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

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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...
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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...
as containing TcDeformed (TcDfd). Southern analysis of HindIII restriction digests of the BAC clones revealed that only one, BDFd3, potentially contained the entire TcScr transcription unit. HindIII 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. HOXAS 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, a-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 abdominal segment (Figure 4B, shown at higher magnification in Figure 4C). 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.

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.
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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 the dermal expression in the thorax.

<table>
<thead>
<tr>
<th>Octapeptide</th>
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<td>KAPTSASQNLSAPSTSTSSST</td>
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</tr>
<tr>
<td>Bm</td>
<td>KAVNQNSPASDSNASNS</td>
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<tr>
<td>Hs</td>
<td>EDAPASSEQAQSEPSPAQPQOTVPMKLIHSNDIPEG</td>
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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 franciscana (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.
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 mxpStmCx5/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 Cx6 (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) mxpStm Cx5/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 lepidopteran 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 (Wallendorf 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 germ band.

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 mzp1 and mas, alleles of the Tribolium orthologs of proboscipedia 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 abd-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 embryonic cDNA library constructed in lambda gt 11 (BRL) was screened at low stringency (McGinnis et al., 1984), using a 32P-labeled Drosophila Scr cDNA containing the homeobox. The resulting partial cDNA, pcCx1, was used to probe another Tribolium embryonic cDNA library constructed in pCMVSPORT 4.0 (Gibco BRL) (Shippy et al., 2000). This screen yielded clone pcCx2, which was determined by sequencing to contain the entire coding sequence. pcCx1 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 HindIII, separated by gel electrophoresis, transferred to GeneScreen (NEN Life Sciences) nylon membrane, and analyzed by hybridization to 32P-labeled pcCx1. Subclones that hybridized to pcCx1 were sequenced to determine exon/intron junctions.

Total RNA and mRNA were isolated using the RNeasy and Oligotex kits (Qiagen), respectively. 32P-labeled pcCx1 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, mxpStm Cx5/Df(HOMC), and RNAi larvae. GA-1 and mxpStm Cx5/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/Onco Therapy predoctoral training grant. M.A.D. was supported by a HFSP postdoctoral fellowship.

Literature Cited


