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Evolution of the insect body plan as revealed by the *Sex combs reduced* expression pattern

Bryan T. Rogers, Michael D. Peterson and Thomas C. Kaufman

Howard Hughes Medical Institute, Department of Biology, Indiana University, Bloomington, IN 47405, USA

SUMMARY

The products of the HOM/Hox homeotic genes form a set of evolutionarily conserved transcription factors that control elaborate developmental processes and specify cell fates in many metazoans. We examined the expression of the ortholog of the homeotic gene *Sex combs reduced* (*Scr*) of *Drosophila melanogaster* in insects of three divergent orders: Hemiptera, Orthoptera and Thysanura. Our data reflect how the conservation and variation of *Scr* expression has affected the morphological evolution of insects. Whereas the anterior epidermal expression of *Scr*, in a small part of the posterior maxillary and all of the labial segment, is found to be in common among all four insect orders, the posterior (thoracic) expression domains vary. Unlike what is observed in flies, the *Scr* orthologs of other insects are not expressed broadly over the first thoracic segment, but are restricted to small patches. We show here that *Scr* is required for suppression of wings on the prothorax of *Drosophila*. Moreover, *Scr* expression at the dorsal base of the prothoracic limb in two other winged insects, crickets (Orthoptera) and milkweed bugs (Hemiptera), is consistent with *Scr* acting as a suppressor of prothoracic wings in these insects. *Scr* is also expressed in a small patch of cells near the basitarsal-tibial junction of milkweed bugs, precisely where a leg comb develops, suggesting that *Scr* promotes comb formation, as it does in *Drosophila*. Surprisingly, the dorsal prothoracic expression of *Scr* is also present in the primitively wingless firebrat (Thysanura) and the leg patch is seen in crickets, which have no comb. Mapping both gene expression patterns and morphological characters onto the insect phylogenetic tree demonstrates that in the cases of wing suppression and comb formation the appearance of expression of *Scr* in the prothorax apparently precedes these specific functions.

Key words: *Sex combs reduced*, evolution, insect wing, Thysanura, Orthoptera, Hemiptera, homeotic genes

INTRODUCTION

Homeotic (HOM/Hox) genes are a group of regulatory loci that control elaborate biological processes and have been implicated in the evolution of animal body plans (Lewis, 1978; Patel, 1994; Carroll, 1995). The examination of homeotic mutant phenotypes and their apparent segment-specific patterns of expression has led to the proposal that the HOM/Hox genes control segment identity through a kind of 'genetic address' system (Lawrence, 1992; Lawrence and Morata, 1983). A large set of experiments on *Drosophila melanogaster* and other metazoans suggest that the HOM/Hox genes control specific cell fates (e.g. Botas, 1993; Peifer et al., 1987) and that specific identity is a unique combination of cell types achieved by a unique mosaic of HOM/Hox gene expression patterns (Castelli-Gair and Akam, 1995). The products of the HOM/Hox genes are a set of transcription factors (Affolter et al., 1990) that control cell fates by binding DNA via the homeodomain and activating and repressing specific sets of targets, or what have been called 'realizator genes' (Garcia-Bellido, 1977). By examining the expression pattern of the *Sex combs reduced* ortholog in non-drosophilid insects, we can test the generality of the identified functions of *Scr* that have been determined from the analysis of *Drosophila*.

The proper development of *Drosophila* requires the ectodermal expression of *Scr* in the most posterior segment of the head, the labial segment, and the most anterior segment of the thorax, the prothorax (T1) (Riley et al., 1987; Carroll et al., 1988; Mahaffey et al., 1989; Pattatucci, 1991; Pattatucci and Kaufman, 1991; Gorman and Kaufman, 1995). *Scr* has quite different roles in these segments. In the embryo, it is required for the formation of the T1 denticle belt and beard (Pattatucci et al., 1991). It is also required for ventral migration and subsequent fusion of the labial lobes (Pattatucci et al., 1991), a general feature of insects, and is the only HOM/Hox gene capable of providing this function (B. Rogers and T. Kaufman, unpublished). In the adult, *Scr* is necessary for the specification of the labial palps (Pattatucci et al., 1991), for the development of the sex combs on the T1 legs of males (Kaufman et al., 1980; Pattatucci et al., 1991) and, as we show here, for the suppression of wing formation on the prothorax.

Previous work has implicated *Scr* in the repression of wings on the prothorax (Carroll et al., 1995). These authors showed that in the primordia of the dorsal discs *Scr* is capable of repressing the expression of *vestigial* and *snail*, which are thought to be necessary for wing formation. In addition, the authors proposed that *Scr* suppresses the formation of wing primordia in other pterygotes. Here we report that certain

hypomorphic alleles of *Scr* result in partial wing formation on the prothorax of adult *Drosophila* and that the expression of *Scr* is in the correct location to suppress prothoracic wing formation in other pterygotes.

The early embryonic expression pattern of *Scr* in *Drosophila* is complex. It initiates early (stage 5) in a jagged dorsolateral stripe around the border of the maxillary and labial segment primordia which, as judged by stripes of *engrailed* expression, is neither segmental nor parasegmental in register (Gorman and Kaufman, 1995). Soon after, it resolves into a parasegmental register (PS2) ventrally (primarily progenitors of the central nervous system) and what is largely a segmental register in its dorsolateral domain (the labial epidermis) (Mahaffey et al., 1989). This early expression includes a small number of cells in the lateral epidermis of the posterior maxillary compartment (Riley et al., 1987; Carroll et al., 1988). Later, the expression of *Scr* expands into the prothorax. This expansion begins in the anterior prothorax and eventually fills the entire prothoracic epidermis but does not expand into the most ventral region of the ectoderm (Riley et al., 1987 ; Carroll et al., 1988; Gorman and Kaufman, 1995). Our observations of *Scr* expression in other insects reveals that the expression pattern seen in *Drosophila* is not entirely conserved. Although the anterior (early) expression pattern of *Scr* in the labium and posterior maxilla is well conserved, expression in the prothorax, the more posterior (late) pattern, shows considerable variation.

MATERIALS AND METHODS

Insect cultures and embryo collection

The collection of *Drosophila*, milkweed bug, and cricket embryos was performed according to Rogers and Kaufman (1996). Firebrats (*Thermobia domestica*) were raised in large (>4 l) glass containers, with a beaker full of water inside, in an incubator kept at 40% humidity and 32°C. Animals of all stages of development are raised together in the same cage and fed dry cat food. Females lay eggs once per molt cycle in cotton placed in the cages (Watson, 1964). Eggs were collected from the cotton every 7 to 10 days and kept in glass Petri dishes at 32°C with moistened tissue paper to keep humidity elevated. Embryogenesis lasts about 12 days. The Scr^{8} and Scr^{Wrv5} alleles of *Drsophila* have been decribed previously (Lindsley and Zimm, 1992).

Cloning partial *Scr* cDNAs

RT-PCR was performed using the *GeneAmp* (Perkin Elmer Cetus) reagents following the manufacturer's recommended protocol. Oligo dT was used to prime cDNA synthesis and degenerate primer pairs made to the amino acid sequences PQIYPWM-MNIVPYHM or PQIYPWM-WFQNRR were used to amplify the target cDNAs. PQIYPWM = 5′ CCR CAR ATH TAY CCR TGG ATG 3′ and MNIVPYHM = 5′ CAT RTG GYA NGG NAC RAT RTT CAT 3′ using IUPAC codes. The design of these primers was based upon *Scr* cDNA sequences of *Drosophila* (Lemotte et al., 1989), honey bee (Waldorf et al., 1989), *Artemia* (Averof and Akam, 1993) and the mouse *HoxA5* gene (Fibi et al., 1988); they target the *Scr*-specific YPWM motif upstream of the homeobox and the 3′ end of the homeobox. The WFQNRR primer is the same as described by Averof and Akam (1993). The initial five thermo-cycles used a 50°C annealing temperature with a 90 second ramp time. The final 35 step-cycles were a 50°C annealing temperature with 30 seconds allowed for each annealing, extension and denaturation step. Potential clones were verified by sequencing using Sequenase 2.0 (U. S. Biochemicals) according to the manufacturer's instructions.

Sequence analysis

Similarity searches were performed using BLAST (Altschul et al., 1990) to search all currently available data bases. Initial alignments and all sequence manipulations were performed using the MacVector (Kodak) software package. The final alignment was determined by the authors.

Detection of RNA and protein

In situ hybridization detection was performed as described by Panganiban et al. (1994) except that 0.2% glutaraldehyde was added to the fixative and protease treatment was performed for up to 1 hour at a concentration of 50 μg/ml. Immunohistochemistry was used to detect SCR and engrailed (EN) protein as described by Gorman and Kaufman (1995) and Rogers and Kaufman (1996).

Microscopy

Embryos were mounted on microscope slides in AquaPolymount (PolySciences Inc.) or methyl salicylate. Slides were examined on a Zeiss axiophot microscope and photographed with Kodak ASA100 print film at 50-200× magnification. Scanning electron micrographs were taken of ethanol preserved specimens as described by Gorman and Kaufman (1995). The accumulation of chromagen was considered to be the signal only if it fulfilled the following criteria: (1) was dependent on specific probes, (2) was confined to the cytoplasm and (3) was reproducible.

RESULTS

In order to evaluate the model of homeotic gene function suggested by previous work on *Drosophila* and to examine the potential role of homeotic genes in insect evolution, we used RT-PCR to clone partial cDNAs of the *Scr* ortholog from three insects: milkweed bugs (*Oncopeltus fasciatus*, Hemiptera), crickets (*Acheta domestica*, Orthoptera) and firebrats (*Thermobia domestica*, Thysanura). We observed the accumulation of *Scr* RNA in embryos by in situ hybridization detection and compared these patterns to the expression of Scr protein in *D. melanogaster* (Diptera) (Riley et al., 1987; Carroll et al., 1988; Mahaffey et al., 1989; Pattatucci, 1991; Pattatucci and Kaufman, 1991; Gorman and Kaufman, 1995; this work). As an aid in determining the domains of *Scr* expression we also compared these patterns with the engrailed protein accumulation pattern of each insect (see also Rogers and Kaufman, 1996).

The *Scr* orthologs

Fig. 1 shows the alignment of the conceptually translated *Scr* partial cDNAs from the milkweed bug, the cricket, and the firebrat with *D. melanogaster Scr, Deformed (Dfd),* and *Antennapedia (Antp)*. The partial cDNAs encompass three conserved regions of the HOM-C genes. In all three, the non-drosophilid insect sequences resemble *Drosophila Scr* much more strongly than *Dfd, Antp* or any other HOM-C gene. In the homeodomain, complete or nearly complete identity is found among the partial cDNAs relative to each other and to *Drosophila Scr*, while they show several differences when compared to *Dfd* and *Antp*.

Further evidence that these partial cDNAs are true *Scr* orthologs is found by comparison of the sequences immediately flanking both sides of the homeodomain. On the amino-terminal end lies the YPWM motif and what we call the 'variable region,' so named because of its variability among members of the HOM-C. The 'variable region' sequence, however, is highly conserved among orthologs of each member of the HOM-C,

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                                                                                                C-Conserved
          YPWM+"Variable Region"                                  Homeodomain                     Domain
DM SCR    PQIYPWMKRVHLGT STVNANG      ETKRQRTSYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEH KMASMNIVPYHM
OF SCR    PQIYPWM------QRR------      Q---------------------------------------------------------- ----MNIVPYHM
AD SCR    PQIYPWM------Q -------      ---------------------------------------------------------- ----MNIVPYHM
TD SCR    PQIYPWM------Q -------      ------------------------------------------WFQNRR

DM ANTP    PL----RSQFGKCQ             -R--G-QT-------------------------------------------------N DQEGRAG
DM DFD      ------KI-VAG   - ---SYQPGM-P-----A---H-I--------Y--------------T-VCS-----------------DN -LPNTKN-RKKT
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Fig. 1. Alignment of the conceptually translated partial *Scr* cDNAs from the milkweed bug, *Oncopeltus fasciatus* (OF), the cricket, *Acheta domestica* (AD) and the firebrat, *Thermobia domestica* (TD), with *Drosophila melanogaster* (DM) *Scr*, *Deformed (Dfd)* and *Antennapedia (Antp)*. Dashes indicate amino acid identity with DM *Scr*. Underlined sequences represent the amino acid motif used to make PCR primers degenerate at the nucleotide level. Underlined sequences, therefore, are not necessarily the sequences found in the proteins of the non-drosophilid insects. The 'variable' region lies between the YPWM motif and the start of the homeodomain. The 'C-Conserved Domain' lies just downstream of the homeodomain and like the variable region is conserved among HOM-C orthologs but differs among other members of the HOM-C. A space has been placed between the end of the homeodomain and the C-Conserved Domain to demarcate the end of the homeodomain. In the variable region, 12/15 residues are conserved between MB and DM *Scr*, while 13/14 are conserved for both AD and TD as compared to DM *Scr*. Only 6/17 are shared for each relative to DM *Dfd* (Regulski et al., 1987) in this region, while no alignment can be made between any of the three non-drosophilid sequences and DM *Antp* (Schneuwly et al., 1986) in this region. With the exception of the first residue of the OF *Scr* homeodomain, complete identity exists between each of these sequences and the DM *Scr* homeodomain.

including *Scr* (Fig. 1 and unpublished data). The 'C- Conserved Domain' lies just downstream of the homeodomain and, like the variable region, is conserved among HOM-C orthologs, but differs among individual members of the HOM-C. In addition, a cross species in situ hybridization of milkweed bug *Scr* (OF*Scr*) to *Drosophila* embryos specifically detected endogenous *Scr* expression in a normal pattern (Fig. 2).

The anterior (early) pattern of epidermal *Scr* expression is conserved

The earliest expression of *Scr* occurs in an anterior domain that corresponds primarily to the labial segment, but also includes ventral and lateral portions of the maxillary segment. In milkweed bugs, crickets and firebrats, this expression initiates in domains that are neither entirely segmental nor parasegmental (Fig. 3D,E, not shown), but soon resolves into a pattern that is parasegmental in register ventrally (central nervous system neuromere) and segmental in its dorsolateral domain (labial epidermis) (Figs 3F-H, 4A,E,I,J).

In all three new species examined, as in *Drosophila*, there is a shift of register of *Scr* expression in dorso-lateral versus ventral domains at both the anterior and posterior limits of expression. The posterior-most limit of dorsolateral expression is at the labial-T1 segment border (Fig. 3F-H; arrow in 4A,E,I,J). The posterior-most border of the ventral expression, however, lies a few cell lengths anterior to this, at the apparent compartmental border (double arrow in Figs 3F-H, 4A,E,I,J) or what in *Drosophila* is called the border between parasegments (PS) 2 and 3. Anteriorly, the shift is the same. The ventral expression extends into the posterior maxillary segment (PS 1-2 border; white arrow in Figs 3F-H,K, 4A,E,I,J), while most of the dorsolateral domain stretches only to the maxillary-labial segment border (Figs 3F-H, 4A,E,I,J). In addition, some maxillary cells in the lateral epidermis express *Scr* (white arrowheads, Figs 3G,I, 4A,F,I,J). As in flies, the maxillary expression in the lateral epidermis of these insects does not fill the entire posterior compartment, but is limited to a subset of cells that become the posterior region of the lacinia, a maxillary structure (see below and Fig. 4).

In addition to the conserved epidermal pattern, the pterygote

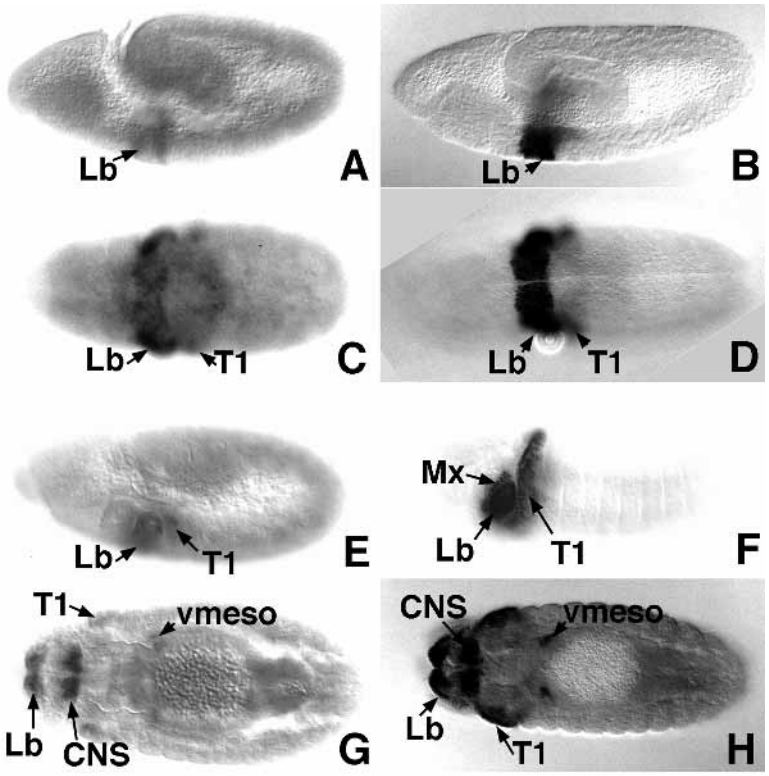


Fig. 2. Cross-species in situ hybridization of *Oncopeltus fasciatus Scr* (OF *Scr*) to *Drosophila* embryos detects endogenous *Scr* (DM *Scr*) expression. A,C,E,G show the result of an in situ hybridization of an OF *Scr* cRNA probe to *Drosophila* embryos. The nucleotide sequence of OF *Scr* (285 nt) is 74% identical to DM *Scr* (282 nt), yet conditions allow the hybridizations to uniquely detect *Scr* expression (dark staining) as determined by comparison with the expression of Scr protein (B,D,F,H). This bio-assay demonstrates that among the genes expressed during *Drosophila* embryogenesis, OF *Scr* is most similar to DM *Scr* and is good evidence that the two genes are truly orthologs. The OF *Scr* probe detects high levels of transcript in the labial and maxillary segment (Lb), prothorax (T1), central nervous system (CNS), and visceral mesoderm (vmeso). An antibody to the DM Scr protein detects expression in the same pattern.

insects, but not firebrats, also have mesodermal expression of *Scr*. In crickets this expression appears broadly over the mesodermal primordia that are located ventrally and subepidermally in the maxilla, labium and thorax (white arrows, Fig. 3E). Later in the development of cricket and milkweed bug embryos, *Scr* expression accumulates in the characteristically spherical cells of the somatic mesoderm (smeso) which underly the columnar epithelial cells of the appendages (white arrows, Fig. 3J; solid white arrowheads, 4A,B,G) and include the progenitors of the leg muscles.

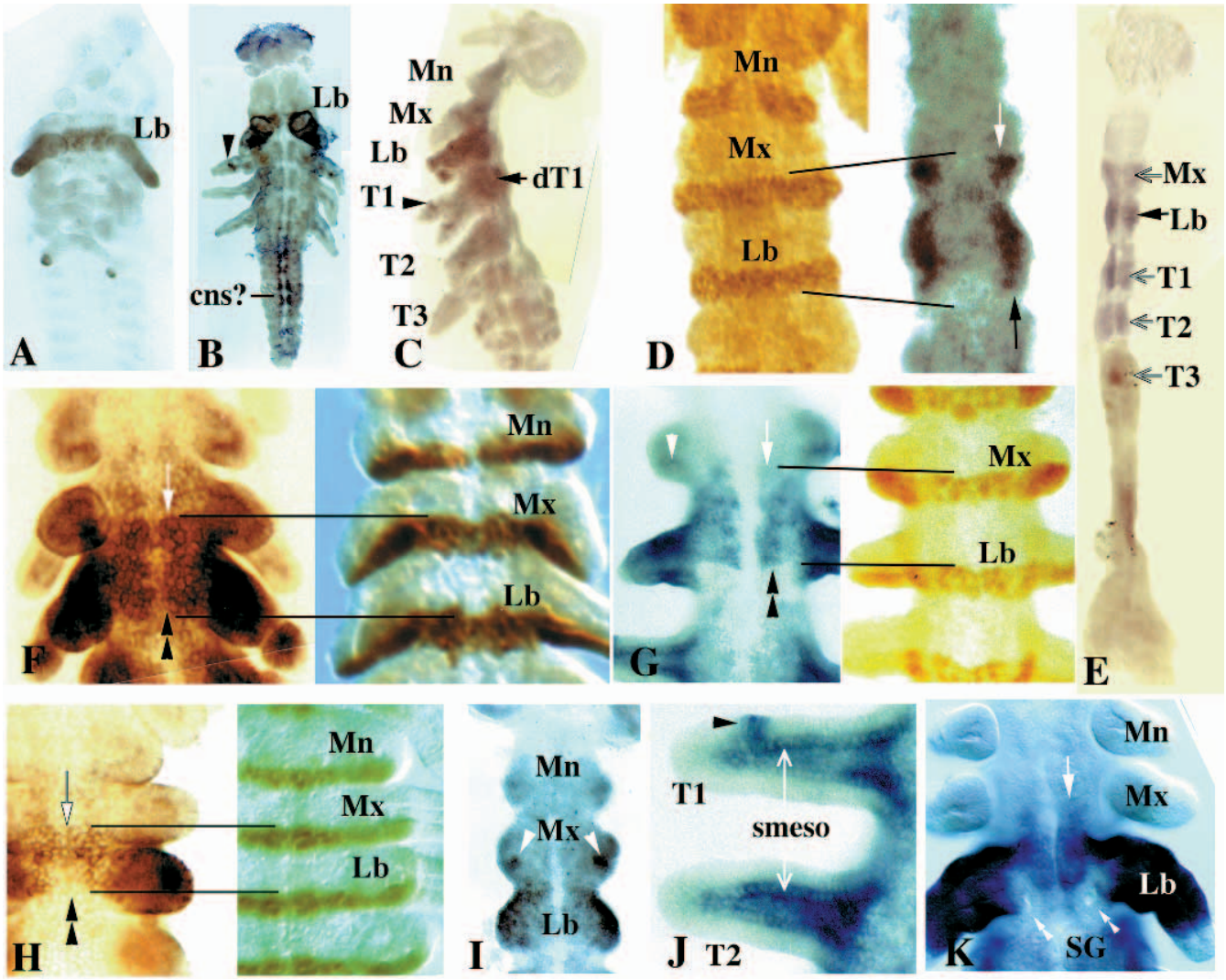


Fig. 3. The expression of *Scr* in insect embryos. Expression of *Scr* is primarily confined to the labial segment (Lb) in the embryos of *Thermobia domestica* (firebrats) (A), *Oncopeltus fasciatus* (milkweed bugs) (B) and *Acheta domestica* (crickets) (C), but also includes portions of the maxilla (Mx) and prothorax (T1). In a lateral view, the expression of *Scr* in a cricket embryo (C) can also be detected in the dorsal regions of the prothorax (dT1) (see Fig. 4. for details of the thoracic expression pattern). Comparing domains of *Scr* expression to engrailed protein (EN) accumulation in the milkweed bug shows that initiation occurs in a domain that is neither segmental or parasegmental (D). At germband condensation the anterior border of *Scr* expression is the compartment border within the Mx segment (white arrow, D), while the posterior border is the Lb segment border (black arrow in D). Early mesodermal expression is prominent in condensed germ bands of cricket embryos (E). *Scr* is expressed broadly throughout the maxillary (Mx), labial (Lb) and thoracic (T1-3) mesoderm (white arrows) and the labial ectoderm (arrow). At germband extension the expression of *Scr* in crickets (F), milkweed bugs (G) and firebrats (H) is in the stereotypical pattern for *Scr* orthologs. This includes a posterior border which, when compared to EN accumulation, is parasegmental ventrally (double arrowhead) and segmental laterally and dorsally (white arrow). Expression in the Mx epidermis is confined to a small cluster of cells (white arrowhead) as shown for the milkweed bug in G and I (see also Fig. 4). Expression of *Scr* in the appendages of crickets and milkweed bugs (J) is primarily confined to the cells of the somatic mesoderm that underly the columnar epithelium. However, during germband extension, expression can also be detected in a small 'patch' (black arrowhead) along the rudiment of the prothoracic legs of crickets (C) and milkweed bugs (B,I). Two dynamic aspects of *Scr* expression in the milkweed bug (K) are the loss of expression from the distal tip of the Lb appendage and the salivary gland (SG, white double arrrowheads). Very low levels of chromagen accumulation can be detected throughout the thorax of firebrats (A) and high levels of expression are also seen in the pleuropodia of firebrats (A) and in the abdominal CNS of milkweed bugs (B). This accumulation may represent *Scr* expression or it may be cross-hybridization of our probe to other homeoboxes (see text).

The posterior (late) pattern of *Scr* expression is variable

As shown in Figure 4, the mature expression of *Scr* in the firebrat, cricket and milkweed bug includes small portions of the prothorax, as well as the labial and maxillary expression described above. The prothoracic expression of *Scr* in milkweed bugs, crickets and firebrats differs from that of *Drosophila* (see Discussion).

In firebrats, milkweed bugs and crickets, *Scr* is detectable in a dorsal anterior region of the prothorax near the apparent base of the prothoracic legs (dT1; Fig. 4C,G,K). In milkweed bugs and crickets, *Scr* expression is also detected in a patch on the anterior side of the leg (large arrowhead, Fig. 4A-C,E-G). These *Scr*-expressing cells appear to lie just proximal to the tibial-tarsal junction (Fig. 4C,G) and are specific to the prothorax. No such patch is seen on the second or third thoracic legs (T2, T3; Fig. 3B,C). In milkweed bugs, a comb strikingly similar to the *Drosophila* sex comb is produced at this location on the prothoracic legs of both first instars (Fig. 4D) and adults (and in both sexes), but not on T2 or T3 legs (not shown). No comb-like entity is present on any of the legs of crickets or firebrats (Fig. 4H,L, and data not shown).

Weak expression has also been observed in the CNS extending from the labial segment posteriorly through the abdomen (Fig. 3B,C). In most cases this expression is weak and may be hybridization to other homeobox-containing

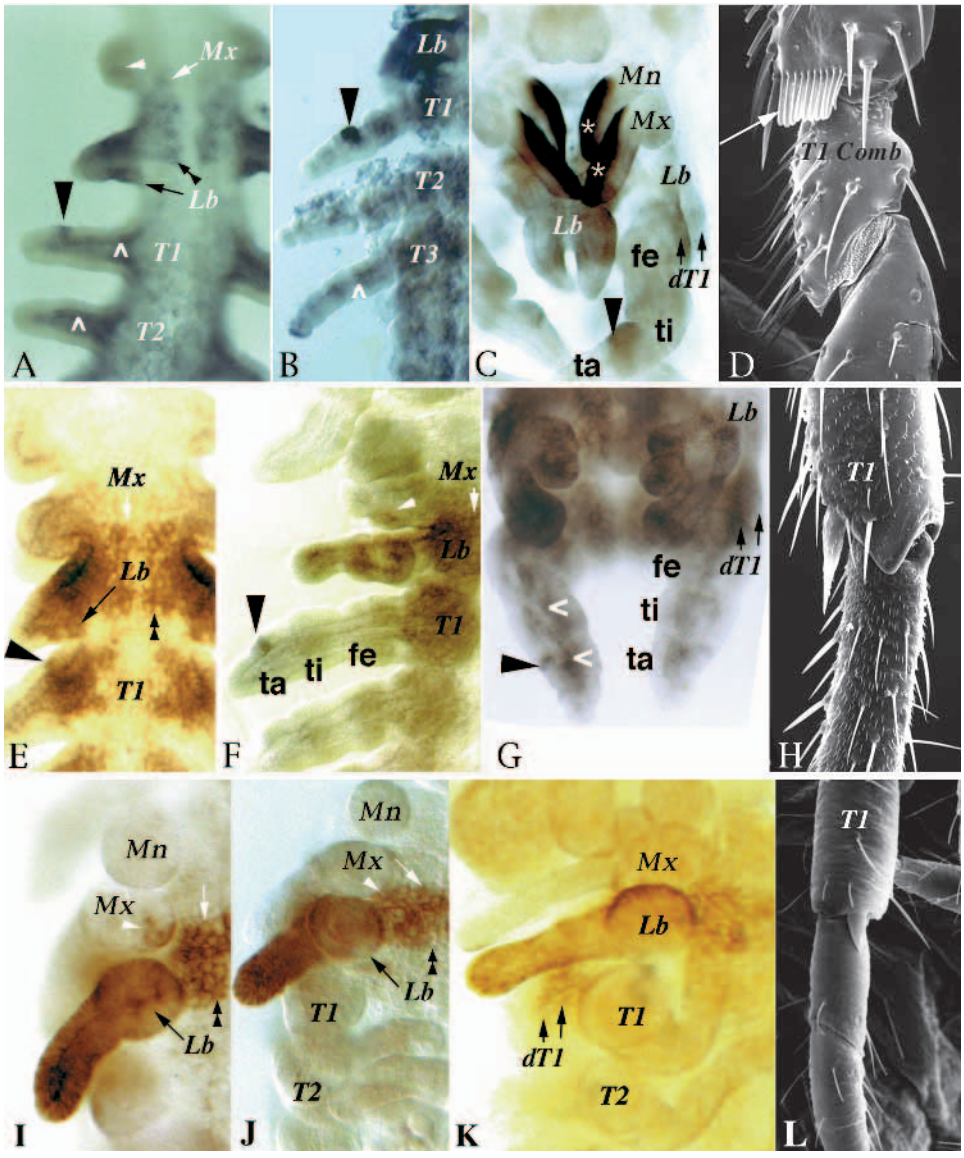


Fig. 4. *Scr* expression in the labial (Lb), maxillary (Mx) and first thoracic (T1) segment of insects and the development of leg combs. (A-D) *Oncopeltus fasciatus* (milkweed bug), (E-H) *Acheta domestica* (cricket), (I-L) *Thermobia domestica* (firebrat). Embryos are shown at progressively later stages from left to right. The final panels (D,H,L) are scanning electron micrographs of the prothoracic leg of first instars. *Scr* expression as determined by in situ hybridization of *Scr* cRNA appears dark blue or brown. The ectodermal expression of *Scr* in the labial and maxillary segments are marked as in Fig. 3. The posterior border of *Scr* expression is parasegmental ventrally (double arrowhead) but segmental laterally and dorsally (arrow). The expression extends anteriorly through the posterior compartment of the maxillary CNS (white arrow) ventrally and, except for a small cluster of lateral cells (white arrowhead), extends to the labial-maxillary segment border laterally and dorsally. As in flies, the maxillary expression in the non-drosophilid insects does not fill the entire posterior compartment but is limited to a subset of cells equivalent to the posterior region of the lacinia in crickets and firebrats (A,F,I), a structure which has been lost in milkweed bugs. As development of the appendages continues, the expression of *Scr* in the prothoracic leg, the 'leg spot,' of milkweed bugs and crickets becomes more distinct (large arrowhead). In later embryos the divisions of the legs, femur (fe), tibia (ti), and tarsus (ta), become clear (C,F,G) and the expression of *Scr* can be mapped to the tibia where a comb forms in the milkweed bug (D). No comb forms on the prothoracic legs of crickets and firebrats (H,L). The spurs evident on the cricket and firebrat legs are not prothoracic-specific structures. Spurs are present on all legs and the number of spurs differs on the T2 and T3 legs of cricket. *Scr* is expressed in the mesoderm of the legs of milkweed bugs and crickets (open white arrowheads) but not firebrats (J,K). During germband retraction expression of *Scr* intensifies dorsal to the prothoracic leg (dT1) (C,G,K). Note that in crickets and milkweed bugs the expression of *Scr* in dT1 is more intense than in the dorsal region of the labium (C,G), where expression has decreased. Also note that in milkweed bugs the expression of *Scr* has faded from the tip of the labium (A,C). The asterisks in C mark non-specific accumulation of chromogen in the Mx and mandibular (Mn) setae which does not represent *Scr* expression.

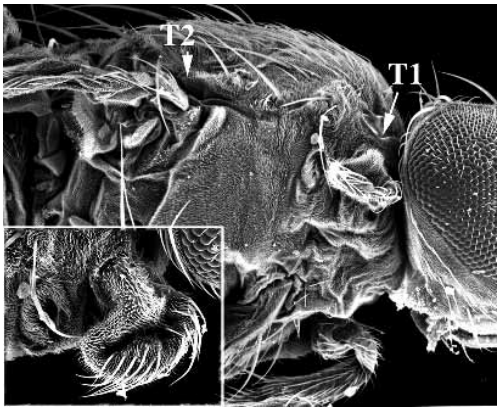


Fig. 5. An *Scr* mutant of *Drosophila* showing the formation of ectopic wings on the prothorax (T1). A pharate adult of the genotype Scr^{8}/Scr^{Wrv5} was dissected from its pupal case before preparation for scanning electron microscopy. Scr^{8} is a hypomorphic allele and Scr^{Wrv5} is a null allele of *Scr* (Lindsley and Zimm, 1992). The ectopic wing forms in the dorsal anterior region of the prothorax just ventral to the tergite in the dorsal pleuron. A normal wing forms in the metathorax (T2).

RNAs. Although apparent low levels of Scr protein accumulation have been detected in the posterior CNS of *Drosophila* (Gorman and Kaufman, 1995), this expression is dependent on the activity of other homeotic genes (ibid; B. Rogers, unpublished) and is likely to be due to cross-reactivity of the antibody used to other homeodomain proteins.

During the later stages of embryogenesis, *Scr* continues to be expressed in the labium and cells of the maxilla. Two dynamic aspects of *Scr* expression in *Drosophila* that are conserved in milkweed bugs are the retraction of *Scr* expression from the distal tip of the labium and from the salivary gland (SG; white double arrowheads, Fig. 3K).

Scr represses wing formation on the prothorax of *Drosophila*

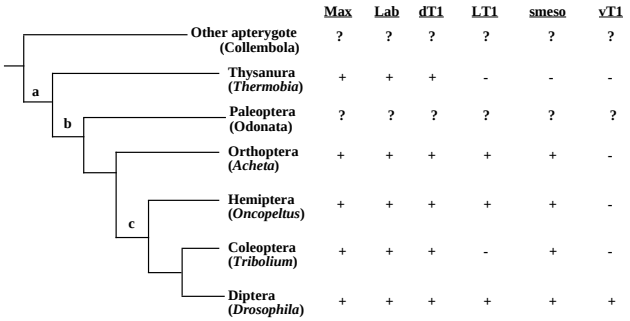
Although null alleles of *Scr* cause embryonic lethality in *Drosophila*, certain allelic combinations allow animals to live to larval, pupal, and even adult stages. The combination of the hypomorphic allele Scr^{8} in *trans* with the null allele Scr^{Wrv5} allows some survival to the pupal stage. An examination of imagos extracted from pupal cases reveals numerous defects of the prothorax. The most striking of these defects is the production of wing tissue. Fig. 5 is a scanning electron micrograph showing wing growth out of the pleuron dorsal to the prothoracic leg of a pharate adult. The presence of ectopic wings in Scr^{8}/Scr^{Wrv5} mutants is a clear demonstration that *Scr* is required for wing suppression on the prothorax of *D. melanogaster*.

DISCUSSION

Elements of the early and late expression of *Scr* are conserved

Comparison of the early embryonic ectodermal expression of *Scr* and the mature derivatives of the domains among the four orders studied here reveals a well conserved pattern. Moreover, due to the structural similarity of the adult *Drosophila* to the larval stages of hemimetabolous and ametabolous insects, e.g., the presence of seta, thoracic and gnathal appendages, and an exposed head with eyes, a comparison of the *Scr* pattern in the anlage of the adult, the imaginal discs, is also informative. The segment-wide expression of *Scr* over the labial (Lab) epidermis and the parasegmental expression in the CNS is observed in all embryos examined ('+' in Fig. 6). Additionally, it would appear that *Scr* expression in the ectoderm of the *Drosophila* labial disc, and a portion of the maxillary anlagen of the eye-antennal disc (Pattatucci, 1991; Pattatucci and Kaufman, 1991), has counterparts in the species studied here. The retention of the labial expression is consistent with a conserved role in labium formation, specifically the control of migration and fusion of the labial lobes, a feature that sets the labial segment apart from the other gnathal segments.

The maxillary expression of *Scr* in firebrats and crickets is clearly homologous, as it is confined to the posterior lacinia of the maxilla. Although the milkweed bug and fruit fly maggot lack lacinia because of the modified structure of the mouthparts, we propose that the observed posterior maxillary expression of *Scr* in a small cluster of cells in these more derived insect embryos is homologous to that seen in the firebrat and cricket. Thus, we conclude that the firebrat and all three pterygotes, including embryonic and adult *Drosophila*, have homologous expression patterns of *Scr* in the maxillary (Max) and labial segments (Fig. 6).



	Max	Lab	dT1	LT1	smeso	vT1
Other apterygote (Collembola)	?	?	?	?	?	?
Thysanura (*Thermobia*)	+	+	+	-	-	-
Paleoptera (Odonata)	?	?	?	?	?	?
Orthoptera (*Acheta*)	+	+	+	+	+	-
Hemiptera (*Oncopeltus*)	+	+	+	+	+	-
Coleoptera (*Tribolium*)	+	+	+	-	+	-
Diptera (*Drosophila*)	+	+	+	+	+	+

Fig. 6. Phylogeny of insects and *Scr* expression patterns. The phylogenetic relationship of *Drosophila*, beetles (*Tribolium*), milkweed bugs *(Oncopeltus*), crickets *(Acheta)*, firebrats *(Thermobia*), Paleoptera, and other apterygotes is shown with a table of the various aspects of *Scr* expression seen in these insects. Max, posterior maxillary expression; Lab, labial expression; dT1, expression in dorsal anterior prothorax; LT1, expression in the lateral prothorax including the leg spot; smeso, expression in the somatic mesoderm of thoracic legs; vT1, expression in the ventral prothorax. Firebrats are apterygotes; that is, they belong to a group that split from the lineage of insects that evolved wings and are believed to be primitively wingless. Collembola are wingless hexapods and represent an outgroup for which the *Scr* expression pattern is unknown. The position marked 'a' is the position on the tree where *Scr* may have gained some control over prothoracic development and may be coincident with the appearance of *Scr* expression in dT1. The evolution of wings is marked on the tree with a 'b' and may also be coincident with the development of the leg spot. 'c' marks the position where leg combs may have evolved. *Scr* expression has also been examined during the embryonic development of *Tribolium* (S. Brown and R. Denell, personal communication). Although no leg spot has been seen, *Tribolium* larvae are combless, but adults have modified leg patches and *Scr* may be expressed in the primordia of these adult structures. The phylogeny of hexapods shown is based upon that of Kristensen (1991).

Although the expression of *Scr* in the prothorax of *Drosophila* differs from the other insects examined, we interpret some individual aspects of the total pattern in the fly as being conserved among either pterygotes or all insects. In addition to being expressed in the embryonic prothorax of *Drosophila*, *Scr* is also expressed in the prothoracic leg disc, the dorsal prothoracic (humeral) disc and in the mesoderm of all of the thoracic leg discs (Pattatucci, 1991; Pattatucci and Kaufman, 1991). Unlike the less derived insects, *Drosophila* embryos do not exhibit patches of dorsal or ventral prothoracic expression. Rather, *Scr* is expressed across the entire prothoracic ectoderm and presumably overlaps both subregions of expression seen in the other insects. This dissimilarity extends to the prothoracic leg discs in which *Scr* is expressed over the epidermis of the anlagen of the entire prothoracic leg rather than in a tibial-tarsal patch. Since the broader domains of expression seen in both embryonic and adult *Drosophila* include the smaller domains of other insects, we interpret expression in the leg patch (LT1) as being conserved in all pterygotes (Fig. 6).

Scr expression in the thoracic somatic mesoderm (smeso) of pterygotes is also conserved (Fig. 6), as demonstrated by the expression seen in the developing legs of milkweed bugs and crickets and in the leg mesoderm of all thoracic leg discs of *Drosophila* (Pattatucci and Kaufman, 1991). Because the *Drosophila* maggot lacks appendages, expression of *Scr* in leg mesoderm is not apparent in the embryo. We did not attempt to examine the visceral mesoderm surrounding the anterior midgut for the expression of *Scr*. This mesodermal expression is known to be required for formation of the gastric caeca in *Drosophila* (Reuter and Scott, 1990). At early stages we have no reliable marker for identifying cells of the visceral mesoderm in the non-drosophilid insects and later when midgut morphogenesis occurs, and the mesodermal cells are identifiable, the deposition of cuticle makes the in situ hybridization detection of expression difficult.

The dorsal prothoracic (dT1) expression of *Scr* differs in embryonic and adult *Drosophila*. In the embryo, *Scr* epidermal expression covers the entire prothorax, but in the dorsal prothoracic disc expression is restricted to the anterior compartment of the disc (Pattatucci, 1991). The *Drosophila* disc pattern more closely parallels the pattern seen in the other insect embryos. This higher degree of similarity between the embryos of less derived species and the imaginal discs that give rise to the adult *Drosophila* is consistent with the idea that the maggot is more highly derived than the adult (Snodgrass, 1953). We interpret the dorsal prothoracic patch (dT1) as being conserved in all insects (Fig 6).

Scr is expressed in the appropriate location to suppress wing formation in the prothorax of pterygotes

All modern winged insects lack wings on the prothorax, but examination of Paleozoic fossil insects has shown that the absence of wings on the prothorax is likely a secondary event following the evolution of wing-like appendages on all abdominal and thoracic segments (Kukalova-Peck, 1978; 1987). Kukalova-Peck (1987) has proposed that wings evolved from side lobes that grew out of the exite of the basal-most limb segment, the epicoxa, on all thoracic and abdominal segments. This hypothetical epicoxal exite would be located just ventral to the tergites and dorsal to the leg (telopodite), exactly where we observe *Scr* expression in modern insects (Fig. 4).

The evidence that *Scr* suppresses wing development comes from three sources: (1) the demonstration by Carroll et al. (1995) that in the embryonic primordia of the dorsal prothoracic discs, *Scr* represses *vestigial* and *snail*, two genes involved in promoting wing development; (2) the formation of wing tissue on the prothorax of flies carrying a hypomorphic allele heterozygous with a null allele of *Scr* (Fig. 5); (3) the formation of elytra (T2 wing derivatives) on the prothorax of adult *Tribolium* carrying a hypomorphic allele heterozygous with a null allele of *Cephalothorax*, the *Scr* ortholog (Beeman et al., 1989). Taken together, the ability of *Scr* to repress wing formation in *Drosophila* and *Tribolium* and the expression of *Scr* in crickets and milkweed bugs just dorsal to the leg argue that in pterygotes *Scr* functions to suppress the production of prothoracic wings.

Expansion of *Scr* expression into ventral and distal domains of the prothorax have produced alterations of the insect body plan

As described, a critical step in the evolution of modern winged insects involved the repression of prothoracic wings, apparently mediated by *Scr* activity. In addition to this role for *Scr*, there are two further cases in which an expansion of *Scr* expression within the prothorax of pterygotes has played a role in insect evolution. Since *Scr* is expressed in the labial and maxillary segments and in the dorsal prothorax of both pterygotes and a Thysanuran apterygote, we infer this to be the *Scr* expression pattern of the last common ancestor of both groups (Fig. 6). However, neither the ventral (vT1) nor lateral (LT1) epidermal prothoracic expression pattern of *Scr* is seen in the firebrat; this expression is observed only in the winged insects (Fig. 6). Further, the *Scr* expression in lateral and ventral prothoracic epidermis is apparently associated with the development of structures uniquely formed in those regions. Thus, the absence of *Scr* ventral T1 epidermal expression in the presumed insect ancestor and the control of the development of T1-specific structures by *Scr* must have involved an expansion of the *Scr* expression pattern in the acquisition of this new role.

The milkweed bug has a patch of *Scr* expression in the epidermis of the tibia in a position where a leg comb forms. Both the patch of *Scr* expression and the comb are specific to the prothoracic leg. In *Drosophila*, proper development of the sex comb requires *Scr* function and ectopic *Scr* expression leads to the production of ectopic sex combs on the T2 and T3 legs (Pattatucci and Kaufman, 1991). This suggests that *Scr* in milkweed bugs may perform a similar role in specifying the prothoracic leg comb. This argument is strengthened by the observations that the combs of *Drosophila* and the milkweed bug form in non-homologous positions along the proximal-distal axis of the leg and that the milkweed bug comb correlates with the position of *Scr* expression, not with the position of the *Drosophila* comb. The *Drosophila* comb forms on the basitarsus, whereas the milkweed bug comb forms on the tibia. One possible test of the relationship between *Scr* and leg combs would be to examine *Scr* expression in other hemipteran species, several of which have multiple combs (Schuh and Slater, 1995). Because the lateral accumulation of *Scr* in the region of the leg patch is not seen in the firebrat, we conclude that this novel domain seen in the other insects represents an expansion of expression that occurred during the evolution of pterygotes ('b' on Fig. 6).

The expression of *Scr* in the epidermis of the prothorax is unique to *Drosophila*. We infer that expression has expanded during the evolution of *Drosophila* to include the ventral and posterior prothorax of the embryo and the ventral epidermis and entire leg of the adult. The ventral expression in the embryo has been shown to be required for the proper development of ventral structures, including specifying denticle types and the elaboration of the prothoracic beard (Pattatucci et al., 1991). Thus, this expansion of the *Scr* expression domain was necessary for *Scr* to evolve control of the development of ventral T1 epidermis.

The evolution of dT1 and leg patch expression of *Scr* occurred prior to the evolution of the comb production and wing suppression functions

It is commonly accepted that apterygotes, modern Thysanurans in particular, are primitively wingless (Boudreaux, 1979), which makes the dorsal prothoracic *Scr* expression in the firebrat curious. However, unlike the evolution of wings, which is thought to have occurred once in the progenitor of all pterygotes, the suppression of wings may have occurred multiple times. Kukalova-Peck (1978, 1987) has reported fossil Neopteran and Paleopteran specimens with wings or proto-wings on the prothorax and has argued for separate loss of this T1 appendage in the two lineages independently. Furthermore, the T1 wings of Paleopteran and Neopteran fossils are morphologically distinct from the T2 and T3 wings (Kukalova-Peck, 1978, 1987). We infer from the existence of dT1 expression of *Scr* in all insects examined that the last common ancestor of Thysanurans and all pterygotes, Paleoptera and Neoptera, also had dT1 expression of *Scr*. Therefore, the *Scr* expression predated the evolution of wings and was in the correct location, perhaps initially, to control the unique development of primitive prothoracic wings. It was subsequently recruited to suppress wing formation in both the Paleoptera and Neoptera. An examination of *Scr* expression in Paleopterans would help to confirm this hypothesis. At present the function of the dT1 *Scr* expression in firebrats is unknown. There are no obvious morphological structures specific to the prothoracic pleuron that distinguish it from the second and third thoracic segments (data not shown) that might shed light on any possible role of *Scr* in dT1 of apterygotes.

Scr is expressed in the tibia of the prothoracic legs of both crickets and milkweed bugs in a patch or hemi-stripe and we infer that this homologous expression existed in the common ancestor of Hemiptera and Orthoptera. Although in milkweed bugs this spot marks the position of the developing comb, no comb is produced on the prothoracic leg (or any other leg) in crickets and the *Scr*-expressing leg spot does not seem to correlate with any morphological structure unique to the prothoracic leg at the tibial-tarsal junction (Fig. 3). Thus, the role of the leg spot in crickets remains unclear. However, because leg combs exist in Diptera, Hemiptera and Coleoptera, but not in Orthoptera or more primitive lineages, we propose that *Scr* expression in the prothoracic leg, like dorsal T1 expression, preceded the adoption of a novel patterning function. We do not have enough data points to infer precisely when leg spot expression evolved. It may have been coincident with the evolution of pterygotes ('b' Fig. 6), with only some pterygote lineages subsequently developing the new regulatory networks necessary to produce leg combs ('c' Fig. 6). Alternatively, the LT1 expression could have evolved several times in the different lineages. Parsimony argues for the former model, but a resolution of the point will require an examination of *Scr* expression in the Paleoptera and other more primitive pterygotes as well as additional apterygotes.

Evolution of *Scr* function

Our observations provide insight into how the activity of *Scr* provides segment identity. Most recent evidence from *Drosophila* supports the idea that HOM/Hox genes control cell-specific functions that depend upon the developmental history of each cell (Castelli-Gair and Akam, 1995). Unique segment identity is provided by a mosaic of cell types. However, an examination of HOM/Hox gene expression patterns in species of different phyla has revealed a striking conservation of their primary anterior to posterior domain of expression throughout the metazoa. The order of expression (with respect primarily to the anterior-most ectodermal expression domain) is generally collinear with the known order of the genes within the homeotic complexes of both chordates and insects (Garcia-Fernandez and Holland, 1994; Manak and Scott, 1994; McGinnis and Krumlauf, 1992). This provides further evidence that the HOM/Hox genes supply positional information and that this function is conserved.

The continuous requirement for HOM/Hox gene activity for certain cell fates (Morata and Garcia-Bellido, 1976) has been used as evidence that they function as a 'genetic address'. However, the branching and irreversible nature of developmental pathways would require that the addresses only be checked early to set in motion pathways that would provide a segment-specific developmental history. In this model, positional information, or the 'genetic address', could be one of the earliest cell-specific functions of HOM/Hox genes that helps to set the unique developmental background upon which future cell-specific functions will depend.

The intense early expression of *Scr* in all the cells of the labial segment in all of the insects examined is consistent with it providing this early positional information. The expression covers all the cells of a single domain, the labial segment epidermis, and provides a common developmental background that by itself or in concert with other gene products could set all the cells of the labium apart from every other segment. This expression of *Scr* could immediately specify functions characteristic of this anterior-posterior position, such as labial migration, or initiate developmental pathways that could lead to the production of unique structures even in the later absence of the Scr protein, such as at the tip of the labium.

In contrast, the later expression of *Scr* in the thorax seems incompatible with providing positional information but instead is required to modulate developmental pathways common to the thorax. Once thoracic identity is established, *Scr* can suppress common features, such as the wing, or promote the production of novel features, such as combs, in a manner that is relatively independent of a cell's recent history of HOM/Hox expression. The production of a unique thoracic identity by mosaic HOM/Hox expression is similar to the situation reported for the abdomen of *Drosophila* (Castelli-Gair and Akam, 1995). However, reports of HOM/Hox gene expression patterns suggest that the degree of mosaicism is much greater in *Drosophila* than in other insects. We have shown that with the exception of *Drosophila*, *Scr* is not expressed broadly over the prothorax, but is generally limited to one or two clusters of cells. This difference is similar to that observed by Hayward et al. (1995) who reported little of the modulation of grasshopper *Antennapedia*

expression that is characteristic of the expression of this gene in the thorax of *Drosophila*. Also, in the grasshopper, *abdominal-A* is expressed much more uniformly over each abdominal segment than is *Drosophila abdominal-A* (Tear et al., 1990).

In our study of the *Scr* expression pattern in three non-dipteran insects, we have shown that although there are conserved elements of expression, including the anterior-most ectodermal domain, expansion of the expression domain posteriorly into the prothorax has directly affected the morphological evolution of insects by allowing specialization of unique prothoracic characters. It is important to establish the degree of variability of HOM/Hox gene expression in a wider group of insects and arthropods in general to determine the relative contribution of this type of diversity of expression on the evolution of segment identity and the arthropod body plan.

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