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The *Tribolium castaneum* Ortholog of *Sex combs reduced* Controls Dorsal Ridge Development

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ABSTRACT

In insects, the boundary between the embryonic head and thorax is formed by the dorsal ridge, a fused structure composed of portions of the maxillary and labial segments. However, the mechanisms that promote development of this unusual structure remain a mystery. In Drosophila, mutations in the Hox genes *Sex combs reduced* and *Deformed* have been reported to cause abnormal dorsal ridge formation, but the significance of these abnormalities is not clear. We have identified three mutant allele classes of *Cephalothorax*, the *Tribolium castaneum* (red flour beetle) ortholog of *Sex combs reduced*, each of which has a different effect on dorsal ridge development. By using Engrailed expression to monitor dorsal ridge development in these mutants, we demonstrate that *Cephalothorax* promotes the fusion and subsequent dorsolateral extension of the maxillary and labial Engrailed stripes (posterior compartments) during dorsal ridge formation. Molecular and genetic analysis of these alleles indicates that the N terminus of Cephalothorax is important for the fusion step, but is dispensable for Engrailed stripe extension. Thus, we find that specific regions of Cephalothorax are required for discrete steps in dorsal ridge formation.

A defining feature of the insect body plan is the grouping of segments into three regions, or tagmata: the head, thorax, and abdomen. In most, if not all, insect species the boundary between the embryonic head and thorax is formed by the dorsal ridge, which develops by fusion of the dorsolateral components of the gnathal segments (post-oral head) (Rogers and Kaufman 1996). In some insects, the dorsal ridge appears as a discrete dorsal lobe between the head and thorax, while in others it is highly reduced and fused to the thorax, or not visible at all (Rogers and Kaufman 1997). In the fruit fly, *Drosophila melanogaster*, the larval head is dramatically reorganized during the process of head involution. A dorsal ridge still forms between the head and thorax but is subsequently internalized to form the dorsal pouch (Younossi-Hartenstein *et al.* 1993).

The initial development of the dorsal ridge is highly conserved among insects (Rogers and Kaufman 1996). The first evidence of dorsal ridge formation is the expression of the segment polarity gene *engrailed* (*en*) along the lateral edge of the anterior compartment of the labial segment. This unusual En domain connects the stripes of En expression in the posterior compartments of the maxillary and labial segments. A single En stripe then extends dorsally from this region as part of the dorsal ridge. In Drosophila, the Hox gene *Deformed* (*Dfd*) is expressed anterior to and in the anterior portion of this En stripe (presumably the maxillary portion of the dorsal ridge) (Rogers and Kaufman 1997). *Sex combs reduced* (*Scr*) is expressed in the posterior portion of the En stripe (Gorman and Kaufman 1995). Mutations in *Dfd* and *Scr* are reported to cause abnormal dorsal ridge formation (Rogers and Kaufman 1997), but no description of these abnormalities has been published. Interpretation of these phenotypes may be complicated by the internalization of the Drosophila dorsal ridge during head involution.

In contrast, head development in the red flour beetle, *Tribolium castaneum*, follows a more generalized plan. Unlike the internalized head of the Drosophila larva, the Tribolium larval head is articulated with the trunk and has external gnathal appendages, allowing the study of genetic regulatory interactions in more typical insect gnathal development. Thus, Tribolium provides an attractive system in which to analyze the regulation of dorsal ridge development. In this work, we first describe formation of the dorsal ridge during wild-type Tribolium development. Then, using a combination of genetic analysis and expression studies, we demonstrate

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that *Cephalothorax* (*Cx*), the Tribolium ortholog of *Scr* (Curtis *et al.* 2001), is essential for two discrete steps in dorsal ridge development: (1) fusion of the maxillary and labial En stripes and (2) extension of En stripes to the dorsolateral edges of the embryo. Furthermore, we find that the N-terminal domain of Cx is required for En stripe fusion, but is dispensable for En stripe extension.

MATERIALS AND METHODS

Genetic analysis: Cx^{E} and Cx^{61} were isolated in an EMS mutagenesis of *Eyeless-lethal free*, a stock created by using the *Eyeless* balancer chromosome to isogenize and homozygose the region of LG2 containing the HOMC.

Stocks were maintained on whole wheat flour supplemented with 5% brewer's yeast (Beeman *et al.* 1989). The balancer chromosomes $Abdominal^{Extra\ sclerite}$ (A^{Es}) and *Eyeless* (*Ey*) and the recessive lethal mutation $Abdominal^{83}$ (A^{83}) were used to facilitate maintenance of mutant lines. The following strains were used: Ga-1 (wild type), Cx^{6}/A^{Es}, Cx^{61}/A^{Es}, Cx^{20}/A^{Es}, Cx^{E}/A^{Es}, mxp^{Stm}/A^{83}, and *Df(HOMC)/Ey*.

After mating, eggs were collected at 2-day intervals and allowed to develop at 30°. Scanning electron micrographs were obtained as described by Curtis *et al.* (2001). For cuticle preps, newly hatched larvae were placed in lactic acid:ethanol (9:1), and after 7 days, all remaining unhatched eggs were placed in lactic acid:ethanol.

Southern analysis: Genomic DNA was isolated from Ga-1 (wild-type) and Cx^{20}/A^{Es} beetles (Brown *et al.* 1990). DNA (2 μg) was digested with *Hin*dIII. The resulting DNA fragments were separated by field inversion gel electrophoresis and transferred to GeneScreen membrane (NEN Life Sciences). The membrane was hybridized with a radiolabeled 3-kb fragment containing the first exon of the *Cx* gene.

Molecular analysis of mutant alleles: In preparation for inverse PCR, genomic DNA (2 μg) from Cx^{20} heterozygotes was digested with *Hin*dIII, purified with the High Pure PCR product purification kit (Roche Applied Science), diluted to ~1 ng/μl, and ligated at 16° overnight. The ligation product (10 ng) was used as template for inverse PCR using Dynazyme EXT (Finnzymes-MJ Research). Amplified products were visualized by agarose gel electrophoresis. DNA from the two resulting bands (wild type and mutant) was ligated into the PCR 4-TOPO vector (Invitrogen, San Diego), and the plasmid inserts were sequenced at the Sequencing and Genotyping Facility (Department of Plant Pathology, Kansas State University).

To amplify the exons of mutant alleles, individual homozygous or hemizygous larvae were homogenized as described by Gloor *et al.* (1993). These homogenates were used as templates for Ready-To-Go bead (Amersham Pharmacia Biotech) or DyNAzyme EXT (Finnzymes) PCR reactions under the conditions described by Gloor *et al.* (1993). Amplified products were purified with the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA) and then directly sequenced with internal primers. Two sequences, one for each exon of the *Cx* gene, were submitted to GenBank for each mutant allele (see results for accession numbers).

Putative translation start codons were identified with the ATGpr program created by Salamov *et al.* (1998). ATGpr is found online at http://www.hri.co.jp/atgpr/.

Expression analysis: Immunostaining of 0- to 96-hr embryos was performed as described by Carroll *et al.* (1988). To detect Tc Engrailed expression (hereafter referred to simply as Engrailed), we used 4D9, a monoclonal antibody to Drosophila Engrailed/Invected developed by Corey Goodman (Patel *et al.* 1989), which was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, Iowa. A cross-reacting polyclonal antibody to Drosophila Scr, α-DmScr (a gift from Thomas Kaufman), was used to detect Cx. After staining, embryos were dissected from yolk and documented using bright-field or differential interference contrast illumination.

Tc hedgehog (*Tc hh*) riboprobe was synthesized from a cDNA clone kindly provided by Yoshinori Tomoyasu. *In situ* hybridization with *Tc hh* and immunostaining with MAb 4D9 were performed as described by Nagaso *et al.* (2001).

RESULTS

Dorsal ridge development in Tribolium: To follow the events of dorsal ridge development in Tribolium, we used a cross-reacting antibody to Drosophila Invected to detect the Tribolium Engrailed (En) protein, which is expressed in the posterior part of each segment (Brown *et al.* 1994). At the extended germband stage, En expression appears along the lateral edge of the anterior compartment of the labial segment (Figure 1, A and D). As has been described for the milkweed bug and cricket (Rogers and Kaufman 1996), En expression appears simultaneously in the entire row of cells and connects the maxillary and labial En stripes. As the embryo begins to dorsally close, the thoracic and abdominal En stripes extend dorsolaterally, such that they eventually encircle the embryo. Likewise, the single fused dorsal ridge En stripe extends dorsolaterally, initially as a broad stripe (Figure 1, B and E), but later becomes much narrower (Figure 1, C and F). The mechanisms underlying dorsal closure are not well understood in Tribolium. It is thought that at least some of the dorsolateral expansion of the embryo is due to cell division, but it is quite possible that cell rearrangements also play a role in this process. Because of this uncertainty, we have chosen the term "extension" rather than "growth" to describe the behavior of the dorsal ridge and trunk En stripes.

In Drosophila, the Hox genes *Dfd* and *Scr* are expressed in portions of the dorsal ridge (Gorman and Kaufman 1995; Rogers and Kaufman 1997). Brown *et al.* (1999) observed expression of the Tribolium *Dfd* ortholog in the dorsal ridge. We have examined expression of Cephalothorax (Cx), the Tribolium ortholog of Scr, and find that, similar to Scr, it is expressed in the posterior region of the developing dorsal ridge (Figure 1, G–I).

***Cx* mutant phenotypes suggest a possible role in dorsal ridge development:** Two classes of *Cx* mutations that affect the boundary between head and thorax in Tribolium embryos have been described (Beeman *et al.* 1993). In one class, the segmental groove between the two tagma is absent, while in the other a supernumerary dorsal segment appears between the head and thorax. These phenotypes, in combination with expression

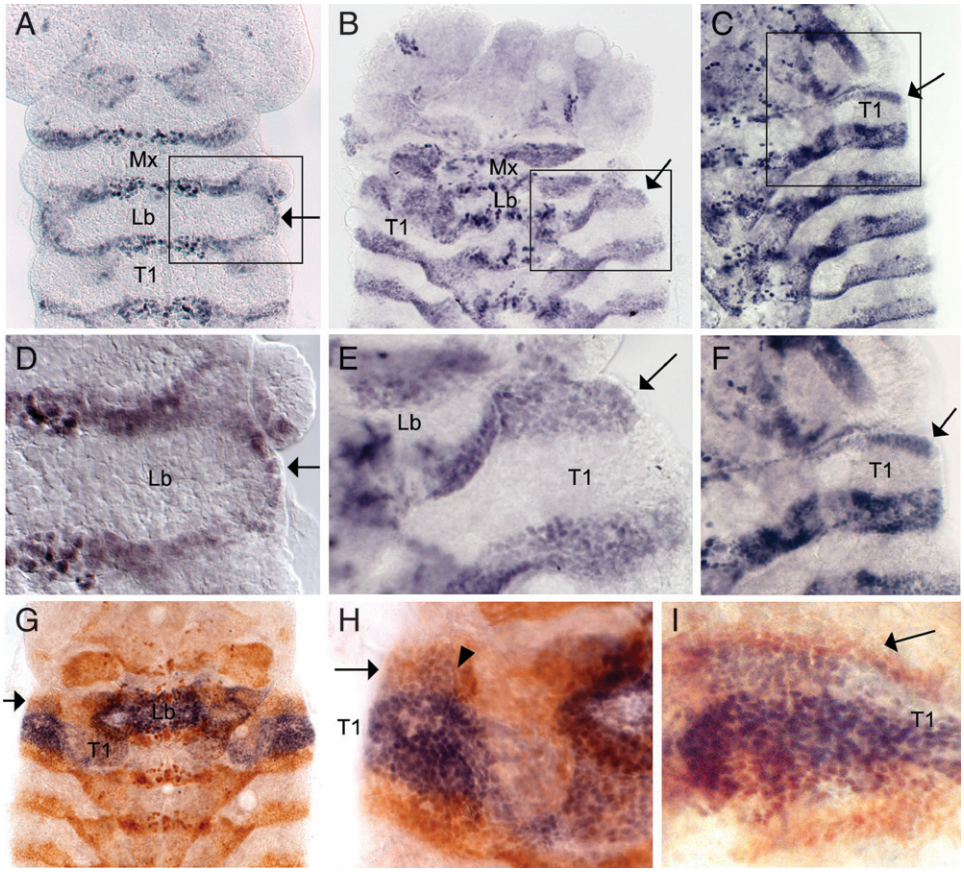

Figure 1.—Dorsal ridge development in wild-type embryos. En expression in the dorsal ridge is indicated by an arrow in A–I. Relevant segments are labeled as follows: maxillary (Mx), labial (Lb), prothoracic (T1). (A–F) En expression (purple) in the wild-type dorsal ridge. (A) An extended germband-stage embryo viewed from the yolk side to provide a clearer view of Engrailed expression along the lateral edges of the labial segment. (B) As dorsal closure begins, a patch of En expression (apparently representing a fusion of the maxillary and labial En stripes) extends dorsally. (C) As dorsal closure continues, the dorsal ridge En stripe becomes significantly narrower than the other extending En stripes. (D) An enlarged view of the region boxed in A. (E) An enlarged view of the region boxed in B. (F) An enlarged view of the region boxed in C. (G–I) Cx expression (purple) and En expression (brown) in the wild-type dorsal ridge. (G) Early in the dorsal closure process, Cx is expressed in a subset of the En-expressing dorsal ridge cells. (H) An enlarged view of G showing the dorsal ridge cells that coexpress Cx and En (arrowhead). (I) As the dorsal ridge En stripe extends and narrows, Cx expression is confined to the posterior half of the En stripe.

data, suggest that *Cx* may be required for normal development of the dorsal ridge. To address this possibility, we have performed phenotypic, molecular, and expression analyses of four Cx alleles, placing particular emphasis on their effects on dorsal ridge development.

Cx null mutants lack the boundary between head and thorax: Beeman *et al.* (1993) described a class of *Cx* alleles that cause fusion of the first thoracic segment (T1) with the head (cephalization), as well as transformation of the larval labial appendages to antennae. These alleles were deemed likely to be nulls, since identical effects were observed in homozygotes and hemizygotes [individuals carrying one copy of the mutant allele and one copy of *Df(HOMC)*, a deficiency that removes *Cx* and several other Hox genes]. We have characterized two alleles in this class: Cx^{6} (Beeman *et al.* 1989) and Cx^{61} (this work). Our analysis of Cx^{6} (Figure 2) and Cx^{61} (data not shown) confirms that the homozygous (Figure 2, C and D) and hemizygous (data not shown) phenotypes of these mutations are identical, indicating that these are null alleles.

We sequenced the exons of the null alleles described above and identified sequence changes associated with each allele. The Cx^{61} allele (GenBank accession nos. AY055845 and AY055848) contains a 7-bp deletion within the homeobox (Figure 3). The resulting frameshift should produce a prematurely truncated protein lacking all but a few residues of the homeodomain. The Cx^{6} allele (GenBank accession nos. AY057858 and AF426395) contains a similar lesion—an 8-bp deletion within the homeobox (Figure 3) that would also result in a truncated protein lacking much of the homeodomain. Thus, the deduced Cx^{61} and Cx^{6} proteins are predicted to have no DNA-binding activity and to be nonfunctional.

***Cx* null mutants are defective in both fusion and dorsal extension of dorsal ridge Engrailed stripes:** One of the most striking abnormalities found in *Cx* null larvae is cephalization of the first thoracic segment, due to the absence of a segmental groove between the head and thorax (compare Figure 2, A and B, with Figure 2, C and D). To determine whether this phenotype results from improper development of the dorsal ridge during

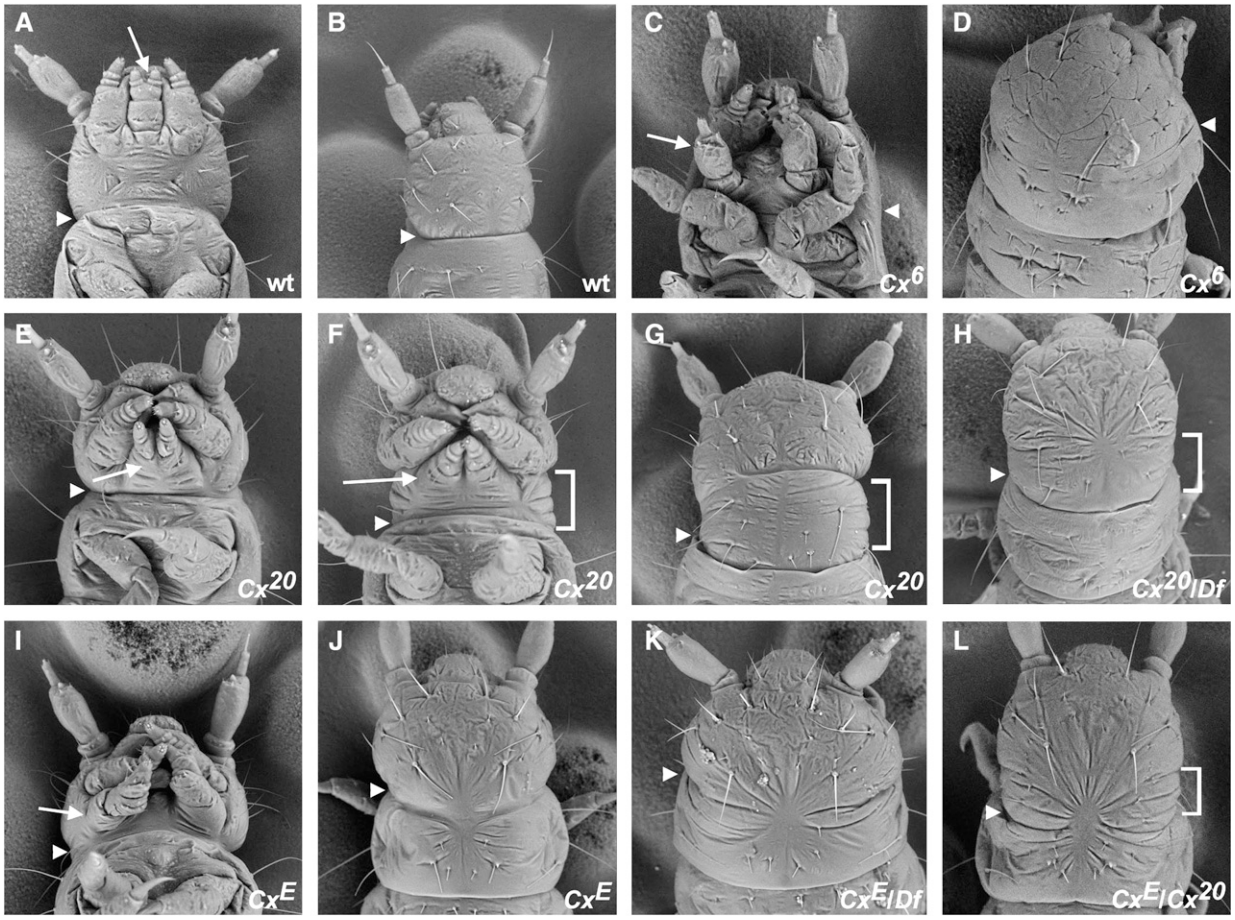

Figure 2.—Scanning electron micrographs of wild-type and *Cx* mutant Tribolium larvae. The boundary between head and thorax (approximated in cephalized mutants) is denoted by an arrowhead in A–L, and the labial appendages are indicated by an arrow in all ventral views. (A) Wild type, ventral view. (B) Wild type, dorsal view. (C) Cx^6 homozygote, ventral view. Note the transformation of labial appendages to antennae (arrow) and the fusion of T1 with the head. (D) Cx^6 homozygote, dorsal view. T1 is fused with the head dorsally as well as ventrally. (E) Cx^{20} homozygote, ventral view. The phenotype of Cx^{20} homozygotes is variable. In this mildly affected individual, the labial appendages are more posteriorly located than in wild type and only the most proximal segment of each appendage is fused. The extra dorsal segment between the head and T1 is not visible from the ventral side. (F) Cx^{20} homozygote, ventral view. In a more severely affected individual, the labial appendages completely fail to fuse. The extra dorsal segment between the head and T1 (bracket) can be seen from the ventral side. (G) Cx^{20} homozygote, dorsal view. Note the extra segment (bracket) between the head and T1. (H) Cx^{20} hemizygote [$Cx^{20}/Df(HOMC)$], dorsal view. An extra segment (bracket) is present, although partly obscured by dorsal closure defects. (I) Cx^E homozygote, ventral view. The labial appendages are positioned laterally, in a similar orientation to the maxillary appendages. Note the triangular head shape. (J) Cx^E homozygote, dorsal view. A segmental groove is present between the head and thorax, but does not appear as deep as in wild type. (K) Cx^E hemizygote [$Cx^E/Df(HOMC)$], dorsal view. T1 is fused with the head. The head has the abnormal triangular shape seen in Cx^E homozygotes. (L) Cx^E/Cx^{20}, dorsal view. An extra segment is present (bracket) although not as distinct as in Cx^{20} homozygotes. Head shape is apparently normal.

embryogenesis, we used En expression in *Cx* null embryos to follow dorsal ridge formation. We found that dorsal ridge development is abnormal (or nonexistent) in *Cx* null embryos. In these embryos, the maxillary and labial En stripes fail to fuse (Figure 4, C and D). Furthermore, although the embryo continues to grow dorsally, the maxillary and labial En stripes do not. Thus, the dorsal material in the maxillary and labial region of *Cx* null embryos apparently originates only from the anterior compartment (and not from the En-expressing posterior compartment) of one or both of these segments. An alternative, but rather unlikely, possibility is that the posterior compartment cells continue to extend dorsally, but stop expressing En.

Extra segment formation in Cx^{20} mutants is due to partial loss of Cx function: Beeman *et al.* (1993) described another *Cx* allele, Cx^1, which has been renamed Cx^{20}. Cx^{20}/Cx^{20} larvae have a very different phenotype from *Cx* null larvae. The labial appendages retain a palp-like morphology, but are abnormal in size and position. In the most mildly affected individuals (Figure 2E), fusion of the labial appendages is abnormal (only the most basal segment, the mentum, is fused) and the position of the labial appendages is more

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   1  GAATGTCCAT CACTTACCCA TAAACCAGCA CGTGATGTGG TCGATGGTAC TTGACGCGGT
  61  GTCATTCGGT GGTACCTGAA GCAGTGATAG TGCCCTTTCG GACCCAGTGC GTGAATGCCA
                                   Cx20
       M  S  S   Y  Q  F   V  N  S  L   A  Q  C   Y  G  Q   Q  A  R
 121  CAATGAGCTC CTACCAGTTC GTCAACTCCT TGGCCCAGTG CTACGGCCAG CAGGCCCGCG
      D  Q  Q  S   S  P  G   T  D  Y  Y   N  P  N   A  N  S   T  Y  P
 181  ACCAGCAGAG CAGTCCCGGG ACAGACTATT ACAACCCCAA CGCGAACTCC ACCTACCCCC
      P  A  C  Y   S  P  P   Q  V  A  P   Q  Y  P   Q  H  P   Y  A  A
 241  CAGCATGCTA CTCGCCCCCG CAGGTGGCCC CCCAGTACCC GCAGCACCCG TACGCGGCCC
      P  A  P  G   H  G  L   Q  P  T  M   G  D  Y   T  Q  L   Q  P  Q
 301  CGGCGCCGGG CCACGGCCTG CAGCCCACCA TGGGCGACTA CACCCAGCTG CAGCCGCAGA
      R  L  A  S   T  H  L   Q  S  P  N   T  Q  T   H  P  S   A  S  C
 361  GGCTGGCCTC GACCCACCTG CAGAGCCCCA ACACCCAGAC CCACCCTTCG GCCAGTTGCA
      K  Y  A  D   S  T  S   S  T  G  V   A  S  P   Q  D  L   S  T  N
 421  AGTACGCCGA TTCGACCTCC TCCACAGGAG TCGCCTCCCC GCAAGATCTG AGCACGAACG
      G  A  P  P   R  S  T   P  P  L  S   T  P  S   N  S  N   A  T  K
 481  GAGCTCCTCC AAGGAGTACT CCGCCGCTTT CAACCCCCAG TAACAGCAAC GCGACGAAGA
      S  S  G  L   T  S  P   L  S  V  S   T  S  P   P  G  K   P  A  T
 541  GCTCGGGACT GACGTCCCCG CTGTCGGTCA GCACTTCGCC ACCAGGCAAA CCCGCGACCT
      S  T  A  S   Q  N  L   S  S  P  A   S  S  T   S  S  T   S  S  T
 601  CCACGGCATC GCAAAACTTA TCCTCGCCGG CTTCTAGCAC TAGTTCTACC TCTAGTACCG
      E  K  A  G   T  N  N   N  N  S  K   S  G  Q   S  S  N   P  P  Q
 661  AAAAAGCCGG CACCAACAAC AACAACTCCA AGAGCGGCCA ATCGAGCAAC CCGCCTCAGA
      I  Y  P  W   M  K  R   V  H  L  G   Q  S  T   V  N  A   N  G  E
 721  TCTACCCCTG GATGAAGAGA GTTCATCTCG GCCAAAGTAC TGTGAACGCA AATGGCGAAA
      T  K  R  Q   R  T  S   Y  T  R  Y   Q  T  L   E  L  E   K  E  F
 781  CGAAGCGGCA GAGGACGTCG TACACGCGCT ATCAGACGTT AGAATTAGAA AAAGAATTCC
                         Cx61
      H  F  N  R   Y  L  T   R  R  R  R   I  E  I   A  H  A   L  C  L
 841  ATTTCAATCG GTACCTGACG AGGAGACGGC GAATAGAAAT AGCTCATGCC TTATGTCTGA
      T  E  R  Q   I  K  I   W  F  Q  N   R  R  M   K  W  K   K  E  H
 901  CCGAAAGACA AATAAAAATC TGGTTTCAAA ACCGCCGCAT GAAGTGGAAA AAAGAGCACA
        Cx6
      K  M  A  S   M  N  I   V  P  Y  H   M  S  P   Y  G  H   P  Y  Q
 961  AGATGGCCAG TATGAATATC GTTCCTTATC ACATGTCTCC TTACGGCCAC CCTTATCAGT
      F  D  L  H   P  S  Q   F  A  H  L   A  T
1021  TTGACTTGCA TCCTAGCCAA TTTGCCCATC TCGCTACTTA GGACGTGTTT ATGTGATTCC
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FIGURE 3.—Molecular lesions associated with *Cx* mutant alleles. The sequence of the *Cx* 5′-UTR and coding region is annotated with locations of *Cx* mutant lesions. The encoded amino acids are written above the nucleotide sequence. Significant motifs are color coded: octapeptide (purple) and homeodomain (blue). The nucleotides deleted in various *Cx* mutant alleles are underlined in red. Two methionine-encoding codons (ATG) that are in frame in the Cx^{20} allele and could serve as alternative translation initiation sites are boxed in black.

posterior than in wild type. The most severely affected larvae (Figure 2F) have larger-than-normal labial appendages that are completely unfused and occupy a more posterior and lateral position, such that they more closely resemble the maxillary appendages. In all of these larvae, the labial appendages are closer than normal to the posterior edge of the head. This may result from reduction of the posterior ventral head, or it may simply reflect the failure of the maxillary and labial palps to migrate anteriorly during development. The abnormal orientation of the maxillary palps is probably an indirect effect of the labial appendage defects. Defects in dorsal closure (evidenced by puckering of the cuticle at the dorsal midline) are also common in Cx^{20} mutants.

The most striking feature of Cx^{20} homozygotes is an extra dorsal segment that forms between the head and T1. This supernumerary segment was previously interpreted as a duplication of the dorsal portion of T1 (BEEMAN *et al.* 1993). However, the data that we present below suggest an alternative explanation for the presence of additional dorsal material.

The size of the extra segment in Cx^{20} homozygotes (denoted by brackets in Figure 2, F and G) is somewhat variable. Individuals with more severe labial appendage abnormalities typically have a more extensive extra segment (compare Figure 2E with Figure 2F). All Cx^{20} effects are completely recessive, since Cx^{20} heterozygotes are phenotypically normal (data not shown).

To further characterize the nature of the Cx^{20} allele, we analyzed its effect in the presence of other *Cx* alleles. Larvae hemizygous for Cx^{20} (Figure 2H) or heteroallelic for Cx^{20} and the hypomorphic allele Cx^{E} (Figure 2L) (see below) develop the extra segment, suggesting that this phenotype is seen only when the Cx^{20} allele is present and wild-type *Cx* function is either absent or reduced. These larvae also show dorsal closure defects and apparent reduction of the posterior head. In addition, the labial appendages of Cx^{20}/Cx^{E} and Cx^{20}/Df larvae (data not shown) are more severely affected than those of Cx^{20}/Cx^{20} larvae in that they are larger and positioned more laterally (*i.e.*, more closely resembling the maxillary appendages). These results suggest that the Cx^{20} phenotype is due to loss-of-function effects. We discuss in greater detail below our conclusion that Cx^{20} is an unusual hypomorphic allele.

Cx^{20} encodes a protein lacking the normal N terminus: To identify the molecular lesion associated with Cx^{20}, we performed Southern analysis of $Cx^{20}/+$ and wild-type genomic DNA using a probe from the 5′-end of the *Cx* transcription unit. We identified a *Hin*dIII restriction fragment length polymorphism (RFLP) (2.6 kb from Cx^{20} *vs.* 2.9 kb from wild type) associated with the Cx^{20} mutation (data not shown). We cloned these fragments using inverse PCR and sequenced them to determine the cause of the RFLP. The Cx^{20}-specific fragment has a 252-bp deletion in the 5′ region of the transcription unit that removes 149 bp of coding region, including the probable translation start site deduced by CURTIS *et al.* (2001) from sequence conservation (Figure 3). We confirmed this deletion by sequencing the *Cx* exons from the Cx^{20} allele (GenBank accession nos.

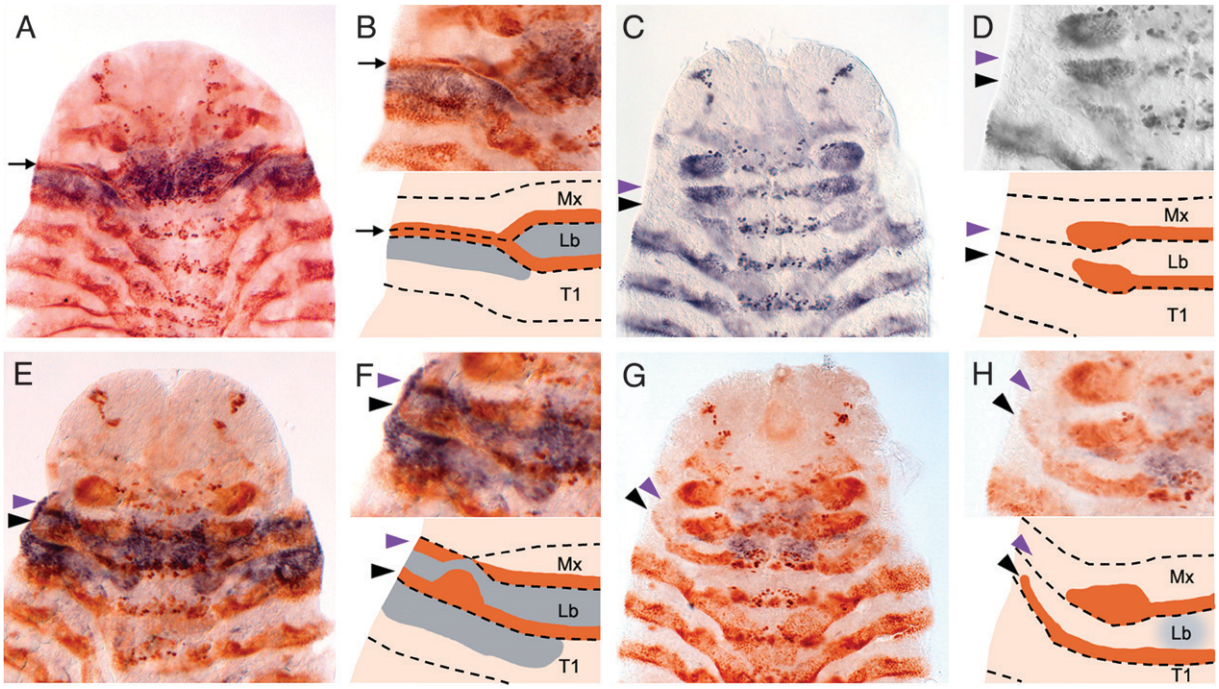

Figure 4.—Expression patterns of En and Cx in wild-type and *Cx* mutant embryos. All embryos are shown in ventral view and have begun the process of germband retraction. In schematic views, brown represents En expression and blue represents Cx. Segments are labeled as follows: maxillary (Mx), labial (Lb), and prothoracic (T1). (A) Wild type, En (brown), and Cx (purple) expression. The maxillary and labial En stripes have fused and the dorsal ridge En stripe (black arrow) extends dorsally. Cx is expressed ventrally in the labial appendages, the anterior compartment of the labial segment, and the posterior compartment of the maxillary segment, as well as dorsolaterally in the anterior compartment of T1. Cx is also expressed in some cells of the developing dorsal ridge. (B) Enlarged view of A (top) and schematic representation of En and Cx expression (bottom). (C) Cx^6 homozygote, En expression (purple). The maxillary (purple arrowhead) and labial (black arrowhead) En stripes have not fused and neither extends dorsally. The apparent contact between stripes is likely due to continued proliferation of En-positive cells, resulting in thickening of the ends of the En stripes. (D) Enlarged view of C (top) and schematic representation of En and Cx expression (bottom). (E) Cx^{20} homozygote, En (brown), and Cx (purple) expression. The maxillary (purple arrowhead) and labial (black arrowhead) En stripes have not fused. Instead, two stripes extend to the dorsal edge of the embryo. Cx is expressed within its normal domain, and additionally in the ectopic dorsolateral material between the two En stripes. (F) Enlarged view of E (top) and schematic representation of En and Cx expression (bottom). (G) Cx^E homozygote, En (brown), and Cx (purple) expression. The maxillary (purple arrowhead) and labial (black arrowhead) En stripes have not fused. Only the labial En stripe extends dorsally. Cx is expressed in the CNS of the maxillary and labial segments, but is absent or very faint in the ectoderm. (H) Enlarged view of G (top) and schematic representation of En and Cx expression (bottom).

AF416772 and AF416773). No additional changes affecting the encoded amino acid sequence were found.

The absence of the putative Cx translation start site in Cx^{20} mutants presents an interesting dilemma. Since Cx^{20} is not a null allele, and immunostaining (see below) indicates that Cx protein is produced, translation must initiate at an alternative site. Two methionine codons in frame with the YPWM-encoding region (one upstream and one downstream of the predicted start) are obvious candidates (Figure 3). We used the ATGpr program (Salamov *et al.* 1998) to analyze the likelihood of each of these codons being used for initiation. The probable wild-type initiation codon received the highest score (64%). The more 3′ ATG received a slightly higher score (56%) than the 5′ ATG (49%). Either, or both, of these sites could conceivably serve as a translation initiation site for the Cx^{20} allele.

In addition to the normal translation start site, the Cx^{20} deletion removes the region encoding the highly conserved octapeptide motif (Zhao *et al.* 1996) (purple in Figure 3). This sequence is found in all known Scr family members, including those of vertebrates, although an additional amino acid is present in the insect orthologs. Another motif shared by the Tribolium, *Bombyx mori*, and *Apis mellifera* orthologs, but not by the known dipteran or vertebrate family members (Curtis *et al.* 2001 and data not shown), is also deleted.

An extra Engrailed stripe extends dorsally in Cx^{20} homozygotes: The presence of an extra segment between the head and the thorax of Cx^{20} homozygotes is suggestive of a dorsal ridge defect distinct from the one seen in *Cx* null homozygotes. Indeed, En expression in Cx^{20} homozygotes is abnormal. As in *Cx* null larvae, the maxillary and labial En stripes do not fuse. In this case, however, two stripes (rather than a single dorsal ridge stripe) extend to the dorso-lateral edges of the gnathal region of the embryo (Figure 4, E and F). One of these stripes is clearly an extension of the labial En stripe

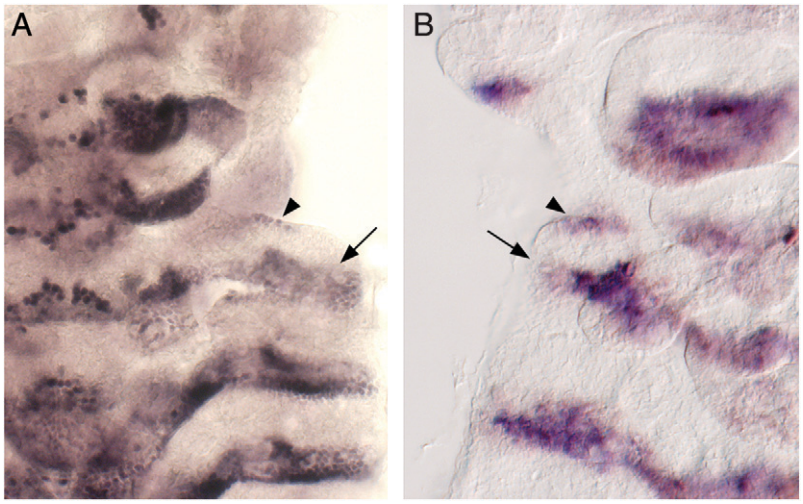

Figure 5.—Expression of En and *Tc hh* in Cx^{20} embryos. (A) En expression in a Cx^{20} homozygote. The labial En stripe extends to the dorsolateral edge of the embryo (arrow). A second stripe of En expression (arrowhead) appears anterior to the labial En stripe. This new expression domain is discontinuous with but roughly parallel to the maxillary En stripe. (B) *Tc hh* and En expression in a Cx^{20} homozygote. *Tc hh* (purple) and En (brown) are coexpressed in the labial segment (arrow) and in the more anterior expression domain (arrowhead).

(Figure 5A). The second stripe, however, appears to arise *de novo* just anterior to the labial En stripe (Figure 5A). The presence of two En stripes correlates with the presence of two segmental grooves (one on either side of the additional dorsal segment) in Cx^{20} larvae. In Drosophila, segmental groove formation requires coexpression of En and Hedgehog (Hh) (Larsen *et al.* 2003). Therefore, we used *in situ* hybridization to examine the expression of the Tribolium ortholog of *hh* (*Tc hh*) at the head/thorax boundary in wild-type and Cx^{20} embryos. *Tc hh* is coexpressed with En, both in the wild-type dorsal ridge (data not shown) and in the two En stripes of Cx^{20} homozygotes (Figure 5B). Thus there is a correlation between the expression of En and *Tc hh* and the number of segmental grooves at the head/thorax boundary.

We also compared Cx expression in Cx^{20} homozygotes and wild-type embryos using an antibody to Drosophila Scr that cross-reacts with Cx protein (Curtis *et al.* 2001). As the dorsal ridge forms in wild-type embryos, En expression at the lateral edge of the labial segment links the maxillary and labial En stripes, forming a ring of En expression that encompasses Cx expression in the anterior compartment of the labial segment (Figure 4, A and B). Some cells in the posterior maxillary segment and the extending dorsal ridge appear to express both Cx and En. As described above, the maxillary and labial En stripes of Cx^{20} homozygotes do not fuse, so the ring of En expression does not form. Instead, the labial En stripe and a second, more anterior En stripe of uncertain origin extend to the dorsal edge of the embryo. Cx is expressed between these two En stripes, suggesting that this region is made up of cells from the anterior compartment of the labial segment.

Cx^E represents a new class of Cx allele: In an EMS mutagenesis, we isolated a new allele of Cx whose phenotype differs from the other Cx alleles. Larvae homozygous for this allele, Cx^E, have labial appendages that resemble maxillary appendages in size and position, but lack the basal endite lobes characteristic of maxillary appendages (Figure 2I). Cx^E homozygotes have a segmental groove between the head and T1 (Figure 2J) but it is not as well defined as that of wild-type larvae. No extra segment is present. Cx^E homozygotes also display posterior head reduction and dorsal closure defects similar to those associated with Cx^{20}. In addition, these individuals have abnormally shaped heads (Figure 2, I and J) that resemble those of *TcDfd* mutants (Brown *et al.* 2000) in that the head is triangular and the labrum is pointed and angled upwards (S. J. Brown, unpublished results). When Cx^E is heterozygous with Cx^{20}, head shape is apparently normal, although these individuals have dorsal closure defects and the extra segment associated with Cx^{20} (Figure 2L) as well as maxillary-like labial appendages and reduced posterior head structures (data not shown). Cx^E hemizygotes have labial appendages similar, if not identical, to Cx^E homozygotes and Cx^E/Cx^{20} individuals (data not shown). They also display the abnormal head shape and dorsal closure defects associated with Cx^E (Figure 2K). But unlike homozygotes, Cx^E hemizygotes exhibit T1 cephalization (Figure 2K). It is somewhat difficult to classify the Cx^E allele on the basis of these phenotypes. The labial appendage phenotype of Cx^E homozygotes and hemizygotes and the cephalization of hemizygotes suggest that Cx^E is a loss-of-function allele. However, the triangular head shape is a novel effect not seen in *Cx* RNA interference (RNAi) (Curtis *et al.* 2001) or in known loss-of-function alleles (see above). We sequenced the exons of Cx^E (GenBank accession nos. AY057859 and AF426396) and found no changes that would alter the amino acid sequence of the encoded protein.

Cx expression and dorsal ridge formation are abnormal in Cx^E homozygotes: Since the other two classes of *Cx* alleles showed dorsal ridge abnormalities, we examined En expression in Cx^E homozygotes. Interestingly, these individuals show yet another type of

dorsal ridge defect. As in the other allele classes, the maxillary and labial En stripes do not fuse. In this case, however, a patchy labial En stripe extends laterally (Figure 4, G and H).

Examination of Cx expression in Cx^E/Cx^E embryos reveals an abnormal expression pattern that is first evident during germband elongation. In wild-type embryos of this stage, Cx is expressed throughout the posterior compartment of the maxillary segment (coincident with the En stripe) as well as in the anterior compartment of the labial segment. In Cx^E homozygotes, posterior maxillary expression is limited to cells near the ventral midline (data not shown). Expression in the labial segment is apparently normal. Later, during germband retraction, Cx expression is faint, or absent, in the ectoderm, but remains detectable in the central nervous system (CNS) (Figure 4, G and H). Since all these changes in the *Cx* expression pattern represent loss of normal expression rather than gain of novel expression, we conclude that Cx^E is a hypomorphic allele. These expression changes, as well as the absence of a molecular lesion within the *Cx* exons, suggest that the Cx^E mutation probably affects transcriptional regulation of *Cx*.

DISCUSSION

Overview: We have identified three classes of *Cx* mutations on the basis of their effects on the labial appendages and dorsal ridge. Since null alleles, by definition, lack all function, we can use this class to infer the normal functions of Cx in these structures. We can gain additional insight by examining which functions are affected by the partial loss-of-function alleles and correlating this information with what is known about the nature of these mutations.

Cx^E appears to be a regulatory mutation that causes diminished levels of Cx expression in portions of its normal domain. Thus, as we discuss below, the Cx^E phenotype may help us to understand the role Cx expression plays in these regions.

The Cx^{20} allele is associated with a deletion that removes the region encoding the N terminus of the protein. There are several conceivable ways in which this lesion could produce a mutant phenotype. The resulting protein could have acquired a new function. However, most gain-of-function mutations are dominant, while the extra segment phenotype produced by Cx^{20} is recessive. Furthermore, the labial appendage abnormalities seen in Cx^{20} homozygotes are reminiscent of those seen in larvae mildly affected by *Cx* RNAi (Curtis *et al.* 2001). This observation might suggest an overall reduction in *Cx* function. Such a reduction could occur if removal of the preferred translation start site leads to decreased levels of protein production, or if loss of the N-terminal region of the protein causes a reduction in protein function or stability. However, not all aspects of the Cx^{20} phenotype can be explained by a general reduction in Cx level or function. The extra segment phenotype is never seen in *Cx* RNAi individuals. For this reason, we favor the hypothesis that the N terminus of the Cx protein is specifically required for a particular subset of Cx functions and that the Cx^{20} phenotype reflects the presence of a protein that retains some Cx functions but is completely deficient in others (see below).

Labial appendage development: Previous studies have implicated both *Cx* and *mxp* in specification of the labial appendages (Beeman *et al.* 1993; Shippy *et al.* 2000; DeCamillis *et al.* 2001). DeCamillis *et al.* (2001) showed that *Cx* regulates transcription of *mxp*, either directly or indirectly, such that removal of *Cx* eliminates *mxp* function as well. Loss of both gene functions results in transformation of the labial appendages to antennae. Weaker *Cx* mutations (Cx^{20} and Cx^E) do not cause transformation to antennae, but rather result in unfused labial appendages that more closely resemble the maxillary appendages in size, shape, and lateral position. The unmistakably palp-like morphology of these appendages probably reflects the presence of *mxp*, given the essential role of *mxp* in maxillary and labial palp formation (Shippy *et al.* 2000). Thus, the level of Cx function in these mutants is apparently sufficient to activate *mxp* expression.

The wild-type labial appendages of Tribolium larvae differ from the other pairs of appendages in that their proximal segments are fused at the ventral midline. This fusion follows the movement of the labial appendage primordia ventrally and anteriorly from their original lateral position to their final location between the maxillary appendages. Labial appendage migration and fusion do not require *mxp* function. The labial appendages of *mxp* null mutants are fully transformed to legs, but are basally fused and occupy their usual location (Shippy *et al.* 2000). In contrast, the labial appendages of *Cx* nulls (which are transformed to antennae) are not fused and remain in a lateral position. The labial appendages are also unfused in larvae carrying weaker *Cx* alleles, although their position is quite variable. These observations suggest that *Cx* is responsible for the migration and fusion of the wild-type labial appendages. A similar role has been suggested for orthologs of *Cx* in other insects (Pattatucci *et al.* 1991; Rogers *et al.* 1997; Hughes and Kaufman 2000).

Dorsal ridge development: Dorsal ridge development, as visualized by expression of the posterior compartment marker En, has been assayed in a number of insects and appears to be relatively well conserved (Rogers and Kaufman 1996, 1997). Very little is known, however, about the regulation of steps involved in this developmental process. Below we discuss how our analysis of Tribolium Cx mutants has provided insight into several aspects of dorsal ridge development.

Engrailed stripe fusion: The first evidence of dorsal ridge formation in wild-type embryos is the connection of the maxillary and labial En stripes by the appearance of En expression along the lateral edges of the anterior compartment of the labial segment. Since this "fusion" does not occur in Cx null embryos, we conclude that Cx is responsible for the unique behavior of these En stripes. The maxillary and labial En stripes also fail to fuse in Cx^{20} and Cx^{E} homozygotes. Thus, this event apparently requires the N terminus of Cx, as well as expression of Cx in at least one of the domains affected in Cx^{E} mutants. At the time the maxillary and labial En stripes begin to fuse, Cx is expressed in the posterior compartment of the maxillary segment and the anterior compartment of the labial segment. Cx^{E} homozygotes have little or no Cx expression in the lateral regions of the posterior maxillary segment, but apparently normal expression in the anterior compartment of the labial segment. This could mean that maxillary Cx expression is required for fusion of the En stripes. Taken together, the phenotypes produced by each of these allele classes indicate that fusion of the maxillary and labial En stripes is an essential step in normal head development. One unresolved issue is the mechanism by which these En stripes fuse. Do En-expressing posterior compartment cells move along the edges of the labial segment? If so, do the migrating cells originate from the maxillary or labial segment, or both? Or does En expression appear *de novo* in anterior compartment cells instead? There have been previous reports of *en* expression in the anterior compartment of Drosophila wing discs, but in that case *hh* expression remains limited to the posterior compartment (Blair 1992; Strigini and Cohen 1997). Likewise, En is expressed in the anterior compartment of the eighth abdominal segment in Drosophila, but *hh* is not (Merabet *et al.* 2005). We have observed that *Tc hh* is coexpressed with En along the edges of the labial segment (data not shown), suggesting that these cells derive from the posterior compartment. However, the simultaneous appearance of En expression along the entire anterior compartment might suggest that "*de novo*" expression is more likely.

Engrailed stripe extension: During dorsal closure in wild-type embryos, En stripes in the thorax and abdomen extend to the dorsolateral edges of the embryo. Likewise, the fused dorsal ridge En stripe (apparently composed of the maxillary and labial En stripes) extends to the dorsolateral edge of the embryo. The maxillary and labial En stripes fail to fuse in all three Cx mutant classes, but the subsequent fate of these stripes differs in each class. In *Cx* null mutants, neither En stripe extends dorsally. This indicates that Cx is required for dorsolateral extension of En in the maxillary/labial region. The ability to promote dorsolateral En expression seems to be a function shared by many Tribolium Hox genes, since in *Df(HOMC)* homozygotes (which lack most of the Hox genes) all of the En stripes fail to extend (T. D. Shippy, unpublished results).

In Cx^{E} homozygotes, the labial En stripe extends, but the maxillary En stripe does not. This difference seems to correlate with the more severe reduction in Cx expression in the posterior maxillary segment in Cx^{E} mutants. In Cx^{20} homozygotes, the labial En stripe extends dorsolaterally to the edges of the embryo, thus behaving like the thoracic and abdominal En stripes. In addition, a second En stripe appears anterior to the labial En stripe and extends dorsolaterally. This suggests that the Cx protein missing its N terminus cannot promote fusion of the maxillary and labial En stripes, but is sufficient for En stripe extension.

Function of the Cx N-terminal domain: The simplest interpretation of our data is that the N-terminal region of Cx includes a domain that controls maxillary and labial En stripe fusion. Interestingly, the region missing from the Cx^{20} protein contains the octapeptide motif that is present in many Hox genes. Zhao *et al.* (1996) reported that deletion of the N terminus of Hox-a5 (a mouse *Scr* homolog) decreased its transactivation ability in *in vitro* assays and its ability to produce homeotic transformations when ectopically expressed in Drosophila. Furthermore, Tour *et al.* (2005) recently showed that Ultrabithorax lacking its N terminus can repress target genes when expressed in Drosophila, but its ability to activate target genes is greatly reduced. Likewise, deletion of the Scr N terminus reduces its ability to activate target genes. Extrapolating from these observations, our current model is that the N-terminally truncated protein produced from the Cx^{20} allele is capable of repression but not activation of target genes. This model is consistent with the loss of particular functions that we see in Cx^{20} mutants and predicts that fusion of the maxillary and labial En stripe during dorsal ridge formation requires activation of target genes, while dorsolateral extension of En stripes requires repression of target genes (perhaps genes that negatively regulate *en*).

Origin and identity of the extra segment in Cx^{20}: In wild-type embryos, the anterior compartment of the labial segment (marked by Cx expression) does not extend dorsolaterally after En expression appears along its lateral edges. Rather, it is restricted to a ventral position encircled by En expression. However, in Cx^{20} homozygotes, the anterior compartment of the labial segment (visible as a Cx-expressing domain between the two En stripes) clearly extends dorsally. Thus we conclude that Cx function is required to prevent dorsal extension of the anterior compartment of the labial segment and that the N terminus of Cx is required for this function. We believe that the dorsal growth or movement of these cells is responsible for the extra dorsal material observed in Cx^{20} homozygotes. The additional *hh*/En stripe that forms at the anterior border of this region probably produces the segmental

groove at the anterior border of the extra segment, while the extension of the labial En stripe seems to correlate with the more posterior segmental groove. This interpretation sheds new light on the identity of the extra segment, suggesting that it is not a duplication of dorsal T1, but rather the uncharacteristic formation of a dorsal labial segment. Morphological similarities to dorsal T1 can be attributed to the expression of Cx in both regions. It is likely that the labial segment also has a dorsal component in *Cx* null and Cx^E homozygotes, but no labial segment-specific marker is currently available to test this possibility. The presence of extra dorsal material could contribute to the abnormal head shape seen in Cx^E homozygotes.

The mechanism responsible for the repression of the dorsal component of the anterior labial segment is unclear. Cx might actively suppress dorsal extension of the anterior compartment, either by preventing cell division and/or migration or by inducing apoptosis. Alternatively, the repression may be an indirect result of En stripe fusion (which we have shown to be dependent on Cx function). Numerous studies in Drosophila have shown that posterior compartment cells (which express En) and anterior compartment cells (which do not express En) segregate from one another (reviewed in Dahmann and Basler 1999). Thus, the En-expressing cells surrounding the anterior compartment might act as a barrier against dorsolateral migration, effectively imprisoning the anterior compartment cells.

Conclusion: We have shown that function of the homeotic gene *Cx* is crucial for formation of the dorsal ridge in Tribolium. Given that the events of dorsal ridge development are conserved in a wide variety of insects, it will be interesting to determine whether the upstream factors governing this process are also conserved. Closer analysis of dorsal ridge formation in Drosophila *Scr* mutants will be a first step, but perturbation of *Scr* ortholog function(s) in other insects (perhaps by RNAi) will be important as well.

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