar in a central ring that corresponds to the presumptive tarsal region (Fig. 3E) (all references here to imaginal fate maps are from Bryant 1978). This ring is transient, and fades out by the late third instar. The *ss* tarsal ring likely corresponds to the tarsal structures deleted in ss^- mutants, which include the distal part of the first tarsal segment and the second through fourth tarsal segments (Fig. 4E). After the tarsal ring fades out, *ss* becomes expressed in a patch in the anterior-proximal portion of the disc in a region that will give rise to structures of the thorax proper (Fig. 3F). *ss* null alleles show no defects in these structures.

The antenna *ss* staining in the antennal disc is first detected during the late second instar (not shown). At all times after this, intense staining is seen in an oval patch in the central (distal) portion of the antennal disc (Fig. 3A,B). After disc eversion, the limits of intense *ss* expression coincide precisely with the boundary between the second (AII) and third (AIII) antennal segments (Fig. 3D).

ss alleles broken in the upstream region of the gene have no effect on *ss* expression in the leg, but alter expression in the antenna to resemble that normally seen in the leg. The effects of a translocation broken at −4 kb to −5 kb ($ss^{D114.3}$) are shown in Figure 3C. *ss* is expressed in a transient ring in the antennal and leg discs in this mutant. This indicates that the antennal expression pattern of *ss* is controlled by the region upstream of −4 kb to −5 kb, whereas the tarsal pattern of expression is likely controlled by a downstream, perhaps intronic, region. Consistent with their effects on *ss* expression, mutants located in the upstream region cause transformation of the distal antenna to tarsus (Fig. 4B). However, only the distal part of AIII and the arista are affected. Detailed examination of one upstream mutant ($ss^{D114.7}$) indicates that the antennal tarsus produced has second leg identity (see legend to Fig. 4), as suggested previously for the ss^a allele (Morata and Lawrence 1979).

In *ss* null mutants, the antenna shows both transformation to leg and tarsal deletion (Fig. 4C). In this case, the entire AIII segment and arista are affected. Strikingly, the AIII segment in *ss* null mutants is unlike any normal appendage segment in that it completely lacks bristles or cuticular hairs. This segment is followed distally by most or all of a fifth tarsal segment terminated by claws.

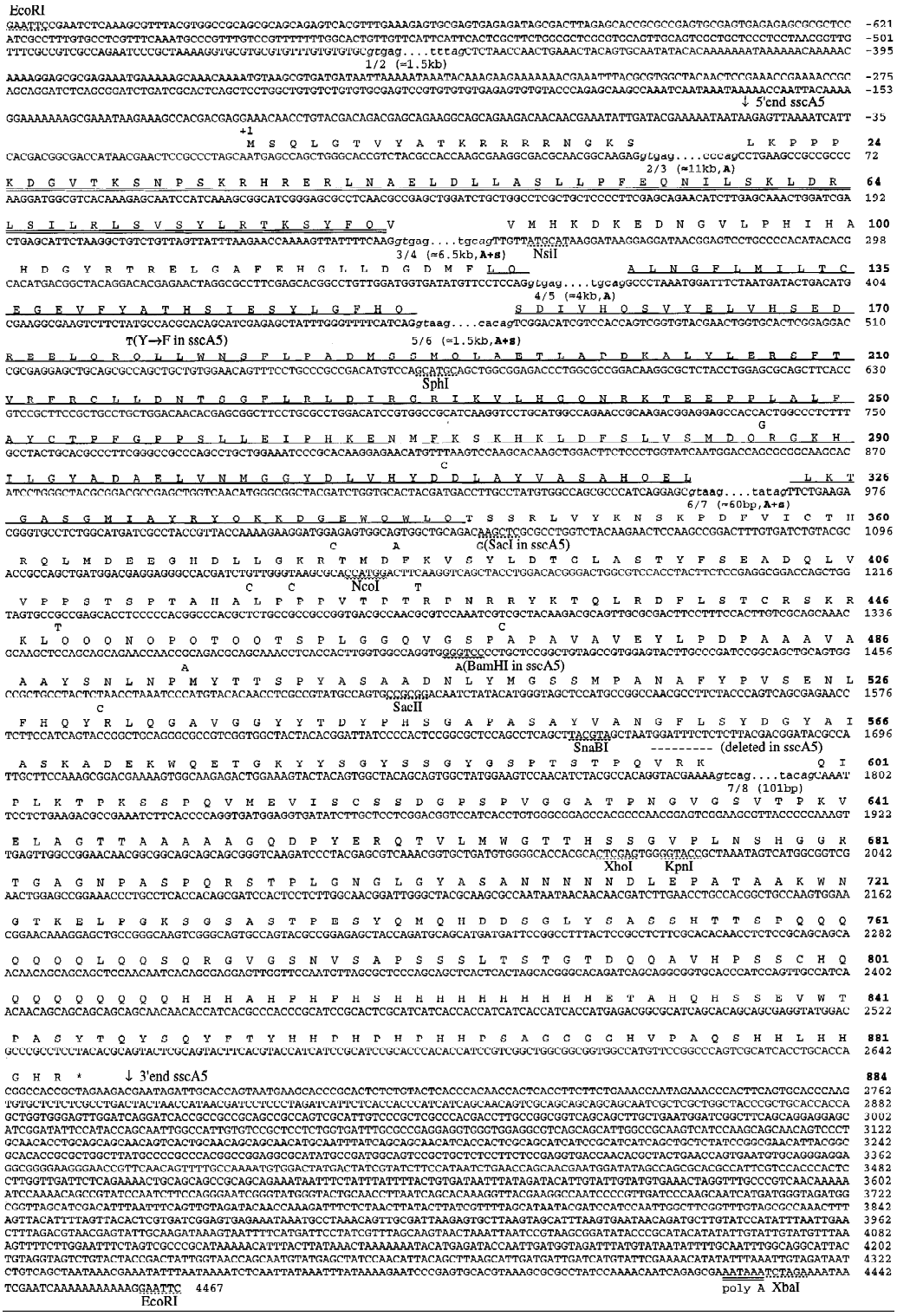

Figure 2. Sequence of the *ss* cDNAs. The sequence and conceptual translation of the *ss* cDNA sscA6 is shown, along with the polymorphisms associated with the sscA5 cDNA. The nucleotide sequence is numbered by plain text at the end of each row; the corresponding amino acid sequence numbering is depicted in boldface type. Downward-pointing arrows indicate the ends of the sscA5 cDNA; broken lines above nucleotides +1789 through +1797 indicate the three codons absent in the sscA5 cDNA. The bHLH region is double underlined; the PAS domain is single underlined. The translation stop is noted by an asterisk. The positions of the seven exon splice junctions are in lowercase text; the donor and splice acceptors are italicized; the three flanking intronic nucleotides are in plain text. The approximate length of each intron is in parentheses, and those splice sites conserved in *Ahr* or *sim* are noted by a bold A and s, respectively.

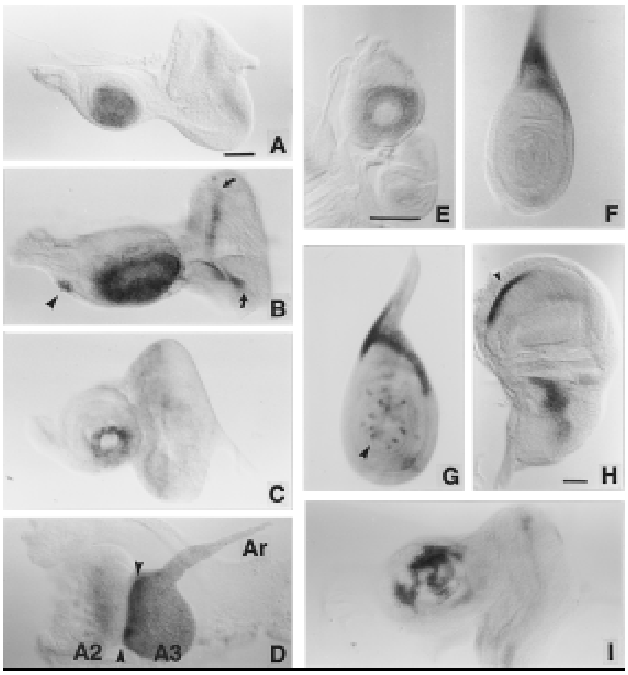

Figure 3. *ss* expression in imaginal discs. Imaginal discs from third instar larvae and pupae were hybridized with RNA probes transcribed from the XB3.2 genomic clone or the sscA6 cDNA. (*A*,*B*) Eye–antennal discs from early and late wild-type third instar larvae. In *B* the arrowhead indicates the maxillary palp anlage and the arrows indicate the morphogenetic furrow in the eye disc. (*C*) *ss* transcript accumulation in ss^{a} mutant ($ss^{D114.3}$/$ss^{D114.4}$) third instar eye–antennal discs. The pattern resembles that in the wild-type early third instar leg (cf. *E*). (*D*) *ss* expression in an everted antennal disc. Arrowheads indicate the boundary between the second (A2) and third (A3) segments. (Ar) The arista. (*E*– *G*) *ss* expression pattern in early *E* and late *F* third instar leg discs, and in a leg disc just prior to eversion (*G*). The arrowhead in *G* indicates bristle precursor cell labeling. (*H*) *ss* expression in a mature third instar wing disc. Expression is seen in an anteroventral stripe (arrowhead) and a patch in the presumptive notal region. (*I*) *ss* antennal expression in a heterozygote for the *Antp* gain-of-function allele $Antp^{73b}$. Variegated reduction in *ss* expression is seen. Scale bars, 50 µm; the bar in *A* refers to *A*– *D* and *I;* the bar in *E* refers to *E, F,* and *G*.

Other adult structures In the late third instar, *ss* becomes expressed in a small patch in the antennal disc that corresponds to the maxillary palp anlage (Fig. 3B). Consistent with this, the maxillary palps of null mutants are truncated (Fig. 4F,G). This suggests a general requirement for *ss* in the development of distal structures in ventral appendages.

In the wing disc, *ss* is expressed in the presumptive notum and wing hinge region, and in a ventral stripe (Fig. 3H). Perhaps related to this expression, the wings of null mutants are held perpendicular to the body and curve ventrally. The haltere disc stains in a similar pattern (not shown). Expression is also detected in the genital and labial discs (not shown), and in the morphogenetic furrow of the eye disc (Fig. 3B). Genital, labial, and eye development appear normal in *ss* mutants.

Bristle precursor cells At pupariation and disc eversion, stereotyped patterns of single large intensely stained cells are seen in most discs. This is shown only for the leg (Fig. 3G). The pattern of labeled cells is identical to that shown by the *neuralized* enhancer trap A101 (Huang et al. 1991), indicating that these late *ss* expressing cells are sensory organ precursors. At later stages, intense staining is seen in developing bristle cells, but not in the associated socket cells.

Embryos As shown in Figure 5, *ss* is also expressed in embryos. *ss* staining first appears at stage 8 (all staging as in Campos-Ortega and Hartenstein 1985) in a crescent just anterior to the cephalic furrow. This staining develops rapidly into an intense patch. As germ-band extension continues, staining develops in the maxillary, labial, and mandibular segments, followed by expression in a ventral patch in all three thoracic segments. These patches were identified as the leg anlage by double-labeling for transcripts from *ss* and *aristaless* (Campbell et al. 1993; Schneitz et al. 1993) (not shown). *ss* staining also appears in cells of the peripheral nervous system in each abdominal segment, as determined by double labeling for the A37 enhancer trap line (Ghysen and O'Kane 1989) (not shown). The *ss* staining pattern is maintained through germ-band retraction and continues until the deposition of larval cuticle makes it difficult to follow further.

To position the *ss* head patch, embryos were stained for both *ss* transcript and engrailed (en) protein. As can be seen in Figure 6, the posterior boundary of the *ss* antennal patch coincides precisely with the posterior edge of the antennal en stripe. There is a one-to-one correspondence between *ss*- and en-expressing cells for some distance along this stripe, although the en antennal stripe extends ventrally several cells beyond the *ss* patch. The anterior limit of *ss* expression lies just posterior to the en head spot, which delimits the posterior border of the ocular segment (Schmidt-Ott and Technau 1992). Thus, *ss* is expressed throughout most or all of the embryonic antennal segment, and is expressed in a segmental, not parasegmental, register.

The expression of *ss* in embryos was unexpected, as no embryonic defects have been described for *ss* mutants. However, examination of null mutant larvae revealed that the antennal sense organ is misshapen and often sclerotized (Fig. 4J). In addition, the dorso-medial papilla of the maxillary sense organ, which is thought to be derived from the antennal segment (Jürgens et al. 1986), fails to migrate completely (Fig. 4K). Examination of ss^{-} embryos stained with the monoclonal antibody 22C10 (Zipursky et al. 1984) failed to reveal any defects in the peripheral nervous system.

Ectopic expression of ss

To assess the developmental potential of *ss*, we used the GAL4/UAS system (Brand and Perrimon 1993) to drive ectopic expression of the sscA5 cDNA. Most GAL4 drivers tested proved to be lethal in combination with UAS–

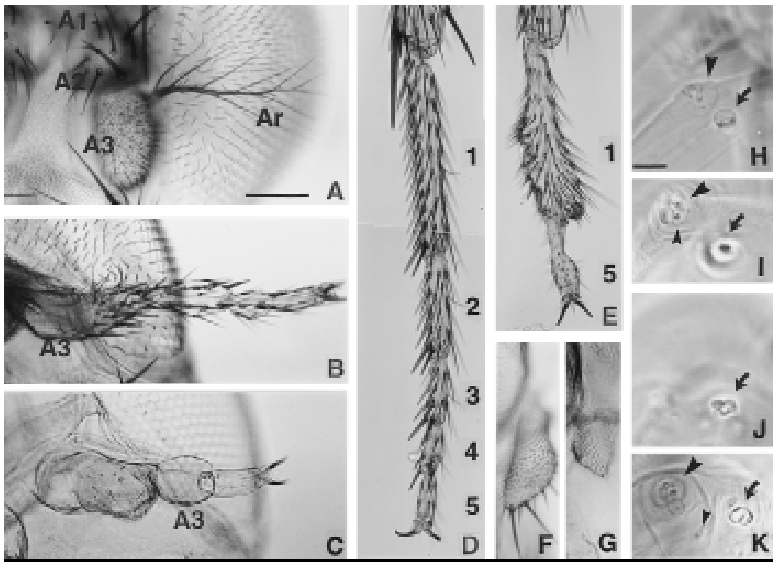

Figure 4. Cuticular phenotypes of *ss* mutants. The antennal and leg phenotypes of *ss* mutants have been described by others [see Lindsley and Zimm (1992) and references therein]. (*A*,*B*) Antennae from wild-type (*A*) and ss^{a} ($ss^{D114.10}/ss^{D114.4}$) (*B*) adults. The first (A1), second (A2), and third (A3) antennal segments and the arista (Ar) are indicated. The antennal tarsus in ss^{a} mutants is judged to have T2 identity by the following criteria: Paired rows of stout bracted macrochaetae present ventrally (Hollingsworth 1964) are like those on the second leg; the most distal bristle pair in each segment is larger than more proximal pairs; on average, there are three pairs of bristles in the fifth tarsal segment (Lawrence et al. 1979); and there are no posterior transverse bristle rows in the second tarsal segment. (*C*) Antenna from a *ss* null mutant ($ss^{D115.7}$). A3 has no bristles or trichomes, and most of the distal tarsal region is absent. (*D–E*) Distal second leg of wild-type and a *ss* null mutant. Tarsal segments 2–4 and part of segment 1 are deleted in *E*. (*F*,*G*) Wild-type and *ss* null mutant maxillary palps. The mutant palps are truncated. (*H– K*) Wild-type (*H*,*I*) and *ss* null mutant (*J*,*K*) first instar antennal and maxillary sense organs (AnSO and MxSO). The AnSO is indicated with a large arrow, the MxSO with a large arrowhead, and the dorsomedial papilla (DMP) with a small arrowhead. In the mutant the AnSO is cauliflower-shaped and has no clear stalk, and migration of the DMP is impaired. Scale bars, *A–G* (shown in *A*), 100 µm; *H–K* (shown in *H*), 10 µm.

ss. Embryos from a cross to the 69B driver (Brand and Perrimon 1993), which is expressed in the embryonic ectoderm and its derivatives (Baylies et al. 1995), were examined in detail. These show a loss of midline structures, as well as tracheal system defects (Fig. 7I,J). These phenotypes are similar to those caused by mutations in the *sim* and *trh* genes, which encode PAS domain proteins. Ectopic *ss* likely interferes with the function of these proteins, perhaps by competition for a common dimerization partner.

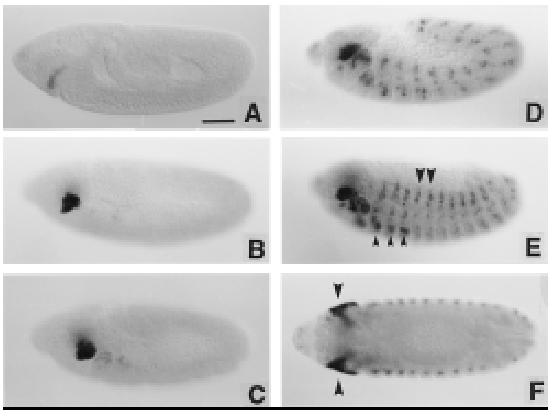

Figure 5. *ss* transcript distribution in embryos. (*A– E*) The pattern of *ss* transcript distribution beginning at stage 8 and continuing through late embryogenesis. Small arrowheads in *E* indicate the leg anlage; large arrowheads indicate expression in the peripheral nervous system. In *F*, an optical section midway through a germ-band retracted embryo shows the extent of expression in the invaginating eye–antennal discs (arrowheads). Scale bar, 50 µm.

A few driver/UAS–*ss* combinations survived to the pharate adult stage, allowing us to determine the effects of ectopic *ss* on adult structures. The results indicate that *ss* is a primary determinant of distal antennal identity. The *ptc*–GAL4 driver (Hinz et al. 1994) has been

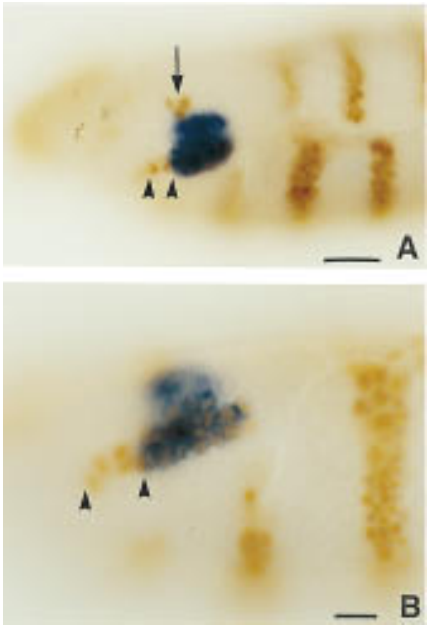

Figure 6. *ss* expression in the antennal segment of the embryo. (*A*,*B*) Wild-type embryos double labeled for *ss* transcript (blue) and en protein (brown). Note that the limits of *ss* expression extend from just ventral to the en head spot (arrow) through the antennal en stripe (arrowheads). The most ventral cells of the latter do not express *ss*. Scale bar in *A*, 20 µm; in *B*, 10 µm.

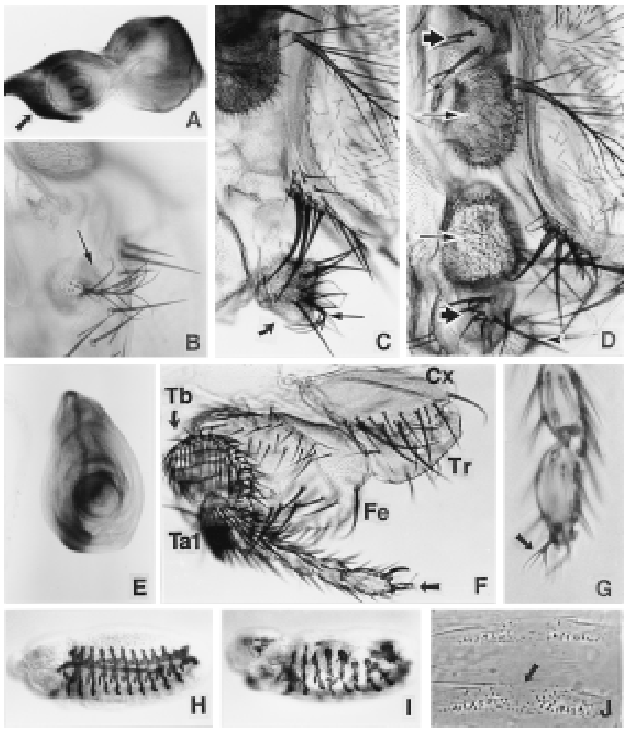

Figure 7. Effects of *ss* ectopic expression. (*A*) Pattern of ectopic expression driven by *ptc*–GAL4 in the eye–antennal disc (β-galactosidase staining). The arrow indicates a zone of high-level ectopic expression that includes the primordia of the palp and rostral membrane. (*B–D*) The effects of ectopic expression of *ss* driven by *ptc*–GAL4 in the head. A partial ectopic antenna (arrow) that has developed in an out pocketing of the rostral membrane is shown in *B,* and partial transformation of the palp (thick arrow) to arista (thin arrow) and AIII is shown in *C*. In *D,* an almost complete ectopic antenna is present in the rostral membrane region. Note that AIII (long arrows) and AII (short arrows) in the normal and ectopic antennae are arranged mirror symmetrically. The palp (arrowhead) is still present. The arista of the ectopic antenna is out of the plane of focus. (*E– G*) The effects of ectopic *ss* in the leg. The pattern of ectopic expression driven in the leg disc by *ptc*–GAL4 is shown in *E.* Deletion of most of the femur (Fe) and tibia (Tb) is shown in *F.* The coxa (Cx), trochanter (Tr), and most of the tarsus (Ta) are unaffected. Aristae are present at the distal tips of the legs shown in *F* and *G* (see arrows). (*H–J*) Effects of ectopic *ss* in embryos. *H* shows a normal embryo stained for the *trh* enhancer trap *1-eve-1* (Wilk et al. 1996). Ectopic *ss* driven by the 69B GAL4 driver causes severe abnormalities in this pattern (*I*). Ectopic *ss* also causes the deletion of midline portions of the denticle belts (arrow in *J*), similar to the effects of *sim* mutants.

studied in most detail. It is expressed in imaginal discs at high level immediately anterior to the compartment boundary. *ss* expression driven by *ptc*–GAL4 causes transformation of the maxillary palp, rostral membrane, and distal leg to antenna. Transformation of the palps varies from essentially no effect to an almost complete transformation to AIII and arista (Fig. 7C). Palps are also often deleted. Surprisingly, ectopic *ss* induces ectopic antennal structures in the rostral membrane between the palp and the normal antenna. These range from small patches of AIII and arista (Fig. 7B) to entire ectopic antennae (Fig. 7D), and are always arranged in mirror symmetry to the normal antenna. Ectopic *ss* expression also causes transformation of the distal leg to arista (Fig. 7F,G). In some cases, aristal-claw intermediates are present, indicating that aristae can arise by transformation of claws. More proximal antennal structures are never present in the leg, and the pulvillus and tarsi are unaffected. Curiously, in most cases the basal cylinder of the arista is suppressed by ectopic *ss.*

The frequency of induction of ectopic antennae in the rostral membrane was 88% (67 half heads scored) in heterozygotes for *ptc*–GAL4 and our standard UAS–*ss* insertion (line A1). In this same genotype, transformations of distal leg to arista occurred at a frequency of 66% (100 legs scored). Because palps are usually absent in this genotype, the frequency of palp transformations was determined in flies carrying a weakly responding UAS–*ss* insertion (line C2). Of 100 palps scored, 27 carried an arista, and 15 showed wavy arista-like bristles.

Ectopic expression of *ss* also causes the deletion of medial leg structures. This occurs in 100% of *ptc*–GAL4; UAS–*ss* legs. Anterior structures from the distal femur and most of the tibia are deleted (Fig. 7F). Much of what appears to be the posterior compartment remains, however, and produces irregular cuticular outgrowths. The distortion these cause makes it difficult to define precisely the limits of the deleted regions. The distal basitarsus is clearly retained, however, because sex combs are present in the first legs of males. More distal tarsal segments are usually unaffected, with the exception of transformation of claw to arista. *ss* expression driven by *ptc*–GAL4 also causes deletion of the central part of the wing, and induces a zone of polarity reversal in the abdominal tergites (not shown). A few scattered bristles are also induced in the wing blade. The significance of these effects is not known.

Position of ss *in the limb development hierarchy*

The *Dll* gene is required for the development of all leg segments distal to the coxa (Cohen and Jürgens 1989; Gorfinkiel et al. 1997). To test whether *ss* lies downstream of *Dll* in limb development, we examined *ss* expression in a weak *Dll* loss-of-function mutant, Dll^{PK}. This allele survives to the pharate adult stage when heterozygous with *Dll* null alleles such as Dll^{B}, and causes the deletion of distal limb structures. We find that *ss* expression is almost completely eliminated in the tarsus, antenna, and maxillary palp of Dll^{PK}/Dll^{B} heterozygotes (not shown). Thus, *ss* lies downstream of *Dll* in all three of these appendages. In the antenna, *ss* expression is reduced in animals that carry only one dose of Dll^{+} (not shown). This presumably accounts for the weak transformation of distal antenna to leg seen in most *Dll* mutant heterozygotes (Sunkel and Whittle 1987).

To monitor *Dll* expression in relation to *ss,* we (in collaboration with Dr. S. Cohen, EMBL, Heidelberg, Germany) isolated a monoclonal antibody against Dll pro-

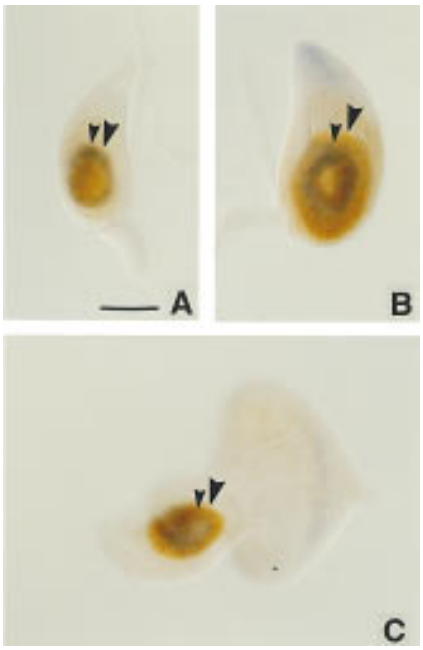

Figure 8. Wild-type imaginal discs double-labeled for *ss* transcript (blue) and *Dll* protein (brown). Small arrowheads indicate the proximal extent of *ss* expression; large arrowheads indicate the proximal extent of *Dll* expression. In early third instar leg discs (*A*), the proximal extents of *ss* and *Dll* expression coincide; later discs show *Dll* expression more proximal than *ss* (*B*). In antennal discs, *Dll* expression is always more proximal than *ss* (*C*). Scale bar, 50 µm.

tein. As shown in Figure 8, *Dll* is expressed uniformly in the central portions of the leg and antennal imaginal discs. In the early third instar, when *ss* is first expressed in the leg, the outer edge of the *ss* tarsal ring coincides precisely with the proximal limit of *Dll* expression. As the leg disc grows, the boundary of *Dll* expression expands beyond the *ss* tarsal ring, so that a proximal zone of cells that express *Dll*, but not *ss*, is created. In the antenna, *Dll* expression extends more proximally than *ss* at all stages we have examined.

The transient early expression of *ss* in the leg suggests that *ss* plays a role in the establishment of the tarsal region. Support for such a role is provided by the finding that *bab* lies downstream of *ss*. In wild type, *bab* expression is initiated in the tarsal region in the mid-third instar, and at disc eversion *bab* expression can be seen to extend from the middle of the first tarsal segment through the fifth segment (Godt et al. 1993). In *ss* null mutants, *bab* expression is abolished in the leg (not shown).

Discussion

Homology to the mammalian dioxin receptor

ss is the closest known relative of the mammalian AHR, also known as the dioxin receptor (for review, see Schmidt and Bradfield 1996). All five splice junctions within the coding region for the ss bHLH and PAS domains are conserved precisely in *Ahr*, indicating that the ss and AHR proteins share common ancestry. The basic regions of the two proteins are identical at 10/13 residues, suggesting they bind similar or identical DNA sequences. AHR functions to activate the transcription of genes that are important in metabolizing polycyclic aromatic hydrocarbons. AHR is normally cytoplasmic, but on binding aryl hydrocarbons, it translocates to the nucleus and forms active heterodimers with another bHLH–PAS protein, the aryl hydrocarbon nuclear translocator. The ligand with the highest affinity known for AHR is dioxin. The similarity of ss to AHR suggests that ss protein might also bind some type of ligand. Despite their close similarity, ss and AHR appear to be unrelated in function. *Ahr* knockouts in the mouse show no obvious developmental defects (Fernandez-Salguero et al. 1995; Schmidt et al. 1996), although, as expected, they are unresponsive to aryl hydrocarbons.

Control of antennal identity by ss

In previous work, we tested a specific model for how *ss* might act indirectly to control distal antennal identity (Burgess and Duncan 1990). Because ectopic expression of the *Antp* gene can cause a distally complete transformation of antenna to second leg (Schneuwly et al. 1987), we suspected that the ss^a antennal transformation might result from the ectopic activation of $Antp^+$. To test this, we examined $Antp^- ss^a$ mitotic recombination clones in the distal antenna. To our surprise, we found these are indistinguishable from $Antp^+ ss^a$ clones, and are transformed to second leg tarsus. This demonstrated that the ss^a antennal transformation is independent of $Antp^+$, and led us to propose that ss^+ controls distal antennal identity directly. The molecular characterization of *ss* provides strong support for this proposal.

In normal development, *ss* is expressed in the primordia of AIII and the arista in the antennal imaginal disc. This corresponds to the portion of the antenna affected in *ss* null mutants. When *ss* is expressed ectopically, we find that it can transform the maxillary palp to AIII and arista, and the distal leg to arista. Ectopic *ss* also induces formation of an ectopic antenna in the rostral membrane ventral to the normal antenna. These effects argue strongly that *ss* is an antennal determinant. However, only certain structures are susceptible to transformation by ectopic *ss*, suggesting that *ss* must interact with other spatially-restricted regulators to promote antennal identity.

Transformations of distal antenna to leg are caused by ectopic expression of a number of different genes, including *Antp*, *Scr*, *Ubx*, *abd-A*, and *Abd-B* (Casares et al. 1996 and references therein) and the mouse genes *HoxA5* (Zhao et al. 1993) and *HoxB6* (Malicki et al. 1990). Transformation of distal antenna to leg is also seen in *Polycomb* heterozygotes (Duncan and Lewis 1982) and in animals having only one dose of *Dll* (Sunkel and Whittle 1987). These effects have led to the view that transfor-

mations of the distal antenna to leg are somehow nonspecific. However, in all of these cases (except ectopic expression of *Abd-B*, which has not yet been tested) we find that distal antennal transformations are correlated with repression of *ss* in the antenna (Fig. 3I; D.M. Duncan and I.W. Duncan, unpubl.). Thus, rather than indicating any lack of specificity for the ss^a transformation, these cases provide strong supporting evidence that *ss* is a primary determinant of distal antennal identity.

The induction of antennae in the rostral membrane by ectopic *ss* was quite unexpected. This region normally produces no appendage and does not express *Dll*, which is thought to be required for ventral appendage formation (Cohen and Jürgens 1989). Moreover, the antennae induced here are often complete, although *ss* is normally expressed only in AIII and the arista. It would appear that ectopic *ss* can initiate an entire antennal limb program in the rostral membrane. Consistent with this, we find that ectopic *ss* induces *Dll* expression in this location (not shown). Ectopic antennae are always oriented in mirror symmetry to the normal antenna, suggesting that they arise from a cryptic head segment that is organized in mirror symmetry to the antennal segment. The existence of such a segment has also been inferred by Gonzáles-Crespo and Morata (1995).

Establishment of the tarsal primordium

Like the antenna, the second leg tarsus appears to develop without input from the ANT-C and BX-C genes. Mitotic recombination clones homozygous for null alleles of the major homeotic genes expressed in the thorax (*Scr, Antp,* and *Ubx*) develop normally in the T2 tarsus (Struhl 1981, 1982b; Abbott and Kaufman 1986), although $Antp^-$ clones in more proximal regions of the T2 leg are transformed to antenna. Struhl (1981) suggested that the lack of effect of $Antp^-$ clones in the T2 tarsus is caused by nonautonomous rescue by nearby wild-type cells in the adjacent compartment. However, our finding that $Antp^-$ ss^a clones in the distal antenna produce apparently normal T2 tarsi (Burgess and Duncan 1990) argues that $Antp^+$ simply plays no role in the second leg tarsus.

The results presented in this report suggest that the T2 tarsus is specified by *ss*. *ss* is first expressed in the tarsal region in the late second instar. Staining increases in this region through the early third instar, and then gradually declines. As far as we are aware, *ss* is the only disc-patterning gene known that shows such transient expression. Consistent with this expression pattern, the temperature-sensitive period of *ss* for tarsal development is in the first half of the third instar (Mglinets 1976). This period probably coincides with when the tarsal primordium is established, as Schubiger (1974) found that when leg discs are forced to undergo premature metamorphosis, the tarsal region first develops competence to differentiate in the early third instar. Significantly, Schubiger found that the tarsal regions differentiated by leg discs of this age are not segmented. Thus, it would appear that tarsal development occurs in two phases: First, a uniform tarsal region is established, and then this region is subdivided into specialized segments. We suggest that *ss* is responsible for the first of these phases, whereas downstream genes are responsible for the second. The timing of maximal *ss* expression, the temperature-sensitive period of *ss*, and the finding that *ss* expression is not segmentally modulated within the tarsal ring are all consistent with this view. Ectopic expression of *ss* in the leg causes the deletion of medial structures. Although it is not clear how these pattern deletions arise, one possibility is that ectopic *ss* specifies medial leg regions as tarsus, causing them to become incorporated into the tarsal primordium.

Our results indicate that *ss* occupies an intermediate position in the leg developmental hierarchy. Tarsal expression of *ss* is abolished in a *Dll* loss-of-function mutant, indicating that *Dll* is an important upstream regulator of *ss*. When *ss* first becomes expressed in the tarsal primordium, its proximal limit of expression coincides precisely with that of *Dll*. This suggests that *Dll* might directly activate *ss* in the leg, thereby defining its proximal limit of expression. *Dll* also appears to be a positive regulator of *ss* in the antenna: *ss* expression here is reduced in animals having only one dose of Dll^+, and is almost eliminated in a *Dll* loss-of-function mutant. In light of these observations, it is surprising that ectopic *ss* induces *Dll* expression in the rostral membrane. Whether this reversal of the expected regulatory relationship between *Dll* and *ss* has any relevance to normal development is not clear, because *Dll* expression appears to be unaffected in *ss* null mutants (not shown). We find that tarsal expression of *bab*, which is required for proper subdivision of the tarsus into segments (Godt et al. 1993), is abolished in *ss* null mutants, indicating that *bab* lies downstream of *ss* in leg development.

What determines whether ss *specifies tarsal or antennal development?*

At least in the antenna, the level of *ss* function appears to determine whether tarsal or distal antennal development is specified. The many *ss* alleles induced by chemical mutagens, which are likely to be mutations in the coding sequence, can be arranged in a phenotypic series in which weak alleles cause only transformations of arista to tarsus, whereas strong alleles also cause tarsal deletions and a loss of AIII identity (Struhl 1982a). These observations suggest that a high level of *ss* function is required to specify aristal identity, whereas lower levels are able to specify tarsus and AIII identity. Consistent with this, we find that the intensity of *ss* staining is much higher in the antennal disc than in the leg disc. The level of function also appears to be critical for the *proboscipedia* (*pb*) gene of the ANT-C: *pb* null alleles cause a transformation of proboscis to tarsus, weak activity of pb^+ causes a transformation to arista, and stronger activity allows normal proboscis development (Cribbs et al. 1995). Whether the tarsal and aristal trans-

formations seen in *pb* mutants result from the activation of different levels of *ss* in the proboscis has not been determined.

It is perhaps surprising that when *ss* expression was driven ectopically in the leg, the tarsal segments were never transformed to antenna, although the distal tip was often transformed to arista. However, we find that the *ptc*–GAL4 driver used does not activate *ss* in the leg nearly as strongly as it does in the antennal disc. Thus, the level of ectopic expression attained in the tarsal region may not have been sufficient to cause a transformation. Alternatively, ectopic *ss* may have no effect in tarsi because of the absence or presence of some critical cofactor in the leg.

The ss$^-$ *antennal appendage may be close to an epigenetic ground state*

The *ss*$^-$ antennal appendage develops in the absence of ANT-C and BX-C, as well as *ss*, gene function, suggesting it is close to a developmental ground state. The prevailing view has been that the limb ground state is T2 leg (e.g., see Struhl 1982b and references therein). However, this has been challenged by the finding that *Antp*$^-$ clones in the proximal second leg are transformed to antenna (Struhl 1981, 1982b; Abbott and Kaufman 1986), and the finding that all trunk body segments develop antennae in Tribolium embryos deficient for the HOM-C (Stuart et al. 1991). A possible resolution was suggested by Struhl (1981, 1982b), who proposed that *Antp*$^+$ functions to preserve the T2 ground state by repressing putative head-determining genes in the second leg. This model predicts that inactivation of these head-determining genes should cause the antenna to develop as second leg. The distal antennal transformation caused by weak *ss*^{a} mutants would seem to conform to this prediction. However, in *ss* null mutants, AIII is not transformed to second leg, but develops as an undecorated segment with no apparent identity. We suggest that this is the fate of an appendage segment that has not been specified by any homeotic gene. As described in Figure 9, we propose that the limb ground state is a four-segmented appendage similar to the *ss*$^-$ antenna, and that *ss*$^+$ and *Antp*$^+$ function to direct the specialization of this appendage to produce the antenna and second leg.

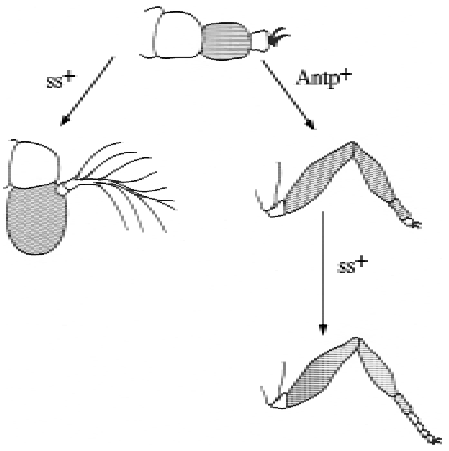

Figure 9. The *ss*$^-$ antennal appendage is close to an epigenetic ground state. The antennal appendage in *ss*$^-$ animals is indicated schematically at the *top*. The third segment of this appendage and its derivatives in the antenna and leg are shaded to indicate homology. As indicated, *ss*$^+$ functions in the antenna to specialize the third and fourth segments of the ground state appendage to produce AIII and arista. In T2, *Antp*$^+$ specifies the identities of the proximal two segments of the ground state appendage as coxa and trochanter, and directs the third segment to expand and subsegment to produce the femur, tibia, and first tarsal segment. These functions of *Antp* are consistent with the phenotype of *Antp*$^-$ clones in the leg (Struhl 1981, 1982b; Abbott and Kaufman 1986), and with the effects of ectopic expression of *Antp* in the antenna (Postlethwait and Schneiderman 1971). In the distal leg, *ss*$^+$ directs the expansion and segmentation of the tarsus. The diagram predicts that the *Antp*$^-$ *ss*$^-$ second leg will look like the *ss*$^-$ antennal appendage. Although this has not yet been tested, the predicted phenotype is consistent with the additive effects of *ss*$^-$ and *Antp*$^-$ single mutants. The proximal two segments of the indicated ground state appendage are normal antennal segments; presumably the identities of these are specified by some other, currently unknown, homeotic gene or genes. Whether the evolutionary ground state of the limb is the same as the developmental ground state is not clear. However, it seems reasonable to suggest that it may be, and that an ancestral four-segmented appendage similar to the *ss*$^-$ antenna became specialized in the head to produce the antenna, and in the thorax to produce legs.

Evolutionary speculations

It is generally considered that the arthropod antenna evolved from a leg-like locomotory appendage (e.g., see Callahan 1979), a view that has received support from work on *Drosophila* homeotic and segmentation genes (Postlethwait and Schneiderman 1971; Cohen and Jürgens 1989; Schmidt-Ott and Technau 1992; this report). It is also generally accepted that unsegmented tarsi are ancestral in the hexapods (Boudreaux 1987), because simple tarsi resembling those present in *ss* mutant *Drosophila* occur in crustaceans and primitive hexapods. Thus, both the transformation of antenna to leg and the tarsal deletions caused by *ss* mutations appear to be atavistic, suggesting that *ss* played an important role in the evolution of distal limb structures in the arthropods.

Because antennal specialization occurred very early in arthropod evolution, we think it likely that the first function of *ss* was antennal specification, and that *ss* was recruited into tarsal development much later, during the evolution of the hexapods. Antennae are often elongated appendages used for probing the environment, and we think it plausible that as part of antennal specification, *ss* came to have an appendage elongation function. Transient expression of this function could then have served to extend other limbs, including the legs. This evolutionary sequence predicts that *ss* homologs will prove to be expressed in the antenna, but not the legs, of crustaceans and primitive hexapods, but in both locations in the insects.

Materials and methods

Screen for new ss *alleles*

Isogenic ss^+ males (either from Canton-S or a $st\ p^p$ line) were irradiated (4000 rads of X rays) and crossed to $sbd^2\ ss^{aM204}/TM1$ females. Non-*TM1* progeny were then screened for the ss^a antennal transformation. ss^{aM204} is a weak *ss* allele kindly provided by Dr. Ernesto Sánchez-Herrero (Universidad Autónoma de Madrid, Madrid, Spain). It shows a good antennal transformation, but little or no bristle or leg effects, when heterozygous with *ss* null alleles.

Cloning

Cloning, Southern blotting, and restriction mapping were by standard procedures (Sambrook et al. 1989). In situ hybridization to polytene chromosomes was as described by Cai et al. (1994). Previously described mutations mapped in this report include *Tp(3;2)P10, In(3LR)P88, T(1;3)ss*^{v}, and *T(2;3)ss*^{a75} (Lindsley and Zimm 1992); *Tp(1;3)sta* (Melnick et al. 1993); and *Df(3R)RS3-32* (Hopmann et al. 1995).

In situ hybridizations

Digoxigenin-labeled RNA probes were used in a modification of the method of Tautz and Pfeifle (1989) (R. Blackman, pers. comm.). After staining, samples were dehydrated through an ethanol series and mounted in 80% Permount (Fisher scientific) : 20% methyl salicylate or wet mounted in 50% glycerol in 50 mM Tris at pH 8.8. Double in situ/antibody staining was essentially as described by Manoukian and Krause (1992).

cDNA analysis

ss cDNA clones were isolated from a λgt10 eye/antennal imaginal disc library kindly provided by G. Rubin. cDNAs corresponding to the centrally located 1.2-kb transcript were obtained from an adult λExlox library (Novagen). Clones were sequenced in entirety on both strands from single-stranded DNA by use of the Sequenase kit (U.S. Biochemical).

Antibody staining

With the exception of imaginal discs, tissue staining was essentially as described by Kellerman et al. (1990). Imaginal discs were found to give unacceptable background when streptavidin-based reagents were used, presumably because of the presence of endogenous biotin. Therefore, prior to incubation with biotinylated secondary antibodies, discs were blocked by a 20-min treatment with unlabeled streptavidin (0.5 μg/ml in PBS) followed by a 20-min treatment with biotin (20 μg/ml in PBS). Tissues were dehydrated, mounted, and photographed as for the in situ samples. The anti-en hybridoma 4D9 (Patel et al. 1989) was obtained from the American Type Culture Collection.

*Monoclonal anti-*Dll *antibody*

Balb/c mice were immunized with bacterially expressed Dll protein and shipped to us by Dr. Stephen Cohen. Spleen cells were fused to Sp2/0 myeloma cells, and supernates of clones surviving HAT selection were screened by staining of 6- to 18-hr embryos. Clones showing positive responses were expanded and used for the production of ascites stocks. The antibody used in this analysis, MAb DMDll.1, stains imaginal discs well, but stains embryos relatively poorly. The antibody is of isotype IgG2b.

Larval and adult cuticle preparations

Larvae were mounted in a mixture of 38% Shandon Immumount, 52% saturated chloral hydrate in water, and 10% lactic acid syrup. Processing of adult tissues was as described in Duncan (1982).

Sequence analysis

All sequence analysis was performed by use of the Wisconsin Genetics Computer Group sequence analysis software (Devereux et al. 1984) on a Vaxstation 3100 computer. Genbank databases were searched by the BLAST program (Altschul et al. 1990). The sequence of the sscA6 cDNA has been deposited with GenBank under accession number AF050630.

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