Abstract

Studies in genetic model organisms such as Drosophila have demonstrated that the homeotic complex (Hox) genes impart segmental identity during embryogenesis. Comparative studies in a wide range of other insect taxa have shown that the Hox genes are expressed in largely conserved domains along the anterior–posterior body axis, but whether they are performing the same functions in different insects is an open question. Most of the Hox genes have been studied functionally in only a few holometabolous insects that undergo metamorphosis. Thus, it is unclear how the Hox genes are functioning in the majority of direct-developing insects and other arthropods. To address this question, we used a combination of RNAi and in situ hybridization to reveal the expression, functions, and regulatory interactions of the Hox genes in the milkweed bug Oncopeltus fasciatus. Our results reveal many similarities and some interesting differences compared to Drosophila. We find that the gene Antennapedia is required for the identity of all three thoracic segments, while Ultrabithorax, abdominal-A and Abdominal-B cooperate to pattern the abdomen. The three abdominal genes exhibit posterior prevalence like in Drosophila, but apparently via some post-transcriptional mechanism. The functions of the head genes proboscipedia, Deformed, and Sex combs reduced were shown previously, and here we find that the complex temporal expression of pb in the labium is like that of other insects, but its regulatory relationship with Scr is unique. Overall, our data reveal that the evolution of insect Hox genes has included many small changes within general conservation of expression and function, and that the milkweed bug provides a useful model for understanding the roles of Hox genes in a direct-developing insect.

Keywords: Oncopeltus; Milkweed bug; Hemiptera; Heteroptera; Hox genes; Hox cross-regulation; Labial; Proboscipedia; Deformed; Sex combs reduced; Antennapedia; Ultrabithorax; abdominal-A; Abdominal-B

Introduction

The Homeotic Complex (Hox) genes have been intensively studied in the fruit fly Drosophila melanogaster ever since the first mutants were found early last century. The Hox genes are required to assign segmental identity along the anterior–posterior (AP) body axis of the embryo, and mutations in Hox genes frequently change the identity of one body region towards another yielding a homeotic phenotype (e.g. Lewis, 1978; Wakimoto and Kaufman, 1981). The Drosophila Hox genes are arranged in two linked clusters, where their chromosomal position reflects their embryonic expression along the AP axis of the germ band. Starting with the most anterior, these are: labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr), Antennapedia (Antp), Ultrabithorax (Ubx), abdominal-A (abd-A), and Abdominal-B ( Abd-B). Orthologous Hox genes have been isolated from diverse animal taxa and encode highly conserved homeodomain-containing transcription factors. This molecular conservation and their central role in assigning segmental identity in model species as diverse as arthropods and vertebrates implies a similarly conserved role in AP patterning throughout the animal phyla.
Although Hox gene expression has been examined in species from several major arthropod groups (reviewed by Hughes and Kaufman, 2002), functional analyses of Hox genes have been limited to the insect models Drosophila melanogaster, Tribolium castaneum (Beeman et al., 1989), and Bombyx mori (Nagata et al., 1996; Ueno et al., 1992). These species are members of the Holometabola, a derived clade that excludes the majority of insect orders (Wheeler et al., 2001). Moreover, the holometabolous insects develop their adult forms through metamorphosis. Given these facts, it is difficult to extrapolate from the data acquired in the holometabolous model species to more primitive insects. Fortunately, the advent of RNA interference (RNAi) technology allows for direct functional studies in these neglected species.

The milkweed bug Oncopeltus fasciatus (Heteroptera) is a member of the Paraneoptera, the sister-clade to the Holometabola (Wheeler et al., 2001), and as such, it is phylogenetically well-positioned for comparisons with the holometabolous insects. Importantly, it is possible to use both zygotic and maternal RNAsi to suppress gene activity in this insect (Liu and Kaufman, 2004a). Moreover, RNAsi for several genes can be combined to give double or triple knockdown phenotypes (Hughes and Kaufman, 2000). These combination phenotypes can reveal the epistatic relationships between genes in a way similar to traditional genetic analysis. (If gene A is necessary for activity of gene B, then the double knockdown of A and B will resemble that of A alone. Genes that act additively or independently will produce a double knockdown phenotype more severe than either alone.) In this way, combinatorial RNAsi can provide information about the functional relationships between genes. Additionally, using a combination of RNAsi injections and in situ hybridization, it is possible to analyze the expression pattern of one gene when another gene has been depleted, directly testing whether one gene is regulating the transcription of a second gene.

Here, we use these techniques to show the expression, function, and interactions of the eight Hox genes in the milkweed bug. In combination with the analyses of Hughes and Kaufman (2000), this provides a complete RNAsi survey of the eight Hox genes in Oncopeltus fasciatus, the first such analysis in a non-model species. We find that despite overall conservation in the specification of body segment identity, there are numerous novel aspects of Hox gene expression and function, particularly in the regulatory interactions between the Hox genes in Oncopeltus.

Materials and methods

Animal husbandry and embryo fixation

Large milkweed bugs, Oncopeltus fasciatus (Dallas), were cultured as described by Hughes and Kaufman (2000). Embryos were raised at 25°C for all experiments. At this temperature the insects hatch after 8 days of development. In the early Oncopeltus germband, the anterior and posterior parasegmental compartments are clearly visible as small and large hemisegments. This segmentation requires engrailed activity (Campbell and Caveney, 1989), as in Drosophila. The structure of hemisegments was used to determine the register of Hox gene expression patterns.

Orthologous gene sequences

Partial cDNA sequences of the Oncopeltus Hox genes have been isolated previously (Hughes and Kaufman, 2000; Liu and Kaufman, 2004b; Rogers and Kaufman, 1997; Rogers et al., 1997). In the course of this study, we isolated larger fragments of Dfd and Antp, which have been submitted to GenBank (AY856073; AY856074). Clones were isolated from embryonic cDNA using standard methods, as described previously (Hughes and Kaufman, 2000). Several independent rounds of PCR did not identify duplicates or transcriptional isoforms. Primer sequences are available on request.

The orthology of cloned sequences was confirmed by neighbor-joining of Hox protein sequences from Drosophila, Tribolium, and Oncopeltus (data not shown). Alignments corresponding to fragments covered by Oncopeltus sequence data were assembled by ClustalW. Gap penalties were distributed proportionally. The tree was rooted using the Drosophila homeodomain protein Homothorax as an outgroup. Oncopeltus clones of Dfd and Scr consist of 3' UTR and only a small protein coding sequence. These sequences could not be resolved in the context of the longer Drosophila and Tribolium orthologs. Therefore, a separate alignment covering only these fragments was used to calculate topology for Dfd and Scr sequences. Putative orthologs formed well-supported clades for all genes except Dfd, Scr, and Ubx, however, orthology of these sequences is supported by expression and phenotypic data. Topology of clades containing the other Hox orthologs is maintained with up to 10,000 bootstrapping iterations.

In situ hybridization

Embryo collection, fixation, and in situ hybridization were performed as previously reported (Liu and Kaufman, 2004b). Anti-digoxigenin, alkaline phosphatase-conjugated antibody F(ab')-fragments (Roche) were used with the chromagens 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Boehringer) and nitro-blue-tetrazolium chloride (NBT; Boehringer). Stained embryos were mounted in Aqua Poly/Mount (Polysciences, Inc.) for imaging.

Expression of the Oncopeltus Hox genes was determined at 72–75 h after egg-lay. At this stage of development, germband elongation is complete and the appendages first show signs of discernible segments, or podomeres. Expression data are summarized for the eight Hox genes of Oncopeltus in Fig. 1I, and a comparison to known patterns from Drosophila is provided in Fig. 1J.

RNA interference

RNA interference was performed by injecting double-stranded RNA into newly oviposited embryos (zygotic RNAi) or into females (maternal RNAi) as previously described (Angelini and Kaufman, 2004; Hughes and Kaufman, 2000; Liu and Kaufman, 2004a). Both zygotic and parental injections produced similar phenotypes. Zygotic RNAi yielded a wider range of severities, including more severely affected individuals, while parental RNAi phenotypes were typically milder but very consistent within broods. Therefore, both methods were used to characterize depletion-associated phenotypes. Table 1 lists the RNAi effects for each gene sequence and injection method. In situ hybridization in RNAi backgrounds was performed using embryos produced through both maternal and zygotic injection in order to capture the full range of phenotypes.

Microscopy and imaging

Photomicrographs of hatchlings and 7- to 8-day embryos were taken with a Nikon Dxm1200 digital camera on a Nikon SMZ1500 dissecting microscope. Younger embryos were photographed using the same camera.
package on a Zeiss Axioshot microscope. Scanning electron micrographs were produced using a Jeol JSM-5800LV electron microscope.

Results

Labial

In Drosophila, expression of the most anterior Hox gene, labial, is restricted to the intercalary segment of the head (Diederich et al., 1989; Mlodzik et al., 1988). Mutations in labial disrupt larval head involution, resulting in defective larval head structures but no clear homeoses (Merrill et al., 1989). While the intercalary segment is present in all insects, head involution is an embryological process unique to the cyclorrhaphous flies, which have a highly modified pattern of gnathal development and dramatic reductions in the contribution of the gnathal segments and appendages to the adult head. Since the morphology and development of these segments in Oncopeltus is more primitive for insects, it was possible that a more ancestral role for labial in the intercalary segment would be revealed. Therefore, we examined the expression and function of labial in Oncopeltus.

In Oncopeltus, as in Drosophila, labial expression is restricted to the intercalary segment (Fig. 1A). We were not, however, able to detect any phenotypic effect on Oncopeltus head development with RNA interference of labial (data not shown). Since lab RNAi was attempted using various concentrations of dsRNA in both maternal and zygotic injection, a regime that has been successful for all other Hox genes tested in this species, this result may suggest that...
labial expression in Oncopeltus is either unnecessary for normal development or has a very subtle phenotype. On the other hand, it may be that RNAi was ineffective in these experiments for some unknown technical reason. Further experiments will be necessary to determine the functional significance of labial expression in Oncopeltus or other insects outside the Cyclorrhapha.

Antennapedia

In Oncopeltus, Antp is strongly expressed in the thorax (Fig. 1E). The register of Hox expression in the Oncopeltus germband was determined in reference to the structure of hemisegments. The anterior boundary of Antp expression is segmental, rather than parasegmental, and does not appear in the posterior labial segment as it does in Drosophila (Carroll et al., 1988; Carroll et al., 1986). Weak expression is also seen throughout the abdomen, except for the posterior-most segment (A11), and the intensity of expression wanes in more posterior segments. This expression pattern in the germband is similar to that seen in other insects (reviewed by Hughes and Kaufman, 2002). In Drosophila, Antp protein appears most strongly in the embryonic thoracic segments (Carroll et al., 1988; Carroll et al., 1986). Each of the early thoracic leg discs also expresses Antp (Casares and Mann, 1998). However, as larval discs mature, factors in the distal region repress Antp, restricting its expression in the T1 and T3 discs to cells that will give rise to only the body wall or notum, while in the T2 disc some Antp protein remains detectible in the proximal regions of the leg itself (Emerald and Cohen, 2004; Wirz et al., 1986). Antp transcripts appear in the legs of Oncopeltus in the proximal half of the limb, including the presumptive femur (Fig. 1E, arrows). However, while expression in the Drosophila leg disc diminishes at later stages, expression in the Oncopeltus legs is still detectable at 95 h, after limb segments (podomeres) have become well established (Fig. 1E’).

RNA interference of Antp activity in Oncopeltus causes a transformation of the thoracic appendages toward antennal morphology. In our analysis, we obtained RNAi phenotypes ranging in severity from mild to severe. Mildly affected animals show a swelling of the distal femur (Figs. 2E–F, arrows), which correlates with the distal limit of Antp expression in the legs (Fig. 1E’). Moderately affected individuals are unable to hatch, and have leg defects in which some legs are transformed towards antennal identity. These transformations are typically more common in the T1 and T2 appendages, than in T3 in the moderate phenotypic class (Fig. 2G). The most strongly affected animals exhibit a complete transformation of all thoracic limbs to antennae (Fig. 2H; compare Figs. 2C and I). These transformed antennae are segmented into 4 podomeres, like normal antennae, with identifiable scape, pedicel, and flagella I and II. However, these ectopic antennae always retain the pretarsal claw (Fig. 2I, pt). Retention of the claw is not surprising since the specification of the pretarsus is regulated by genes other than Antp in Drosophila (reviewed by Kojima, 2004). Indeed, it has been shown that tiptop is required for distal leg identity and pretarsal development in Oncopeltus (Herke et al., 2005; see Discussion).

Ultrabithorax

Ubx expression in the Oncopeltus germband is very similar to that seen in the Drosophila embryo (Akam and Martinez-Arias, 1985). Ubx shows a parasegmental register of expression in both species, strongest in the posterior T3 and anterior A1 compartments. In Oncopeltus, weaker expression is also detected throughout the abdomen, but staining intensity decreases towards the posterior, leaving the A11 segment devoid of any expression (Fig. 1F). Additionally, the T3 legs bear a domain of Ubx expression near the presumptive femoral–tibial joint (Fig. 1F, arrows).

Ubx-depleted animals can be subdivided into two groups as in Table 1: a milder class that hatches (Fig. 3A), and a more severe class that does not (Fig. 3C). Regardless of whether or not specific individuals hatched, all affected

### Table 1

<table>
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<th>dsRNA</th>
<th>Mode</th>
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<th>Nonspecific defects (%)</th>
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animals showed the same segmental transformations. In the RNAi embryos, the first abdominal segment adopts dorsal pigmentation resembling the T3 pattern and an ectopic pair of leg-like appendages (Fig. 3A). The ectopic appendages consist of three podomeres, resembling the proximal segments of the legs (Fig. 3B).
The transformations of Ubx-depleted embryos resemble Ubx mutant phenotypes in Drosophila (Lewis, 1978), Tribolium (Lewis et al., 2000), and Manduca sexta (Zheng et al., 1999). Tribolium larvae with mutations in the Ubx ortholog display leg-like appendages on A1, also bearing three podomeres (Bennett et al., 1999). A mutation reducing Ubx expression in Manduca also results in ectopic A1 legs (Zheng et al., 1999). In the Drosophila embryo, Ubx mutations or deficiencies develop ectopic ventral pits and Keilin’s organs on abdominal segment A1. (Keilin’s organ is a reduced larval appendage derivative, and both structures are normally found on the thorax.) Thus, the homeosis observed in all these species is a transformation of A1 toward a thoracic (and presumably T3) identity.

**Abdominal-A**

In Oncopeltus, abd-A is expressed from the posterior compartment of A1 through A10 (Fig. 2G; Liu and Kaufman, 2004a) with expression strongest from A2 through A7. This expression pattern more closely resembles the patterns of abd-A expression in Thermobia domestica and Tribolium castaneum (Peterson et al., 1999; Stuart et al., 1993) than the pattern seen in Drosophila, where its posterior limit is the anterior compartment of A9 (Harding et al., 1985; Regulski et al., 1985).

In Oncopeltus, individuals showing mild abd-A RNAi phenotypes hatch and display ectopic black pigmentation on segments A3–A6. Pigmentation appears in small, paired patches on the dorsolateral surface of these tergites (Figs. 4A, F, arrowheads), and resembles wild type T3 pigmentation (Fig. 4E).

More moderately affected individuals display ectopic appendages on abdominal segments A2–A5 (Figs. 4A–B), but limbs were never observed on A1. In situ hybridization reveals that expression of the appendage marker Distal-less (Dll) appears in paired lateral domains in the abdomen in an abd-A RNAi background, indicative of the formation of limb primordia (Fig. 4D, arrows). While Dll expression in the wild type A1 segment is associated with pleuropodia (Fig. 4C), in the A2–A5 segments of RNAi embryos, these domains are instead associated with ectopic legs.

The most strongly affected abd-A-depleted individuals failed to hatch, and developed ectopic appendages on all abdominal segments from A2 to A8 (Fig. 4B, arrows). However, segments A9–A11 were consistently free of ectopic appendages. Melanin is deposited shortly after hatching in Oncopeltus, thus no pigmentation was observed in these unhatched abd-A-depleted individuals. Unlike the A1 appendages seen in Ubx-depleted animals, the ectopic appendages seen in moderate and strong abd-A-depletions consist of only two, rather than three, podomeres, and also resemble proximal leg segments. Thus depletion of abd-A results in the ectopic production of appendages on the second through eighth abdominal segments as well as dorsal pigmentation patterns that resemble a T3-like identity.

The formation of legs on the abdomen, as well as the ectopic thoracic pigmentation, indicate a transformation of A2–A8 towards thoracic identity. Moreover, the anterior abdomen was most sensitive to abd-A depletion, as weakly affected animals showed ectopic limbs only on A2, while stronger depletions caused ectopic appendages to form on more posterior segments, although limbs were never observed on A1.

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![Fig. 4. abd-A RNAi defects. (A) abd-A RNAi causes ectopic appendages to develop on abdominal segments (arrows). (B) Strongly abd-A-depleted individuals fail to hatch and bear ectopic appendages on segments A2 through A9 (arrows). (C) The abdomen of a wild type 72-h embryo. Dll expression in the abdomen is limited to paired lateral domains in the A1 segment. (D) An abd-A-depleted embryo showing ectopic Dll expression in paired domains of segments A2 –A6. (E) Dorsal view of a wild type hatchling showing that melanin pigmentation appears in paired lateral domains on T3 and in medial domains of A5 and A6. (F) Moderate abd-A-depleted individuals display ectopic pigmentation, resembling a reduction of the normal T3 pattern, on A2–A5 (arrowheads).](image-url)
Simultaneous RNA interference of Ubx and abd-A

Both Oncopeltus and Drosophila lack appendages on the abdominal body segments during all juvenile and adult stages. No Dll expression is detected in the A1 segment of Drosophila, and Ubx and abd-A act redundantly to repress Dll transcription and limb development in the abdomen (Castelli-Gair and Akam, 1995; Vachon et al., 1992). In contrast, Dll is expressed in paired lateral domains in the A1 segment of Oncopeltus (Fig. 4E), although no external appendages are produced, the primordia are internalized to produce the pleuropodial glands. Among insects that do possess juvenile abdominal appendages, Ubx and abd-A may be absent from areas of Dll expression in the abdomen, as in the moth Manduca sexta, thus allowing the establishment of abdominal limb primordia (Warren et al., 1994). Alternatively, Ubx and abd-A may not act to transcriptionally repress Dll, as with Ubx in Tribolium and both genes in Collembola (Palopoli and Patel, 1998). Because Ubx and abd-A overlap in their expression, but apparently not in their ability to repress ectopic legs in the abdomen of Oncopeltus, we wished to test the degree to which these genes are functionally independent through simultaneous RNAi.

As in the Ubx and abd-A single RNAi experiments, coinjection of Ubx and abd-A dsRNA resulted in abdominal transformations with a range of severity. Two phenotypic classes were used to group affected individuals. Mild individuals were able to hatch, and exhibited ectopic limbs on A1, A2 and less frequently on more posterior segments (Fig. 5A, arrows). All ectopic appendages resemble those produced through Ubx RNAi, and consist of three podomeres. Some individuals also have defects in the dorsal scent-producing glands on tergites A5 and A6 (Fig. 5A, black arrowheads). Some individuals also show ectopic T3-like dorsolateral pigmentation (Fig. 5A, white arrowhead), similar to the mild phenotype of abd-A RNAi alone. More strongly affected Ubx+abd-A-depleted individuals fail to hatch, and display ectopic appendages on abdominal segments A1–A8 (Fig. 5B, arrows). As in abd-A-depleted individuals, segments A9–A11 are consistently free of ectopic limbs. Ectopic leg-like appendages have been obtained by simultaneous removal of Ubx and abd-A activity in Tribolium (Lewis et al., 2000) and Bombyx mori (Ueno et al., 1992).

These results show that Ubx and abd-A function independently to repress leg development within their respective domains of strongest expression. Ubx is required to repress the formation of legs on A1, irrespective of abd-A activity, while abd-A acts similarly to repress legs on A2–A8. Therefore, the double RNAi effect is additive, producing legs...
on A1–A8. The dorsal scent glands appear to represent a different regulatory paradigm. Since defects in these organs are only obtained through coincident RNAi, Ubx and abd-A must both be required for the proper development of these structures.

**Abdominal-B**

The expression of *Abd-B* in *Oncopeltus* is strongest in abdominal segments A10 and anterior A11, with less intense expression in more anterior segments up to and including A4 (Fig. 1H). This pattern of expression is similar to that seen in the *Drosophila* embryo, where two non-overlapping transcriptional isofoms of *Abd-B* appear in the abdomen from the posterior A4 compartment to the anterior of A10 (Delorenzi and Bienz, 1990). Although *Drosophila* lacks an eleventh abdominal segment, both *Drosophila* and *Oncopeltus* maintain a terminal posterior compartment free from *Abd-B* activity (pA10 for *Drosophila* and pA11 in *Oncopeltus*; compare Figs. 11 and J).

In *Drosophila*, loss of function mutations in *Abd-B* cause posterior segments including A10 to adopt more anterior identities (Karch et al., 1985). A similar phenotype is seen in *Tribolium* larvae mutant for the *Abd-B* ortholog, *extra urogomphi*. This mutation results in an ectopic pair of the A9 larval appendages, urogomphi, on the A10 segment (Beeman et al., 1989). RNA interference of *Abd-B* in *Oncopeltus* also produces a loss of posterior abdominal characteristics in favor of more anterior identities. Among mildly affected individuals, the most obvious defect is a lack of melanic pigment on the A10 tergite (Figs. 6C, D, F, arrow), which suggests a transformation to a more anterior abdominal identity. The abdomen is also abnormally shaped. Rather than the smooth, rounded appearance of the wild type (Figs. 6A, B), the posterior abdominal segments of these individuals retain deeply furrowed segment boundaries, like those normally seen between more anterior segments. This is most noticeable between segments A8 and A11, and gives the posterior of the animal a pointed appearance (Fig. 6C).

Additionally, moderately affected animals also show ectopic black pigmentation on the posterior edge of the A7 tergite (Fig. 6D, arrowheads). This may indicate a partial transformation of this tissue towards an A5 or A6 identity. In Fig. 6E, a scanning electron micrograph illustrates that an ectopic external gland opening (arrow) also appears in this area. Ectopic spiracles can also be found on abdominal segments A10 and A11 (Fig. 6F, arrowheads), again indicating a transformation to a more anterior abdominal identity. The most strongly affected individuals fail to hatch, and do not produce melanic pigments. Therefore, we were unable to score the presence of scent glands. However, the abdomen in these individuals is abnormally pointed, as in the other *Abd-B* phenotypic classes, which indicates posterior segments have adopted a more anterior identity.

**Analysis of Hox cross-regulation**

In general, RNA interference of abdominal Hox genes in *Oncopeltus* consistently causes a transformation of abdominal segments to more anterior identities (summarized in Fig. 9). This resembles the posterior prevalence effect observed in *Drosophila*, whereby posterior Hox proteins transcriptionally repress the activity of other more anterior Hox genes (Carroll et al., 1986; Hafen et al., 1984; Macias et al., 1990; Riley et al., 1987; Struhl and White, 1985).

To explore posterior prevalence in *Oncopeltus*, we examined potential cross-regulation between the Hox genes by performing in situ hybridization for putative downstream genes in embryos injected with dsRNA to a putative upstream gene. We examined several potential Hox interactions summarized in Fig. 7A. Expansion or reduction in the expression of a target gene would be evidence of negative or positive transcriptional regulation, respectively, by the depleted gene. However, if a transformation phenotype is produced (indicating successful RNAi depletion), but the expression pattern of the putative downstream gene does not change, this argues that the depleted gene does not transcriptionally regulate the putative downstream gene.

We should keep in mind that the level of reduction in gene activity provided by RNAi is variable and does not necessarily provide a null state. It remains possible that these experiments may fail to identify weak or indirect interactions. However, depletion of putative upstream genes that result in a morphological phenotype indicates that RNAi can deplete gene function enough to have a developmental, and presumably genetic, effect. Moreover, we successfully detected evidence for transcriptional cross-regulation in some cases, indicating that RNAi suppression is sufficient to reveal interactions.

In this way, we tested several possible regulatory interactions between *Antp*, *Ubx*, *abd-A*, *Abd-B*, as well as more anterior genes (Fig. 7A). RNAi of any of these Hox genes did not alter the expression patterns of any of the remaining genes. This suggests that none of thoracic or abdominal Hox genes regulate each other at the transcriptional level, in contrast to the implications from the RNAi phenotypes.

We did however, detect transcription-level regulation for the gnathal Hox genes, *pb* and *Scr*. In *Oncopeltus*, the *Scr* and *pb* single RNAi phenotypes as well as the *Scr*+*pb* double phenotype are distinct, implying that the two genes do not genetically interact in *Oncopeltus*, but independently contribute to different aspects of gnathal specification (Hughes and Kaufman, 2000). It was therefore surprising to find that in 72- and 96-h *Scr* RNAi embryos, *pb* was reduced or absent in the labial appendages (Fig. 8B, 17 of 24 or 71% of the embryos scored), indicating that *Scr* function is required for *pb* expression at this stage of embryogenesis. Notably, *pb* expression was always retained in domains that do not normally overlap with *Scr* expression, such as in the posterior compartment of the mandibular body wall. Zygotic RNAi embryos sometimes showed a left/right asymmetry due to the lateral injection of dsRNA (Hughes and Kaufman, 2000). Although an injection artifact, this left/right asymmetry conveniently serves as an internal control, since these individuals have both affected and unaffected regions of the same body segment. Thus, some embryos depleted for *Scr* through zygotic injection exhibit asymmetrical expression of
pb in the labial appendages. In these embryos, appendages that retained pb expression exhibit wild type morphology, while those lacking pb expression show a transformation towards a leg-like identity. These results show that in 72- and 96-h embryos, pb expression in the labium is dependent on Scr function, which is in conflict with the phenotypic analysis discussed above.

This paradox was resolved by the analysis of older embryos. In wild type embryos at 120 h, the left and right labial appendages have elongated and fused medially, to form the mid-ventral labium (Fig. 8C). In contrast, Scr-depleted embryos at this stage do not have fused labial appendages. Instead, the left and right labial appendages remain separate and adopt a mixed leg- or antenna-like appearance. These transformed appendages clearly express pb transcript at this stage (Fig. 8D). This is in marked contrast to the result from earlier stages (compare Figs. 8B and D). This suggests that in 120-h embryos, pb expression is not dependent on Scr function. Thus, we have identified two phases of pb expression showing different regulatory paradigms. Up to 96 h of development, pb expression in the labium...
is dependent on Scr function, but by 120 h, pb becomes Scr independent (Fig. 8F). Reciprocal experiments (Scr staining in pb-depletions) showed no evidence of interaction. These interactions are summarized in Fig. 8E.

Discussion

The data presented here have shown that the Hox genes of Oncopeltus function to specify the identity of specific body segments in which they are normally expressed. These RNAi effects are similar in this respect to homeotic Hox mutations recovered from Drosophila and Tribolium. Despite their conserved expression patterns and high degree of conservation at the molecular level, we have found some striking dissimilarities in some functions and interactions in Oncopeltus.

Antp is required throughout the thorax for leg identity in Oncopeltus

RNA interference of Antp in Oncopeltus causes a transformation of all thoracic appendages toward antennal morphology. In this respect, Oncopeltus Antp function more closely resembles that of the Tribolium ortholog than Drosophila Antp. In Tribolium (Beeman et al., 1989), Antp is required in all thoracic segments to specify leg identity, and reduction of Antp activity transforms all three pairs of legs to antennae. In the Drosophila adult, clonal analysis of Antp in the imaginal leg discs (Struhl, 1982) has shown that all legs require Antp for some aspects of patterning, such as bristle arrangement. However, only the proximal T2 leg is transformed to antennal identity when the discs lack Antp activity. However, triple mutant clones lacking Scr, Antp, and Ubx are transformed to
antennal fate in all Drosophila leg discs. Therefore, while Antp is required in all thoracic segments, it acts to determine the identity of only the adult T2 legs in Drosophila. Drosophila Antp cooperates with Scr and Ubx to specify T1 and T3 leg identity, respectively. It has been presumed that Drosophila Scr and Ubx are also capable of determining leg fate in the thorax (Struhl, 1982). Scr orthologs in Tribolium and Oncopeltus are capable of specifying at least partial leg fate in the labial appendages (DeCamillis et al., 2001; Hughes and Kaufman, 2000). However, Scr and Ubx apparently lack this ability in the thorax of these species.

It was previously reported by Herke et al. (2005) that Oncopeltus Antp RNAi resulted in a loss of medial leg joints, rather than a transformation of legs to antennal identity. The Antp RNAi defects reported by Herke et al. (2005) resemble only the mild phenotypic classes obtained in this study, in which medial leg segments (femur and tibia) are transformed to a pedicel, flagella I, or a fusion of these antennal podomeres. Examination of more severely affected individuals (such as those shown in Fig. 2) shows a clear transformation towards antennal morphology, in addition to a loss of the medial leg joints. Antp RNAi may result in a loss of the femoral–tibial joint, however, this is clearly due to a transformation of these podomeres to antennal structures, and a comparison of wild type leg and antenna with the thoracic appendages of Antp-depleted embryos shows that the affected appendages become antennal-like (compare Figs. 2C, D, and I). Since loss-of-function Antp mutations in Drosophila (Struhl, 1982), Tribolium (Beeman et al., 1993, 1989), and Bombyx (Nagata et al., 1996) cause transformations of legs to antennae, our interpretation for Oncopeltus is more consistent with these other insects.

While Antp RNAi transforms thoracic appendages to antennae, these limbs retain a distal pretarsal claw. The persistence of the claw in these individuals suggests that the specification of this structure is independent of Antp regulation. Herke et al. (2005) have shown that the Oncopeltus tiptop ortholog is required for formation of the pretarsal claw on thoracic appendages. In Drosophila, spineless, and distal antenna also function in the specification of distal appendage structures (Emerald et al., 2003), and could act similarly in Oncopeltus. Therefore, while pretarsus specification appears to be independent of Antp, the more proximal podomeres (coxa, trochanter, femur, tibia, and tarsi) require Antp for leg identity and in the absence of Antp, antennal development ensues.

Ubx and abd-A act independently to repress pigmentation in the abdomen

A model for the regulation of melanic pigmentation in the Oncopeltus abdomen is presented in Fig. 9O. Except the area surrounding the dorsal scent glands, abdominal segments A1–A9 are normally free of melanic pigmentation. Depletion of either Ubx or abd-A results in the appearance of T3-like pigmentation in non-overlapping regions of the abdomen. Ubx is expressed most intensely in A1, and this segment gains pigmentation when Ubx is depleted (Fig. 3A). Similarly, abd-A represses pigmentation in segments A2–A5, where it is most strongly expressed. While Ubx and abd-A expression overlaps across the entire abdomen, these genes apparently act to suppress pigmentation independently within the domains of their most intense expression. For example, Ubx is still present in A2 in abd-A RNAi (Fig. 7A), and yet does not prevent ectopic pigmentation in that segment.

Ectopic pigmentation is more frequent on anterior segments and it scales with the severity of other defects.
Therefore, it seems likely that the activator of pigmentation is expressed in a gradient from anterior to posterior. However, this activator cannot be Antp, since Antp-depleted embryos still display wild type thoracic pigmentation. At the posterior of the abdomen, Abd-B seems to act independently to promote pigmentation throughout A10 and A11.

Ubx and abd-A act redundantly and antagonize Abd-B to specify the dorsal abdominal scent glands

Depletion of Abd-B activity causes posterior abdominal segments to adopt more anterior fates, demonstrated by the fact that the A7 tergite acquires the medial black pigmentation and dorsal scent gland structures normally seen on A5–A6. This
suggests that Abd-B normally acts to repress genes responsible for the development of the dorsal scent glands and their associated pigmentation (Fig. 9N). As shown by Ubx + abd-A double RNAi, these genes act redundantly in the development of the dorsal scent glands. Thus it would appear that high levels of Abd-B activity are capable of suppressing the action of both Ubx and abd-A.

In summary, the interactions of Ubx and abd-A in the regulation of abdominal appendages, dorsal scent glands, and abdominal pigmentation illustrate three different paradigms (compare Figs. 9M–O). These genes function independently to repress leg development in the abdomen, but do so through different mechanisms:Dll expression is normally repressed by abd-A in most of the abdomen, but not in A1 in the presence of Ubx. Similarly, either high levels of Ubx or abd-A activity can accomplish the repression of T3-like pigmentation, but low levels are ineffective to this end. In contrast, the proper development of the dorsal scent glands requires either Ubx or abd-A, implying that these genes are functionally redundant for the specification of this organ.

Posterior prevalence of Hox genes is observed in the Oncopeltus abdomen

The “rule” of posterior prevalence is the general tendency of posterior Hox genes to repress the activity of anterior genes (Struhl and White, 1985). Miller et al. (2001) have systematically examined posterior prevalence in Drosophila. In their study, the ectopic expression of Hox genes resulted in the transcriptional repression of Hox genes that are normally expressed in more anterior domains of the AP body axis. However, the ectopic expression of anterior Hox genes produced no apparent change in the expression of genes normally found in more posterior body segments. The epistatic interactions of Hox mutations in Drosophila also demonstrate posterior prevalence (Carroll et al., 1986; Hafen et al., 1984; Macias et al., 1990; Riley et al., 1987; Struhl and White, 1985). Fig. 7B presents a schematic representation of the Hox interactions known from the Drosophila literature.

Posterior prevalence is also evident in the Oncopeltus abdomen at the phenotypic level. As we have shown, RNA interference of Antp, Ubx, abd-A, and Abd-B results in the homeotic transformation of body segments towards more anterior identities (summarized in Figs. 9F–I). For example, Ubx RNAi transforms A1 toward T3 identity. Since Antp is normally required for the identity of T3 and weak Antp expression is present in A1, Ubx activity must be necessary to repress Antp function in A1. Thus, when Ubx is depleted, Antp presumably directs development of T3 fate in A1. Similarly, abd-A RNAi transforms A2–A8 toward T3 fate, implying that abd-A normally represses activity of Antp and Ubx in A2–A8. Depletion of Abd-B transforms A7, A10, and A11 towards more anterior abdominal identities, suggesting that Abd-B must suppress abd-A function in wild type embryos.

Posterior prevalence among Ubx, abd-A, and Abd-B does not appear at the transcriptional level

Posterior prevalence in Drosophila is thought to be caused by transcription-level repression of target Hox genes, either directly or indirectly, by more posterior Hox proteins (Macias et al., 1990; Struhl and White, 1985). Among Oncopeltus Hox genes expressed in the thorax and abdomen, we have shown that RNAi results in the homeotic transformation of segments toward more anterior identities. Therefore, it is plausible that in wild type embryos posterior Hox genes act to prevent transcription of more anterior Hox genes.

Fig. 7A schematically presents the potential Hox interactions that were tested in Oncopeltus. Based on RNAi phenotypes, it was predicted that transcription of Antp, Ubx, and abd-A, would be up-regulated in the absence of Abd-B, Antp and Ubx expression would expand in abd-A RNAi, and that Ubx RNAi would cause expanded expression of Antp. Surprisingly, these genes were all expressed normally in these RNAi backgrounds. Numerous other potential Hox interactions were tested, but did not indicate any regulatory relationship (Fig. 7B). It must be noted that the sensitivity of this method is limited by the potentially incomplete suppression of gene activity induced by RNAi. However, we were able to detect Hox regulation of the limb-patterning genes Dll and dac using the same method (Angelini and Kaufman, in press). Furthermore, regulation of pb by Scr was detected at early stages (see below). Therefore, despite the observation of posterior prevalence between Antp, Ubx, abd-A, and Abd-B at the phenotypic level, it is likely that these genes do not interact through transcriptional regulation, either directly or indirectly.

One possible explanation of these paradoxical results is that phenotypic posterior prevalence in the Oncopeltus abdomen may result from the competition of Hox proteins. A Hox gene with preferential binding to target DNA sequences would exert a prevailing influence on the identity of that cell, as has been shown for Antp, Ubx, and Abd-A proteins in Drosophila (Appel and Sakonju, 1993). Alternatively, differential-binding affinities for cofactors, such Homothorax or Extradenticle could also lead to a similar effect. It is also possible that preferential translation of different Hox transcripts could lead to the phenotypic prevalence of more posterior Hox proteins in Oncopeltus.

The interaction of Scr and pb in the Oncopeltus labium

Among the insects in which cross-regulation between pb and Scr has been examined (Drosophila, Tribolium, and Oncopeltus), all three species show temporal variation in the interaction of these genes (summarized in Fig. 8E). Early embryonic phases of pb expression are dependent on activation by Scr in all three species (DeCamillis et al., 2001; Rusch and Kaufman, 2000), implying that this conserved aspect of their expression may be ancestral. In Drosophila, this phase of expression seems to be without phenotypic consequences since pb expression in the embryo
has no discernable function (Rusch and Kaufman, 2000). Interactions are reversed later in the Drosophila labial imaginal disc, when pb is required for Scr expression and these Hox genes repress different appendage patterning genes to specify the proboscis (Abzhanov et al., 2001). Similarly in Tribolium, early expression of the pb ortholog is dependent on the Scr ortholog (DeCamillis et al., 2001). As in Drosophila, this regulation is later reversed, so that pb activates Scr. However, Scr is also required to maintain pb expression (DeCamillis and ffrench-Constant, 2003), which is not true of their Drosophila orthologs. Tribolium is unique in that pb is epistatic to Scr. Double mutants in pb and Scr produce the same transformation of labium to antennae that is seen in Scr mutants, while pb mutations result in a labium-to-leg transformation (DeCamillis et al., 2001). It is not known at what stage(s) these genes are required for labial development, but the early requirement for Scr expression in order to see pb accumulation correlates with their epistatic interactions, suggesting that unlike Drosophila, the early phase of Tribolium Scr induction of pb expression may be required for proper labial development.

Based on the observed transformations of the labium seen in RNAi experiments (Hughes and Kaufman, 2000), it was concluded that pb and Scr do not interact epistatically in Oncopeltus. Depletion of pb results in a transformation of the distal labium to legs, while Scr RNAi transforms the labium to a mixed leg/antenna identity. Simultaneous pb + Scr RNAi produces a transformation of the labium to antennae. Therefore, both genes apparently cooperate to specify labial appendage identity. Alone each gene has the ability to specify leg identity, at least partially, over a “default” antenna fate. Despite the absence of phenotypic epistasis, early expression of pb is dependent on Scr in Oncopeltus as it is in Drosophila and Tribolium (Fig. 8B). Notably, later in embryonic development, these genes are expressed independently of another (Fig. 8D).

Drosophila, Tribolium, and Oncopeltus share the ancestral insect organization of the head, including three gnathal segments. However, the mouthpart morphologies, Hox gene expression patterns, and genetic interactions in the gnathal segments of these species differ dramatically (Hughes and Kaufman, 2000; the present study). It is clear that further investigation in these and other species will be required to (1) determine the ancestral condition and (2) to map out the evolutionary changes that underlie the observed variability and how it might relate to the evolution of form.

The evolution of Hox gene function and regulatory interactions

The Hox genes have been remarkably well conserved in sequence and expression across the arthropods and other animal phyla. However, it would be dangerous to overextend assumptions of functional and regulatory conservation based on expression data alone. From our examinations of Hox gene expression, function, and cross-regulation in Oncopeltus, conclusions can be drawn at two levels. With regard to the expression domains and general role of Hox genes in the specification of segment identities along the AP body axis, our data show that the Hox genes are well conserved. However, considering specific tissues, interactions, and target genes the Hox genes have changed significantly since the divergence of the various insect groups studied functionally to date.

Several of the regulatory interactions of Oncopeltus Hox genes differ from model organisms: pb and Scr do not interact late in development, as they do earlier; while Ubx, abd-A, and Abd-B do not appear to interact at the transcriptional level, despite posterior prevalence. It is not clear whether these changes have been due to adaptive evolution or random drift in these regulatory networks (True and Haag, 2001). They are nonetheless significant differences in the orchestration of Hox gene expression in this insect lineage. We must now ask how a set of seemingly conserved if not invariant loci, such as the Hox complex, can evolve different regulatory paradigms and nonetheless result in the production of the relatively conservative insect body plan. These results underscore the need for wider phylogenetic investigation into the function and interactions of the Hox genes, as well as other important developmental regulatory genes, in a wider range of insect and animal species.

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