2001

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Published in *genesis:The Journal of Genetics and Development* 30 (2001), pp. 12–20.

Submitted January 8, 2001; accepted February 28, 2001; published online May 9, 2001.

Molecular Characterization of *Cephalothorax*, the *Tribolium* Ortholog of *Sex Combs Reduced*

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Abstract

Sex combs reduced (*Scr*), a Hox gene located in the Antennapedia complex of *Drosophila melanogaster*, is required for the proper development of the labial and first thoracic segments. The *Tribolium castaneum* genetically defined locus *Cephalothorax* (*Cx*) is a candidate *Scr* ortholog based on the location of *Cx* in the beetle Homeotic complex and mutant effects on the labial and first thoracic segments. To address this hypothesis, we have cloned and characterized the *Tribolium* ortholog of *Scr* (*TcScr*). The transcription unit is less complex and encodes a smaller protein than *Scr*. The predicted amino acid sequence of the *Tribolium* protein shares motifs with orthologous proteins from multiple species. In addition, we have analyzed the *TcScr* expression pattern during embryonic development. *TcScr* is expressed in parts of the maxillary, labial, and first thoracic segments in a pattern similar to but not identical to *Scr*. Furthermore, *TcScr* RNA interference results in a phenocopy of the *Cephalothorax* (*Cx*) mutant phenotype in which the labial palps are transformed into antennae and the head and first thoracic segment are fused. All of the available results indicate that *Cx* is the *Tribolium* ortholog of *Scr*.

Keywords: genetic regulation, homeotic, Hox genes, morphological evolution, expression pattern

Introduction

Hox genes are widely conserved among eukaryotic animals and in model systems have been shown to be important in the establishment of developmental commitments. Mutations of these genes in *Drosophila* result in homeotic phenotypes in which one body part develops in place of another. Since Hox genes regulate the expression of genes that are necessary for region-specific development, differences in targets or expression domains of homeotic genes may be involved in morphological diversity (McGinnis and Krumlauf, 1992). We use comparisons of *Tribolium castaneum* Hox genes with those of *Drosophila* and other insects to assess this hypothesis. Although Hox genes have been identified in many insects, *Tribolium* is an exceptional choice for comparative studies because it offers the use of genetic as well as molecular and developmental approaches. In addition, although beetles and flies show morphological specializations, they represent variations of a common insect body plan. These characteristics provide a context for understanding the relationship between genetic and morphological change.

In both *Tribolium* and *Drosophila*, the labium develops an appendage important for feeding. Larval and adult *Tribolium* have mandibulate mouth parts, in which the gnathal appendages are specialized for grinding (mandible) and manipulating (maxilla and labium) food. This is considered to be the ancestral condition in insects (Rogers and Kaufman, 1997). In flies, the adult labium (proboscis) is highly derived and specialized for ingesting liquified food. (The *Drosophila* maggot has no gnathal appendages.) *Sex combs reduced* (*Scr*) activity is necessary for normal proboscis development (A. Abzhanov, S. Holtzman, T. C. Kaufman, personal communication; Percival-Smith et al., 1997; Pattatucci et al., 1991). Thus comparison of the *Tribolium Scr* ortholog to *Drosophila Scr* may reveal a relationship between differences in the regulation or function of these genes and the morphological specializations seen in each insect. However, it is first necessary to identify the *Tribolium* ortholog of *Scr*.

In *Drosophila* embryos, *Scr* loss-of-function (LOF) mutations result in a transformation of prothoracic to mesothoracic identity as well as abnormalities of the labium that have been interpreted as a transformation to maxilla (Sato et al., 1985; Pattatucci et al., 1991) or conversion to a generic gnathal segment (Pederson et al., 1996). Analysis of *Scr* adult clones and hypomorphic alleles shows that *Scr* is

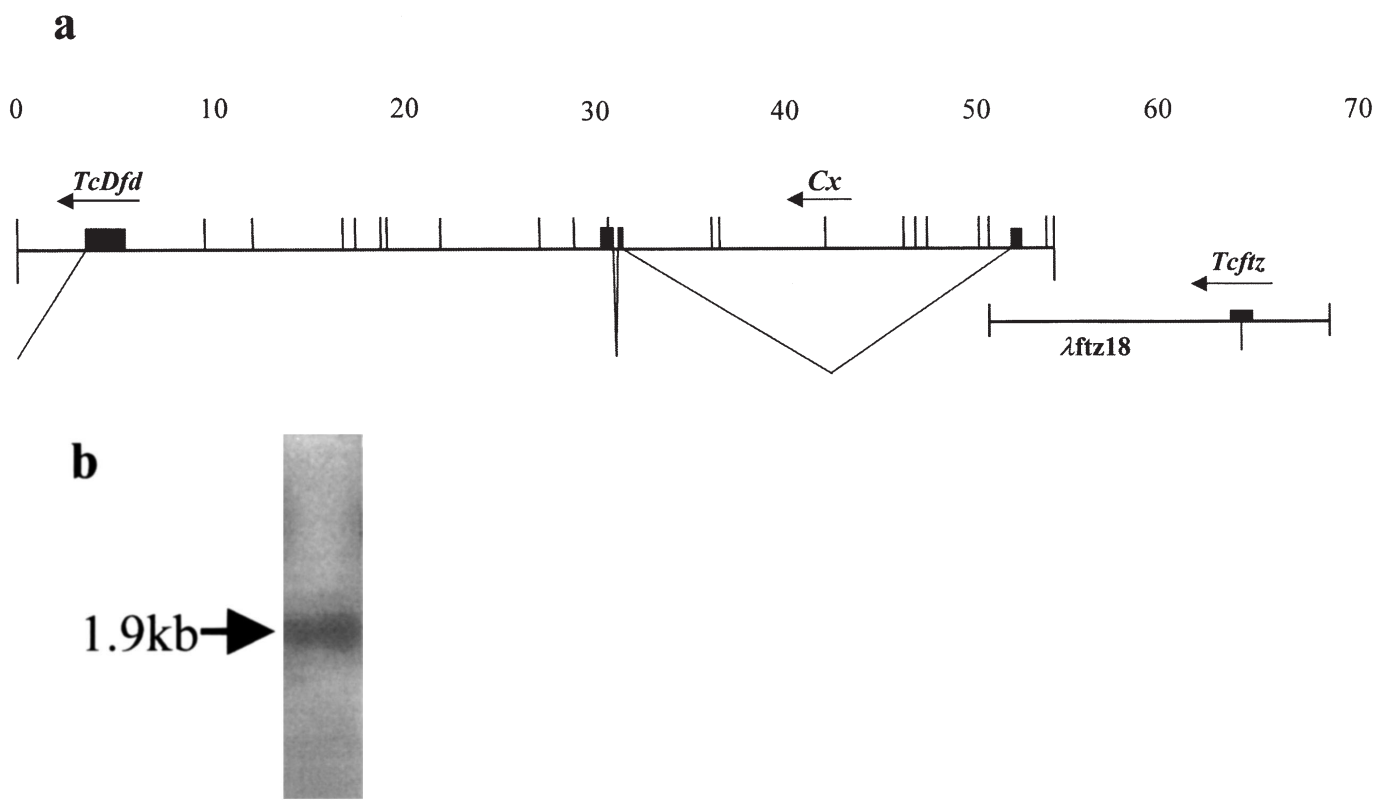

Figure 1. *TcScr* transcription unit. **(a).** *Hin*dIII restriction map of a BAC clone, BDfd3, is shown with an overlapping lambda clone, □ftz18. Black boxes denote exons and thin lines denote introns. Arrows show the direction of transcription. **(b).** Northern blot of embryonic (0-72 h) mRNA probed with pcCx1.

also required for normal development of the imaginal first thoracic (T1) and labial segments (Wakimoto et al., 1984; Sato et al., 1985; Pattatucci and Kaufman, 1991).

Based on the mutant phenotype of *Tribolium Cephalothorax* (*Cx*) and the location of this gene in the Homeotic complex, Beeman et al. (1989) suggested that *Cx* is the ortholog of *Drosophila Scr*. In homozygous and hemizygous condition, strong LOF *Cx* alleles result in a transformation of the larval labial appendages to antennae as well as a fusion of the head and T1 segment (Beeman et al., 1993). In heterozygous adults, subtle abnormalities of the pronotum appear. When an allele of this type is heterozygous with the hypomorphic allele $Cx^{alate\ prothorax}$, the pupal prothorax elaborates mesothoracic elytra (wing covers) (Beeman, 1987). To further address the possibility that *Cx* and *Scr* are orthologous, we have cloned and characterized the *Tribolium* ortholog of *Scr*. For the purposes of discussion, we will call the molecularly ascertained ortholog *TcScr*.

In this article, we describe the molecular organization, sequence, and expression pattern of *TcScr*. In addition, we demonstrate that RNA interference using *TcScr* double-stranded RNA phenocopies strong *Cx* LOF mutant individuals, supporting the hypothesis that the genetically defined *Cx* locus corresponds to *TcScr*.

Results

Molecular Characterization of TcScr

A low-stringency screen of a *Tribolium* embryonic cDNA library with a probe containing the *Drosophila Scr* homeobox (Gibson et al., 1990) yielded a 1.3-kb cDNA, pcCx1. This cDNA encodes a protein that most resembles that of *Scr* including an identical YPWM region and homeodomain. Thus we have identified the *Tribolium* ortholog of *Scr*. A single band of approximately 1.9 kb is observed by Northern analysis, using pcCx1 as a probe (Figure 1b). Since pcCx1 is smaller than the predicted transcript size and its reading-frame is open at the 5′ end, we concluded that pcCx1 is incomplete. A screen of another cDNA library using pcCx1 as a probe resulted in a 1.9-kb cDNA clone, pcCx2. This clone was sequenced and determined to encode a protein sharing N-terminal identity with other *Scr* family members and thus appears to contain the entire coding sequence. The Genbank accession number for the *TcScr* cDNA is AF227628.

To obtain genomic sequence for intron and exon mapping, a BAC library (Brown and Denell, unpublished) was screened with pcCx1. Four unique BAC clones hybridized to the cDNA, three of which had been previously identified

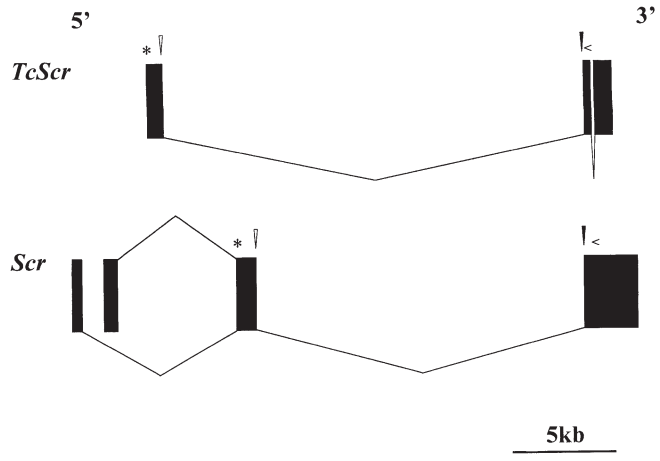

Figure 2. Comparison of *TcScr* and *Scr* transcripts. The single known *TcScr* transcript is shown above two alternative *Scr* transcripts with different first exons. Andrew (1995) has suggested that there are at least two additional, as yet uncharacterized, transcripts that are not pictured. Exons and introns are denoted as in Figure 1a. For each gene an open triangle represents the YPWM-encoding region and a filled triangle indicates the homeobox. The putative translational start site is denoted by an asterisk and < marks the translational stop.

as containing *TcDeformed* (*TcDfd*). Southern analysis of *Hin*dIII restriction digests of the BAC clones revealed that only one, BDfd3, potentially contained the entire *TcScr* transcription unit. *Hin*dIII subclones of BDfd3 were ordered as previously described (Shippy et al., 2000) to obtain a physical map of the region.

Southern analysis and sequencing were used to define *TcScr* exons and place them on the physical map. The *TcScr* transcription unit spans approximately 22 kb and includes three exons (Figure 2). The first intron, approximately 20 kb, lies between the YPWM-encoding region and the homeobox. In addition, there is a 160-bp intron in the 3′ UTR. Previous work demonstrated that one end of BDfd3 lies within the *Tribolium Deformed* (*TcDfd*) gene, and the other end overlaps a lambda genomic clone including the *Tribolium* ortholog of *fushi tarazu* (*Tcftz*) (unpublished observations) (Figure 1a). Thus *Tribolium* resembles *Drosophila* and the mosquito *Anopheles gambiae* in that *Scr* is flanked by *Dfd* and *ftz* (Powers et al., 2000).

The *TcScr* putative translational start site was defined as the 5′-most methionine codon in the open-reading frame (ORF) that encodes the YPWM motif (see below). *TcScr* encodes a predicted protein of 312 amino acid residues. In addition to the YPWM motif and homeodomain, *TcScr* shares a number of additional regions of sequence identity with other members of the *Scr* family. The octapeptide (MSSYFVNS), first described in mammals (Odenwald et al., 2000), is conserved among mammals and insects except for an additional amino acid (glutamine) between the fourth and fifth residues of the insect motif. Insects share extended identity (shaded in Figure 3) following the octapeptide. In previously described insect genes, there is a potential translation start site upstream of the region encoding the octapeptide. However, in these cases the functional start site has not been biochemically defined. Similar to the mammalian ORFs, the *Tribolium* ORF begins at the octapeptide, raising the possibility that translation starts at this methionine in all *Scr* cognates.

Previously, a potential PEST sequence just upstream of the YPWM motif was identified in *DmScr* (Andrew, 1995). PEST sequences are stretches of 12 or more amino acid residues, enriched in proline (P), glutamic acid (E), serine (S), and threonine (T), which have been implicated in targeting proteins for rapid degradation (Rogers et al., 1986; Rechsteiner and Rogers, 1996). We used the PESTfind program (see "Materials and Methods") to identify potential PEST sequences in other *Scr* orthologs. Scores ranging from +5 to +50 are considered potential PEST sequences. The PEST sequence identified by Andrew (1995) was properly predicted by PESTfind. *Anopheles* and *Drosophila* have similar scores of +12.68 and +12.45, respectively. *Tribolium* has the highest PEST score at +15.07. Although Kokubo, et al. (1997) reported that *BmScr* does not contain a PEST sequence, PESTfind identifies a potential PEST sequence with a score of +5.26. HOXA5 has two nearly juxtaposed putative PEST sequences with scores of +7.76 and +6.20, one of which encompasses the YPWM motif. All the predicted PEST sequences are located immediately upstream of the YPWM motif.

Expression Pattern

We examined the expression of *TcScr* by in situ hybridization and by immunostaining with a cross-reacting polyclonal antibody, α-DmScr. The expression patterns revealed by these two methods are identical. TcEngrailed (TcEn), a marker of posterior compartment ectoderm, was used to identify the stage of embryonic development and to provide a register for assessing the expression pattern of *TcScr*. As the germband elongates, TcEn stripes appear in the trunk in anterior to posterior progression. Initially each TcEn stripe is interrupted by mesoderm at the ventral midline and then forms a continuous stripe after the mesoderm invaginates.

TcScr expression first appears after the germ rudiment condenses. At this stage, two TcEn stripes are present (Figure 4a). Expression is seen in the ectoderm coincident with and just posterior to the second TcEn stripe. With the appearance of the third TcEn stripe, it is clear that *TcScr* expression is limited to parasegment 2 (PS2), posterior maxillary and anterior labial segment (Figure 4b, shown at higher magnification in Figure 4c). As the germband begins to elongate, *TcScr* is expressed at higher levels in PS2. Expression also appears in the T1 segment mesoderm, which has not yet invaginated (Figure 4c). In slightly older embryos, *TcScr* also accumulates in the mesoderm of T2 and T3 but does not reach the intensity seen in T1 (Figure 4d). A lateral view of *in situ* hybridization (Figure 4e) clearly shows ectodermal expression in PS2 versus meso-

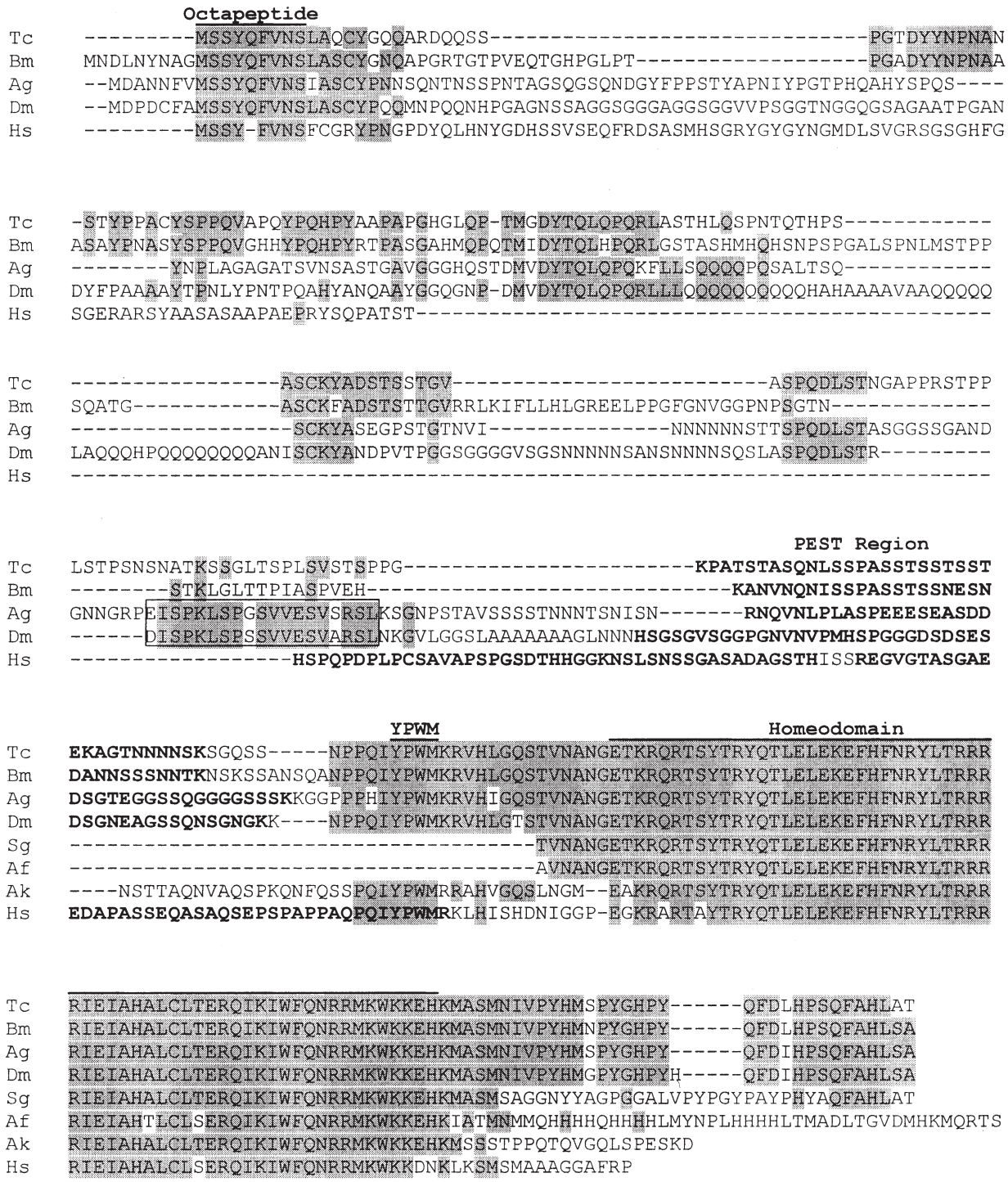

Figure 3. Comparison of *Scr* cognates. *Scr* cognates from *Tribolium castaneum* (*Tc*) (Genbank accession #AF227628), *Schistocerca gregaria* (*Sg*) (S36448), *Bombyx mori* (*Bm*) (BAA76868), *Anopheles gambiae* (*Ag*) (AAC31944), *Drosophila melanogaster* (*Dm*) (AAD19795), *Artemia fransiscani* (*Af*) (X70080), *Acanthokara kaputensis* (*Ak*) (AAB92411), and *Homo sapiens* (*Hs*) (AAB97946) are compared. Regions of identity matching the consensus are shaded gray. The octapeptide, YPWM motif, and homeodomain are overlined. The dipteran-specific motif is boxed. Potential PEST sequences are in bold and include the positively charged flanking residues.

dermal expression in the thorax. As limb buds form, *TcScr* is expressed in the ectoderm of the labial appendage primordia, and the thoracic signal is redistributed to reflect the association of mesoderm with developing limbs there (Figure 4f & 4j). As the limb buds elongate, protein accumulates at higher levels at the base of each labial appendage (Figure 4g). *TcScr* is also expressed in a few cells within the mesoderm of the mandibular appendage and within

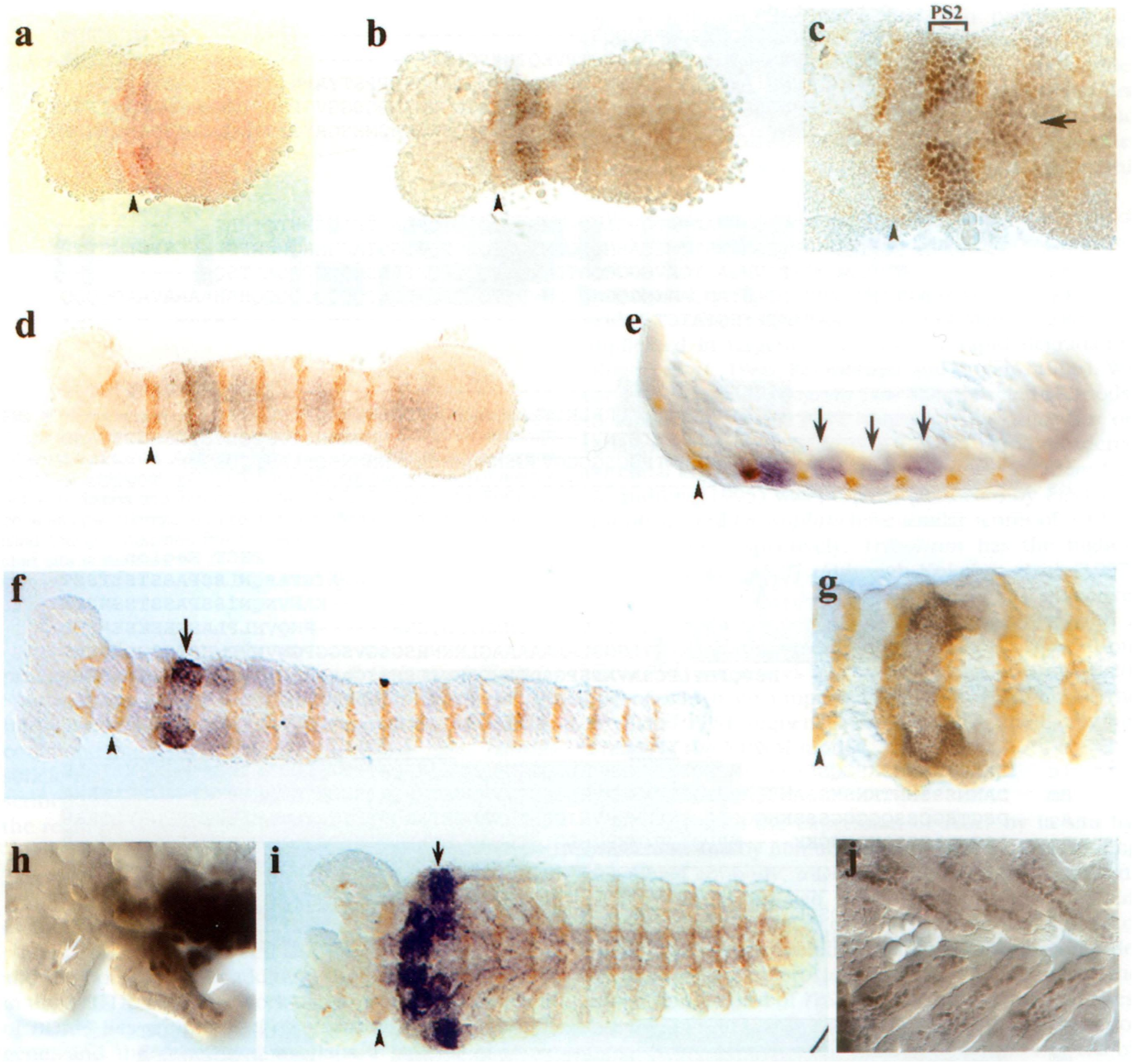

Figure 4. Expression pattern of the *TcScr* transcript and protein. All panels, with the exception of e (*TcScr* transcripts in a lateral view of the germband), show *TcScr* protein localization in ventral views of dissected embryos. The black arrowhead points to the mandibular Engrailed stripe (gold). *TcScr* gene products are visualized by purple/black staining, **(a-d)** *TcScr* expression in germ rudiment and early elongation stages, **(c)** A high-magnification view of the embryo shown in **b**, with an arrow pointing to the T1 ventral mesoderm. **(e)** The stained thoracic mesoderm (arrows) has invaginated and lies above the unstained ectoderm. **(f)** At the extended germband stage, *TcScr* expression is most intense in the labial palps (arrow) and in the mesoderm of the thoracic limb buds. **(g)** As the labial appendages develop, *TcScr* expression is more intense in the basal portion of the limb bud. **(h)** In this high-magnification view of mandibular and maxillary appendages, two out of three cells within the mandibular mesoderm that express *TcScr* are in focus (white arrow). Although *TcScr* expression in the base of the maxillary limb is obscured by the intense staining of the underlying body wall, *TcScr* expression is evident in the posterior compartment of more distal regions of this appendage (white arrowhead). **(i)** By the retracted germband stage, additional *TcScr* expression is evident in dorsal T1a (arrow) and the central nervous system. **(j)** *TcScr* is expressed throughout the mesoderm of the embryonic legs.

a few cells of the posterior compartment of the maxillary limb (Figure 4h). It is uncertain whether the *TcScr*-positive cells in the mandible are of mesodermal or peripheral nervous system origin. By the completion of germband retraction, *TcScr* expression is present in the anterior dorsal T1 ectoderm and in a segmentally repeated pattern in the developing central nervous system in PS1 and all posterior parasegments (Figure 4i).

RNA Interference

Pioneering studies in *C. elegans* have shown that injection of double-stranded RNA representing a particular gene results in a phenocopy of that gene's mutant phenotype (Fire et al., 1998). This methodology has been shown to work in several insect species as well (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Hughes and Kaufman, 2000), including *Tribolium* (Brown et al., 1999; Shippy et al., 2000; DeCamillis et al., 2000). If *Cx* and *Scr* are orthologous, we predict that injection of *TcScr* dsRNA will phenocopy *Cx* LOF variants.

Double-stranded RNA was synthesized using pcCx2 as a template and injected into precellular *Tribolium* embryos. In an experiment involving about 300 injected eggs, ca. 60% hatched and all were abnormal. The phenotype of some of these individuals was documented by scanning electron microscopy (Figure 5). The abnormal larvae appeared to display a common syndrome of variable severity. The most severely affected individuals displayed a complete phenocopy of the LOF mutant phenotype of *mxp^{Stm}Cx5/Df(HOMC)*, including a transformation of labial appendages to antennae and a fusion of the labial and T1 segments (Figure 5b & 5c). Many larvae had less complete transformations of the labial appendages and incomplete fusion of the labial and T1 segments, resembling the range of effects characteristic of larvae homozygous for the hypomorphic allele *Cx^{6}* (C. Curtis, unpublished observations). These data argue that *Cx* variants result from mutation of the molecularly defined *TcScr* gene and that *Cx* and *Scr* are orthologous.

Discussion

In *Drosophila* and other higher flies, the larval appendages are reduced to vestiges and the head involutes through the presumptive mouth to form specialized internal structures. Many of the ancestral functions of the anterior Hox genes have presumably been modified during the evolution of these developmental events (Rogers et al., 1997). In contrast, head development and morphology in *Tribolium* are much more typical of ancestral insects. In this work, we characterize the *Tribolium* ortholog of *Scr* and compare its organization, sequence, and expression pattern with those of *Scr* family members in *Drosophila* and other organisms. We also provide evidence from RNAi analysis that supports the assignment of *TcScr* to the genetically defined *Cephalothorax* locus.

The transcription units of *TcScr* (22 kb) and *Scr* (27 kb) are similar in size but differ somewhat in intron/exon organization. In *Drosophila*, a 15.5-kb intron separates the YPWM-encoding region and the homeobox. *TcScr* has a 20-kb intron in the same position. The positions of other introns are not conserved. For example, *TcScr* contains an intron in the 3′ UTR, but no intron has been reported in the 3′ UTR of *DmScr*. A single *TcScr* transcript has been identified thus far. However, *Drosophila* may have

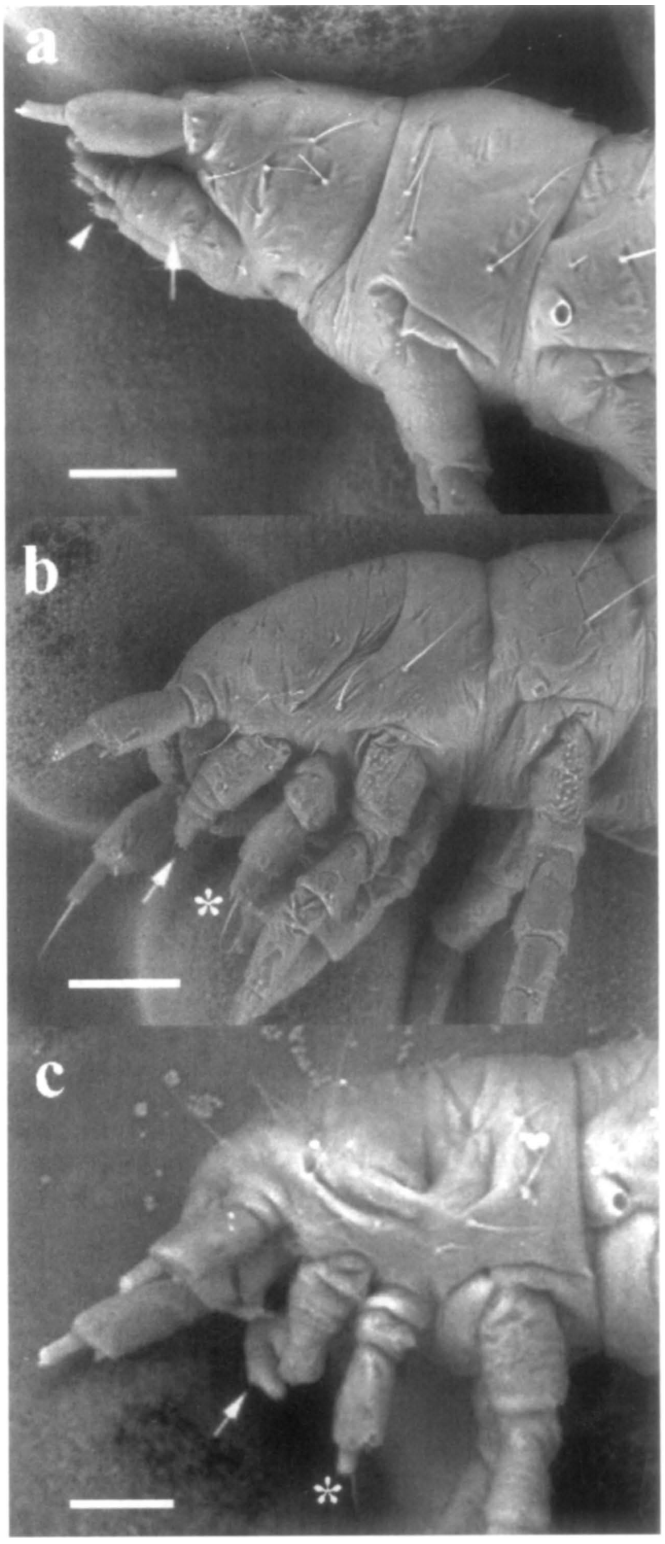

Figure 5. RNA interference with *TcScr* phenocopies a *Cx* mutant These scanning electron micrographs show anterior portions of **(a)** wild-type, **(b)** *mxp^{Stm} Cx^{5}/Df(HOMC)*, and **(c)** RNAi larvae. In each panel the arrow points to one of the maxillary appendages. In **a** the arrowhead points to the labial palps, while in **b** and **c** the asterisks denote labial appendages that are homeotically transformed to antennae. Note the fusion of the T1 segment with the head in **b** and **c**. Scale bars = 50 μm.

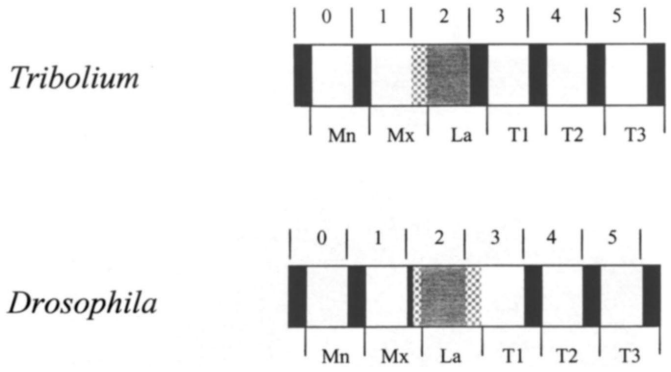

Figure 6. Early ectodermal expression domains of *Scr* in *Tribolium* and *Drosophila*. This schematic shows the differences in the expression pattern of *TcScr* and *Scr*. Larval gnathal (Mn, mandible; MX, maxillary; La, labial) and thoracic segments are denoted below each figure. Parasegments are listed above. Thick black lines show expression of Engrailed protein in the posterior compartment of segments. Gray boxes show *Scr* expression, and hatched lines represent overlap of *Scr* and Engrailed expression.

as many as four *Scr* transcripts, two of which are known to originate via the use of alternative 5′ exons (Andrew, 1995).

By comparison of *Tribolium Scr* with available full-length orthologous sequences from insects and a representative mammalian sequence (Figure 3), we show that *TcScr* contains the three apparently ancestral motifs (octapeptide, YPWM, and homeodomain) common to *Scr* cognates (Odenwald et al., 1987). Several previously identified insect-specific motifs are conserved in *TcScr* (Figure 3). *Tribolium* and *Bombyx Scr* are more similar to one another than either is to the dipteran proteins. This similarity includes conserved residues within the PEST region. The *Bombyx*- and *Tribolium*-specific motifs may have been present in the insect ancestor but lost in the dipteran lineage. The dipteran proteins share an amino acid sequence upstream of the PEST region that is not found in the *Tribolium* or *Bombyx* orthologs, suggesting that it arose after the separation of the lepidoteran and dipteran lineages.

In the long-germ insects *Apis mellifera* (honeybee) and *Drosophila*, *Scr* is expressed at the cellular blastoderm stage just posterior to *Deformed* (Walldorf et al., 2000). *TcDeformed* is similarly expressed at the cellular blastoderm stage in the short-germ insect *Tribolium* (Brown et al., 1999). However, *TcScr* expression is delayed until after the formation of the germband.

Early ectodermal expression of *TcScr* is clearly parasegmental and restricted to PS2, while in other insects early expression appears neither segmental or parasegmental (Rogers and Kaufman, 1997). *DmScr* is expressed in a few cells of the posterior maxillary compartment, while *TcScr* is expressed throughout this compartment (Figure 6). In contrast to all other insects studied to date, *TcScr* is not expressed in the posterior labial compartment at this time. Thus, in being parasegmental, the initial expression of *TcScr* more closely resembles that of the posterior Hox genes.

During development, ectodermal expression of *TcScr* is dynamic. Although the anterior border of expression in the body wall remains the same, posterior expression appears in the dorsal region of anterior T1. In having only dorsal T1 expression, *Tribolium* resembles all other insects studied to date except *Drosophila*, for which there is ventral T1 expression as well. In the gnathal segments, *TcScr* is expressed in the basal region of the posterior compartment of the maxillary appendages and throughout the labial appendages. As noted above, *TcScr* expression is not observed in the posterior labium prior to appendage formation and appears *de novo* in the posterior compartment of the appendage. Thus *TcScr* seems to be regulated differently in the labial appendage and the body wall.

Similar to other pterygotes with embryonic appendages (Rogers et al., 1997), *Tribolium* displays *TcScr* expression in the early mesoderm of all thoracic segments. (Such expression is absent in the T2 and T3 mesoderm of the limbless maggot [Mahaffey and Kaufman, 1987]). Later in development, *TcScr* expression is present in the mesoderm of all three pairs of larval thoracic legs. Percival-Smith et al. (1997) have suggested that during imaginal leg development in *Drosophila*, mesodermal expression of *Scr* induces events necessary for normal tarsal development. This does not appear to be the case in *Tribolium*—except for the orientation of the T1 limbs, the morphology of all three pairs of larval legs is normal in *Cx* (*TcScr*) LOF mutants.

We have shown here that *TcScr* differs from *Scr* in being expressed to a greater extent in the posterior maxillary segment, in being quiescent in the early posterior labium, and in being expressed in the T2 and T3 mesoderm. Nevertheless, the effects of LOF mutations in both insects are restricted to the labial and T1 segments. The larval mutant phenotypes are, however, quite different. A more detailed description of the mutant phenotypes of *Cx* variants is underway, and combining such observations with gene interaction studies will allow a better understanding of the differences in the functions of beetle and fly *Scr* orthologs.

Several lines of evidence lead to the conclusion that the genetically defined *Cx* locus corresponds to *TcScr*, the molecularly defined *Scr* ortholog. Here we show that *TcScr* lies between *TcDfd* and *Tcftz*. That is, it occupies the same relative position in the *Tribolium* Homeotic complex as does *Scr* in the *Drosophila* Antennapedia complex. Previous evidence indicates that the *Cx* locus maps to a similar position. Beeman (1987) mapped a *Cx* mutant allele between mxp^1 and *mas*, alleles of the *Tribolium* orthologs of *probocipedia* and *abdominal-A* (*abd-A*), respectively. Moreover, *Cx* mutant alleles fail to complement *Df(HOMC)*, a deficiency that removes a region extending from the *Dfd* through a*bd-A* orthologs. Mutations of *Cx* and *Scr* are similar in that they each affect the development of the labial and T1 segments (Wakimoto et al., 1984; Sato et al., 1985; Pattatucci and Kaufman, 1991). In addition, depletion of *TcScr* transcripts phenocopies *Cx* LOF mutants. Finally,

we have evidence that a *Cx* variant is associated with partial deletion of *TcScr* (C. Curtis, unpublished observations). Thus we conclude that *Cx* is the *Tribolium* ortholog of *Scr*.

Materials And Methods

Cloning and Molecular Analysis of TcScr

A *Tribolium* embronic cDNA library constructed in lambda gt 11 (BRL) was screened at low stringency (McGinnis et al., 1984), using a ^{32}P-labeled *Drosophila Scr* cDNA containing the homeobox. The resulting partial cDNA, pcCx1, was used to probe another *Tribolium* embryonic cDNA library constructed in pCMV●SPORT 4.0 (Gibco BRL) (Shippy et al., 2000). This screen yielded clone pcCx2, which was determined by sequencing to contain the entire coding sequence. pcCxl was used to screen a BAC genomic library (Denell and Brown, unpublished). A physical map of one BAC clone, BDfd3, was constructed as described by Shippy et al. (2000). The resulting subclones were linearized with *Hin*dIII, separated by gel electrophoresis, transferred to GeneScreen (NEN Life Sciences) nylon membrane, and analyzed by hybridization to ^{32}P-labeled pcCxl. Subclones that hybridized to pcCxl were sequenced to determine exon/intron junctions.

Total RNA and mRNA were isolated using the RNeasy and Oligotex kits (Qiagen), respectively. ^{32}P-labeled pcCxl was used for northern analysis as described by Brown et al. (1994b).

Sequence Analysis and Alignment Programs

Sequencing was performed using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Pharmacia) or by automated fluorescent sequencing (Iowa State University DNA Sequencing Facility and Kansas State University Veterinary Medicine). BLAST searches were used to identify similar genes and proteins. DNA analysis was performed using Lasergene (DNASTAR). Protein alignments were computer generated using Vector Nti (Informax) and then manually adjusted. PEST sequences were identified using the PESTfind computer program (Rogers et al., 1986). This program is accessible at http://www.at.embnet.org/embnet/tools/bio/PESTfind/.

Immunostaining and In Situ Hybridization

Immunostaining and *in situ* hybridization were performed as described by Carroll et al. (1988). For both protocols, 0-96 h wild-type embryos were dechorionated and fixed (Brown et al., 1994a). A cross-reacting, polyclonal antibody to *Drosophila Scr* (a gift from Dr. Thomas Kaufman) was used to stain *TcScr*. TcEngrailed, a posterior compartment marker, was detected with the Mab 4D9 antibody (Patel et al., 1989).

For *in situ* hybridization, the Dig RNA Labeling Kit (Boehringer Mannheim/Roche) was used to generate a digoxigenin-labeled antisense riboprobe from the pcCx2 template. Hybridization was performed as described by Brown et al. (1994a).

RNA Interference

Sense and antisense RNAs were transcribed from pcCx2 as described previously (Brown et al., 1999; Shippy et al., 2000). The strands were annealed and the double-stranded RNA injected into wild-type embryos (Brown et al., 1999). Embryos were incubated at 25°C for eight days. Larvae that hatched during this time were either transferred to lactic acid/ethanol (Brown et al., 1999) for cuticle preparation or analyzed by scanning electron microscopy. Eggs that had not hatched were also processed for cuticle preparations.

Scanning Electron Microscopy

Scanning electron micrographs were taken of wild-type, mxp^{Stm} $Cx^5/Df(HOMC)$, and RNAi larvae. GA-1 and mxp^{stm} Cx^5/Es stocks were placed on Gold Medal flour (General Mills) with 5% yeast. Stocks were incubated at 25°C for 48 h. Eggs were collected and washed in 2.5% bleach to remove any residual flour. Eggs were then laid out on microscope slides and incubated at 25°C for 8 days. Newly hatched larvae were stored at −70°C. Frozen larvae were then mounted on a sample stub on double-coated carbon conductive tape and then placed on a cold stage (Oxford Instruments Microanalysis Limited) in a Hitachi S-3500N scanning electron microscope (Hitachi Science Systems) at the Kansas Agricultural Experiment Station Scanning Electron Microscope Laboratory, Kansas State University Entomology Department. The internal chamber was cooled to –180°C with vacuum pressure of 80 Pa. The sample was then warmed to −70°C to decrease frost. Images were captured with a backscatter detector (ETP-USA/Electron Detectors). An accelerating voltage of 25 kV was used.

Acknowledgments

We give thanks to Barbara Van Slyke for constructing the BAC library, Kay Hummels for assistance with expression experiments, Richard Beeman and Sue Haas for identifying the *Cx* mutants, and Marcé Lorenzen for performing the RNAi injections. We also thank Thomas Kaufman for supplying the α-DmScr antibody and Nipam Patel for providing the antibody to Engrailed. This work is supported by NSF and NIH grants to R.E.D. and S.J.B. C.D.C. is supported by an NIH Virology/Oncology predoctoral training grant. M.A.D. was supported by a HFSP postdoctoral fellowship.

Literature Cited

Andrew DJ. 1995. The *Sex combs reduced* gene of *Drosophila melanogaster* has multiple transcripts. *Gene* **152**:149-155.

Beeman RW. 1987. A homoeotic gene cluster in the red flour beetle. *Nature* **327**:247-249.

Beeman RW, Stuart JJ, Brown SJ, Denell RE. 1993. Structure and function of the Homeotic gene complex (HOM-C) in the beetle, *Tribolium castaneum*. *BioEssays* **15**:439-444.

Beeman RW, Stuart JJ, Haas MS, Denell RE. 1989. Genetic analysis of the homeotic gene complex (HOM-C) in the beetle *Tribolium castaneum*. *Dev Biol* **133**:196-209.

Brown SJ, Hilgenfeld RB, Denell RE. 1994a. The beetle *Tribolium castaneum* has a fushi tarazu homolog expressed in stripes during segmentation. *Proc Natl Acad Sci USA* **91**:12922-12926.

Brown SJ, Mahaffey JP, Lorenzen ML, Denell RE, Mahaffey JW. 1999. Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. *Evol Dev* **1**:11-15.

Brown SJ, Patel NH, Denell RE. 1994b. Embryonic expression of the single *Tribolium* engrailed homolog. *Dev Genet* **15**:7-18.

Carroll SB, DiNardo S, O'Farrell PH, White RAH, Scott MP. 1988. Temporal and spatial relationships between segmentation and homoeotic gene expression in *Drosophila* embryos: Distributions of the *fushi tarazu, engrailed, Sex combs reduced*, *Antennapedia* and *Ultrabithorax* proteins. *Genes Dev* **2**:350-360.

DeCamillis MA, Lewis DL, Brown SJ, Beeman RW, Denell RE. 2001. Interactions of the *Tribolium Sex combs reduced* and *proboscipedia* orthologs in embryonic labial development, (submitted)

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**:806-811.

Gibson G, Schier A, LeMotte P, Gehring WJ. 1990. The specificities of sex combs reduced and Antennapedia are defined by a distinct portion of each protein that includes the homeodomain. *Cell* **62**:1087-1103.

Hughes CL, Kaufman TC. 2000. RNAi analysis of *Deformed*, *proboscipedia* and *Sex combs reduced* in the milkweed bug *Oncopeltus fasciatus*: Novel roles for Hox genes in the Hemipteran head. *Development* **127**:3683-3694.

Kennerdell JR, Carthew RW. 1998. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. *Cell* **95**:1017-1026.

Kokubo H, Ueno K, Amanai K, Suzuki Y. 1997. Involvement of the *Bombyx Scr* gene in development of the embryonic silk gland. *Dev Biol* **186**:46-57.

Mahaffey J, Kaufman TC. 1987. Distribution of the *Sex combs reduced* gene products in *Drosophila melanogaster*. *Genetics* **117**:51-60.

McGinnis W, Krumlauf R. 1992. Homeobox genes and axial patterning. *Cell* **68**:283-302.

McGinnis W, Levine MS, Hafen E, Kuroiwa A, Gehring W. 1984. A conserved DNA sequence in homeotic genes of the *Drosophila* Antennapedia and bithorax complexes. Nature **308**:428-433.

Misquitta L, Paterson BM. 1999. Targeted disruption of gene function in *Drosophila* by RNA interference (RNAi): A role for *nautilus* in embryonic somatic muscle formation. *Proc Natl Acad Sci USA* **96**:1451-1456.

Odenwald WF, Taylor CF, Palmer-Hill FJ, Friedrich JV, Tani M, Lazzarini RA. 1987. Expression of a homeodomain protein in noncontact-inhibited cultured cells and post-mitotic neurons. *Genes Dev* **1**:482-496.

Patel NH, Kornberg TB, Goodman CS. 1989. Expression of *engrailed* during segmentation in grasshopper and crayfish. *Development* **107**:201-212.

Pattatucci AM, Kaufman TC. 1991. The Homeotic gene *Sex combs reduced* of *Drosophila melanogaster* is differentially regulated in the embryonic and imaginal stages of development. *Genetics* **129**:443-461.

Pattatucci AM, Otteson DC, Kaufman TC. 1991. A functional and structural analysis of the *Sex combs reduced* locus of *Drosophila melanogaster*. *Genetics* **129**:423-441.

Pederson J, Kiehart D, Mahaffey J. 1996. The role of HOM-C genes in segmental transformations: Re-examination of the *Drosophila Sex combs reduced* embryonic phenotype. Dev Biol **180**:131-142.

Percival-Smith A, Hayden DJ. 1998. Analysis in *Drosophila* melanogaster of the interaction between *Sex combs reduced* and extradenticle activity in the determination of tarsus and arista identity. Genetics **150**:189-198.

Percival-Smith A, Weber J, Gilfoyle E, Wilson P. 1997. Genetic characterization of the role of the two HOX proteins, *Proboscipedia* and *Sex Combs Reduced,* in determination of adult antennal, tarsal, maxillary palp and proboscis identities in *Drosophila melanogaster*. *Development* **124**:5049-5062.

Powers TP, Hogan J, Ke Z, Dymbrowski K, Wang X, Collins FH. 2000. Characterization of the Hox cluster from the mosquito *Anopheles gambiae* (Diptera; culicidae). *Evol Dev* **2**:311-325.

Rechsteiner M, Rogers SW. 1996. PEST sequences and regulation by proteolysis. *TIBS* **21**:267-271.

Rogers BT, Kaufman TC. 1997. Structure of the insect head in ontogeny and phylogeny: A view from *Drosophila*. *Int Rev Cytol* **174**:1-84.

Rogers BT, Peterson MD, Kaufman TC. 1997. Evolution of the insect body plan as revealed by the *Sex combs reduced* expression pattern. *Development* **124**:149-157.

Rogers S, Wells R, Rechsteiner M. 1986. Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* **234**:364-368.

Sato T, Hayes PH, Denell RE. 1985. Homeosis in *Drosophila*: Roles and spatial patterns of expression of the *Antennapedia* and *Sex combs reduced* loci in embryogenesis. *Dev Biol* **111**:171-192.

Shippy TD, Brown SJ, Denell RE. 2000. *Maxillopedia* is the *Tribolium* ortholog of *proboscipedia*. *Evol Dev* **2**:145-151.

Wakimoto BT, Turner FR, Kaufman TC. 1984. Defects in embryogenesis in mutants associated with the *Antennapedia* gene complex of *Drosophila melanogaster*. *Dev Biol* **102**:147-172.

Walldorf U, Binner P, Fleig R. 2000. Hox genes in the honey bee *Apis mellifera*. *Dev Genes Evol* **210**:483-492.