RNAi analysis of *Deformed*, *proboscipedia* and *Sex combs reduced* in the milkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the Hemipteran head

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Accepted 20 June; published on WWW 9 August 2000

SUMMARY

Insects have evolved a large variety of specialized feeding strategies, with a corresponding variability in mouthpart morphology. We have, however, little understanding of the developmental mechanisms that underlie this diversity. Until recently it was difficult to perform any analysis of gene function outside of the genetic model insects *Drosophila melanogaster* and *Tribolium castaneum*. In this paper, we report the use of dsRNA-mediated interference (RNAi) to dissect gene function in the development of the milkweed bug *Oncopeltus fasciatus*, which has specialized suctorial mouthparts. The Hox genes *Deformed* (*Dfd*), *proboscipedia* (*pb*) and *Sex combs reduced* (*Scr*) have previously been shown to be expressed in the gnathal appendages of this species. Strikingly, the milkweed bug was found to have an unusual expression pattern of *pb*. Here, by analyzing single and combination RNAi depletions, we find that *Dfd, pb* and *Scr* are used in the milkweed bug to specify the identity of the mouthparts. The exact roles of the genes, however, are different from what is known in the two genetic model insects. The maxillary appendages in the bug are determined by the activities of the genes *Dfd* and *Scr*, rather than *Dfd* and *pb* as in the fly and beetle. The mandibular appendages are specified by *Dfd*, but their unique morphology in *Oncopeltus* suggests that *Dfd*’s target genes are different. As in flies and beetles, the labium is specified by the combined activities of *pb* and *Scr*, but again, the function of *pb* appears to be different. Additionally, the regulatory control of *pb* by the other two genes seems to be different in the bug than in either of the other species. These novelties in Hox function, expression pattern and regulatory relationships may have been important for the evolution of the unique Hemipteran head.

Key words: RNAi, Hemiptera, Milkweed bug, *Oncopeltus*, Head, Stylate-haustellate mouthpart, Mandible, Maxilla, Labium, Hox, *Deformed,* *Dfd,* *proboscipedia,* *pb,* *Sex combs reduced,* *Scr*

INTRODUCTION

Our current understanding of insect development is deep, but narrow. While important insights have come from the extensive developmental genetic studies in the fruit fly *Drosophila melanogaster*, we have little idea of how generally applicable these mechanisms and pathways are to other insects. Especially in terms of head development, it is very difficult to generalize from flies to other orders of insects, since both the *Drosophila* larva and adult have very specialized head appendages. Work in *Tribolium* is helping to remedy this situation. *Tribolium castaneum*, the red flour beetle, has mandibulate mouthparts, which are both ancestral and typical of most insects. Genetic analysis of head development in *Tribolium* provides a useful foil for comparison to *Drosophila*, but these two species alone cannot represent the diversity of morphology within the insects. To understand the morphological and developmental variability of the insects, we need to study representatives of additional orders.

The Hemiptera represent a basal outgroup to both flies and beetles, and as hemimetabolous insects they exhibit a very different developmental pattern, which is well suited to phenotypic studies, since most adult structures are present at hatching (Fig. 1A,B). They are particularly interesting in terms of head development. In the Hemiptera, the mandibular and maxillary segments give rise to two similar pairs of long, thin stylets (Fig. 1D). The paired maxillary appendages form channels for liquid flow and the piercing mandibles lie on either side. These four interlocked stylets run down a groove in the long, fused labium, which provides support. Although in typical mandibulate insects the maxillary and labial appendages are very similar (Fig. 1C), in the Hemiptera, it is the mandibular and maxillary appendages that share a highly unusual morphology very different from the labium (Fig. 1D). These specialized mouthparts represent an important evolutionary innovation that allowed this order of insects to feed by extracting fluids from other organisms. The question then arises: what developmental mechanisms changed to promote such novel morphology?

Based on work in *Drosophila* and other animals, the anterior Hox genes are undoubtedly important regulators of identity for the head appendages. Each Hox gene controls the
developmental fate of one or more segments, to the extent that removal of that gene creates a homeotic transformation to the fate of another segment. For instance, the proboscipedia (pb) mutation in flies causes transformation of the proboscis (the fused labial appendages) to a pair of legs (Kaufman, 1978). We can think of a Hox gene mutant phenotype as reflecting the removal of an entire developmental module. This is because Hox genes are transcription factors that specify segmental identity by controlling perhaps hundreds of target genes simultaneously (Botas and Auwers, 1996; Mastick et al., 1995). In addition, since Hox genes also regulate each other, mutant phenotypes are often complicated by a concomitant shift in expression of another Hox gene which is normally excluded from or promoted in a given segment. Analysis of the mutant phenotype of a Hox gene can thus provide information about the domain of activity, the type of identity conferred through targeted effector genes and regulatory interactions with other Hox genes.

Because of their importance in development, the Hox genes are thought to be important for evolutionary change in development as well. Since the DNA-binding homeodomain is highly conserved among animals and orthologs from very distant species can rescue fly mutations (e.g., Lutz et al., 1996), it is thought that the influence of Hox genes on evolution of the arthropods is generally not due to changes in biochemical function (for an exception, see Falciani et al., 1996). Rather, the function of a Hox gene within the arthropods is likely to change primarily via three possible mechanisms: changing expression pattern, changing regulatory relationships, or changing suites of target genes. A shift in the expression pattern of a Hox gene, for instance, would carry a whole module of developmental instructions into a new tissue. A change in regulatory interactions between Hox genes would have a similar effect. These shifts in Hox expression could result in either dramatic or relatively subtle morphological changes in evolution (Averof and Patel, 1997; Stern, 1998). Finally, changing suites of target loci activated or repressed by a given Hox gene would change the effective function of that Hox gene in terms of developmental fate. Because of the ‘relaxed’ requirement for binding, whereby the homeodomain can bind many variations of sites that include the core TAA T, ‘relaxed’ requirement for binding, whereby the homeodomain is limited to a dorsal patch, it is relatively easy for the Hox genes to acquire new target loci (Hayashi and Scott, 1990).

Previous work has revealed the expression patterns of Dfd, pb and Scr in Oncopeltus, which have some interesting differences compared to Drosophila and other insects (Rogers and Kaufman, 1997; Rogers et al., 1997). For instance, unlike the broad bands of prothoracic (T1) expression in the Drosophila embryo, in Oncopeltus, the T1 ectodermal expression of Sex combs reduced is limited to a dorsal patch, which is presumed to suppress wing formation, and a spot on the leg, which is thought to control comb development (Rogers et al., 1997). The expression pattern of proboscipedia is especially unusual in the bug. Unlike other insects, pb is not expressed in the maxillary appendage, but only in a small dorsal maxillary spot. Interestingly, this lack of pb expression in the maxillary appendage is correlated with the Hemipteran stylate-haustellate mouthparts (Rogers and Kaufman, 1997).

While these differences in Hox expression patterns between flies and bugs are provocative, they also raise additional questions, since we cannot know the roles of these genes without functional testing. A particular domain of expression may not necessarily reveal a gene’s function. For instance, although pb has a discrete expression pattern in the fly embryo, due in part to regulation by Dfd and Scr, loss-of-function mutations in pb do not affect fly embryonic development (Rusch and Kaufman, 2000; Pultz et al., 1988). Therefore the best way to infer gene function is to analyze the phenotype resulting from a disruption of gene activity. Until recently, the ability to knockout gene function in non-model insects seemed to be a distant possibility. With development of the dsRNA-mediated inhibition technique (RNAi), however, we can potentially bring loss-of-function studies to additional arthropods (Fire et al, 1998; Kennerdell and Carthew, 1998; Brown et al., 1999).

To examine the role of Hox genes in Hemipteran mouthpart specification, we used the RNAi technique to create loss-of-function phenocopies of the genes Dfd, pb and Scr in the milkweed bug. Analysis of the resulting morphological transformations provides insight into the roles of these Hox genes in the Hemipteran head and how they differ from other insects. Despite the reputation of Hox loci as some of the most highly conserved developmental genes, we find notable differences in the roles, functional domains and regulatory interactions of Deformed, proboscipedia and Sex combs reduced in the milkweed bug as compared to Drosophila and Tribolium.

MATERIALS AND METHODS

Husbandry

A strain of milkweed bugs (Oncopeltus fasciatus) adapted to feed on sunflower seeds was supplied by Carolina Biological Supply. Bugs were reared at room temperature, fed on organically grown sunflower seeds and provided with sterilized cotton for laying eggs.

Cloning

Eggs of mixed stages were collected and RNA was prepared using Trizol (GibcoBRL/Life Technologies). Poly(A) RNA was selected using the Oligotex mRNA minikit (Qiagen). cDNA was prepared using the 5'3' RACE kit (Boehringer Mannheim). Primers to Dfd and Scr were designed using homeobox sequences cloned previously (Rogers and Kaufman, 1997; Rogers et al., 1997). 3' RACE was done using the RACE kit in combination with either Taq Polymerase (Fisher) or a blend of Taq and Pfu polymerases from the TaqPlus Long PCR System kit (Stratagene). PCR products were viewed on standard agarose gels, gel-extracted (Qiagen), blunt-ligated into Bluescript and sequenced. For specificity, the homeobox and poly(A) region were trimmed off and the remaining portion of the 3' end was subcloned into Bluescript. Clones with identical sequences were isolated from independent PCRs. For pb, a previously isolated clone was used (Rogers and Kaufman, 1997). Although it contains the homeobox region, the sequence identity with Scr is only 63% with, at most, 24 contiguous identical nucleotides. The sequence identity between the Dfd and Scr 3' end clones is negligible. Final clones consisted of 358 bp (Dfd), 262 bp (Scr) and 258 bp (pb). GenBank accession numbers are AF279336, AF279337 and AF279338.

Preparation of dsRNA

RNA was prepared using the MEGAscript kit (Ambion). Plasmids were linearized by restriction digestion, and sense and antisense strands were transcribed in two separate reactions. The RNA was phenol-chloroform extracted and isopropanol precipitated. Then the resuspended RNA strands were combined and annealed in a thermal
RESULTS

Injection of dsRNA creates loss-of-function phenocopies in the milkweed bug

Double-stranded RNAs corresponding to portions of the genes Deformed (Dfd), proboscipedia (pb) and Sex combs reduced (Scr) were produced and injected into freshly laid Oncopeltus fasciatus eggs. Injections included individual genes as well as all possible combinations. Eggs were injected either anteriorly or laterally; point of injection did not affect the resulting phenotypes, except that laterally injected embryos show slightly more asymmetrical defects (data not shown). Negative controls were injected with buffer and dye only. The negative controls resulted in a set of defects present in all experiments, which are presumed to be caused by the injection process itself. These include developmental arrest (50% of injected negative controls, 54-91% of injected experimental animals) as well as non-specific developmental defects, e.g., undifferentiated head appendages (17% of developed negative controls, 6-17% of developed experimental animals). No appendage transformations were seen in the negative controls (Table 1).

For the experimental animals, RNA injection results in a clear suite of transformations. Left and right appendages were generally affected similarly and were scored as pairs. The spectrum of transformation from mild to severe is probably due to differences in the volume of RNA injected, which is necessarily variable due to inconsistencies in needle size and egg turgor.

Double or triple depletion analysis was possible by injecting a mixture of dsRNAs corresponding to the different genes. These multiple depletions are extremely effective; in the Dfd Scr double, for example, just 8% of the developing animals have a phenocopy of only one of the genes, while 42% exhibit the double phenocopy. The facility of the double depletion in Oncopeltus provides a great advantage for the analysis of genetic interactions.

Except for the aforementioned non-specific effects, the phenotypes produced by each injected RNA were unique. Moreover, based on previously determined expression patterns, they primarily affected the expected appendages (Rogers and Kaufman, 1997; Rogers et al., 1997). Finally, each RNAi depletion resulted in homeotic transformations, the hallmark of Hox gene mutations. These results give us confidence that the

![Fig. 1. Wild-type Oncopeltus morphology and mouthparts.](image)

(A,B) Two wild-type embryos from negative control injections, at hatching. Stylistes are visible in B at the top of the labium. (C) Typical mandibulate insect mouthparts versus (D) Hemipteran stylate-haustellate mouthparts. In the Hemiptera, the mandibular and maxillary segments form long thin styli, which run down a groove in the fused labium (the haustellum). Mn, mandibles (red); Mx, maxillae (blue); Lb, labium (green). SEMs by Rudi Turner.

(C,D) Adapted from Daly et al. (1998), with permission.
RNAi technique produces true loss-of-function phenocopies of the targeted gene.

**Deformed RNAi transforms the mandibular and maxillary appendages**

Injection of dsRNA corresponding to a portion of the *Deformed* gene resulted in transformation of the mandibular and maxillary appendages (penetrance=62% of developed animals; Fig. 2). The mandibular appendage takes on distal antennal identity, with a short fused base (98% of affected animals). The resulting short, plump, bristled appendages are quite distinct from the thin, wire-like stylets usually formed from this segment (Fig. 1). The form of the appendage, as well as the type of bristle, is very similar to the most distal segment of the antenna, and is clearly recognizable as such in the vast majority of affected embryos. Moreover, 12% of these transformed mandibular appendages are partially fused to the genuine antennae rather than attached directly to the head capsule (Fig. 2C). Of the majority that emerge directly from the head, it is not clear precisely which segment(s) are associated with the base of the appendage, as the more proximal portion is distorted. Based on the relative position of this antenna-like appendage, however, we conclude that it is derived from the mandibular segment. Interestingly, despite the consistent and obvious distal transformation, the proximal antennal segments are missing and a full-length mandibular antenna is never seen in the *Dfd* depletions.

The transformed fate of the maxillary appendages in the *Dfd* depletions is much less clear. They range from short, bristled appendages (93% of affected animals) to larger fused structures with short, bristled lumps at the tip (7%). In both cases, the appendages resemble the distal antenna, but the overall shape is curved and distorted, generally curving towards the posterior. Overall, in the *Dfd* depletions the maxillary appendages seem to take on a unique form, but which is nevertheless largely antennal in identity. The labium is not affected by *Dfd* RNAi depletion.

**proboscipedia RNAi transforms the labium to leg identity**

Injection of dsRNA corresponding to a portion of the *proboscipedia* gene results in transformation of the distal labium to prothoracic legs (penetrance=71% of developed animals; Fig. 3). The mandibular and maxillary appendages are unaffected. Injection of 20 μM RNA results in a high penetrance of an extreme tuning-fork-like phenotype (52% of developed animals, 73% of affected animals), in which the proximal first two sections of the labium are wild type, while the third section is split midway into a pair of tibia, tarsi and pretarsi, complete with T1 combs, claws and pulvilli (Fig. 3A,B). Transformation of the proximal labium is never seen.

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**Fig. 2.** The *Dfd* RNAi phenotype. (A,C) Two typical individuals, exhibiting the transformation of the mandibular and maxillary stylets to antenna-like appendages. (B) The mandibular and maxillary appendages, removed from a third individual. The mandibular appendages (Mn) resemble the distal antenna. The mandibular appendages usually originate from the head capsule (A,B), but are sometimes fused basally to the genuine antennae (Ant) (C, asterisks). The maxillary appendages (Mx) are short and generally curl posteriorly (A,C). The labium (Lb) is wild type in the *Dfd* depletion animals.

**Fig. 3.** The *pb* RNAi phenotype. (A) A light micrograph and (B) SEM of typical individuals exhibiting the tuning-fork-like phenotype. While the first two segments of the labium are wild type (I, II), the third (III) splits into a pair of distal legs composed of tibia (ti), tarsus (tr) and pretarsus (pt), complete with claws and pulvilli. The mandibular and maxillary stylets are wild type (B, arrow). Compare (C) a wild-type distal labium and (E) a wild-type distal leg, with (D) the distal labial appendage of a *pb* depletion animal. Note the claws (arrowheads) and pulvilli (asterisks) present in D,E. Scale bar, 10 μm.
Occasionally, only one side of the labium produces a prothoracic leg (19% of developed animals); this mosaic effect is seen with lateral injections but not anterior injections (data not shown). The mandibular and maxillary styles are present and properly extended in the majority of the affected animals, even in conjunction with a strongly transformed labium (87% of affected animals; Fig. 3B). The strong pb phenotype is associated with surprisingly high viability: 80% of affected animals hatch and tan, although they are unable to feed.

**Sex combs reduced RNAi transforms the labium to mixed identity**

Injection of dsRNA corresponding to a portion of the Scr gene results in transformation of the labium to a pair of appendages of mixed identity (penetrance=51% of developed animals; Fig. 4). These range from basally fused, leg-like appendages (Fig. 4A,E) to well-separated appendages, which are more antenna-like (Fig. 4C). Invariably, however, the distal tip of the appendages bear claws and pulvilli. Typically the appendages are well-separated, short and wide, with a club-shaped distal segment bearing claws, pulvilli and often a tibial comb (83% of affected animals). The large distal segment is borne on a thick base of one or more compressed segments of uncertain identity. Thus, the typical appendage resembles a partially extended leg with segment fusions. The occasional animals with more leg-like appendages (Fig. 4A,E) probably represent a partial loss-of-function, as they often hatch. While these appendages clearly contain regions of leg identity, they are not as leg-like as the pb depletion animals.

The transformation of the labial appendage is accompanied, in a high percentage of embryos, by undifferentiated tissue or lack of appendages from the mandibular and maxillary segments (78% of affected animals). The remaining embryos, however, carry wild-type styles accompanied by a strongly transformed labium (Fig. 4F).

In wild-type bugs, the legs bear a particular pattern of bristles on the inner side of the distal end of the tibia. On the prothoracic (T1) legs of both sexes, a distinct comb is formed from a unique type of blunt-ended bristle (Fig. 4D top). This T1 comb is transformed in the Scr depletions. While a line of bristles is still present, the combs on the transformed T1 legs are formed from the pointed, ridged type of bristle (Fig. 4D bottom), similar to those present on more posterior legs. Due to the difficulty of scoring the subtle T1 comb phenotype, only 10 Scr depletion animals, each with a transformed labium, were scored. Of those 20 T1 legs, 16 bore transformed T1 combs (80%). In contrast, negative control embryos always have wild-type combs (100%; n=20 legs).

Of the Scr depletion animals that have the labium transformed to a pair of legs with combs, these combs are often transformed as well; i.e., the leg-like appendages from the labial segment bear distorted combs formed of pointed bristles (Fig. 4B). Sometimes, however, the leg-like appendages bear fairly normal T1 combs (Fig. 4F). This suggests that some animals have incomplete repression of Scr, and that the requirement for Scr to make a T1 comb is lower than that necessary to specify labial identity. In other words, Scr has apparently been reduced sufficiently to transform the labium to partial leg identity, but there is still enough Scr activity to confer T1 leg identity on the comb-forming cells. Alternatively, since it is not known how long into development the RNAi effect persists, it may be that a relatively late requirement for Scr activity in the combs allows those cells to reinitiate Scr activity in time for T1 comb differentiation.

**Double RNAi depletion of Dfd and Scr transforms the maxillae completely**

The double depletion of Dfd and Scr produces a dramatic phenotype, which is not simply a combination of the effects of the single depletions (Fig. 5A). The mandibles take on distal antennal identity as in Dfd depletion alone (100% of affected animals), and the labium is transformed to leg-like appendages like Scr alone (92%). Instead of forming the mixed-identity lumps, however, of the Dfd single depletion, the maxillary appendages form full-length antennae in the majority of cases (54% of affected animals), or antennae slightly shorter than wild type in most of the remainder (26%).

**Double RNAi depletion of Dfd and pb**

The double depletion of pb and Dfd is similar to the singles in the mandibular and labial appendages (Fig. 5B); i.e., like the Dfd depletion, the mandibular appendages are transformed to distal antennae (96% of affected animals) and, like the pb depletion, the labium is transformed distally to a pair of legs (82%). The transformation of the maxillary is different, however, from either the Dfd depletion (in that the Mx produces short curled appendages) or the pb depletion (in which the Mx produces normal wild-type styles). In the Dfd
Double RNAi depletion of \( pb \) and \( Scr \) transforms labium to antennae

Injection of dsRNAs corresponding to \( pb \) and \( Scr \) results in an extreme transformation of the labium to a pair of antennae (Fig. 5C), which is unlike the effects of either \( pb \) or \( Scr \) alone. While the affected appendages fall into a range between leg and antennal identity; the majority are clearly antenna-like and lack claws (65% of affected animals). Very few of the appendages are overall truly leg-like (5%), and the remainder are unclear or have mixed identity between leg and antenna (29%).

The labial transformation is nearly always accompanied by undifferentiated or unelongated mandibular and maxillary appendages (97% of affected animals). In addition, the T1 legs sometimes exhibit a knobby appearance, due to a thickened tibia (20%). Finally the genuine antennae are usually undifferentiated on their distal ends, often with their tips pinched off or fused to each other (79% of affected animals; Fig. 5C). Rather than being a direct effect, this phenotype likely represents an indirect, physical effect of the transformed labium on the antennae, since in the egg these appendages lie in close proximity.

Triple RNAi depletion transforms all gnathal appendages to antennae

By combining preparations of dsRNA corresponding to \( Dfd \), \( pb \) and \( Scr \), all three genes were depleted simultaneously. This is helpful for the analysis, as it produces a ‘default state’ for the fate of the appendages in the absence of all three genes. A few animals (6%) were affected in only a subset of the three appendages, but 67% of developed animals had transformations of the Mn, Mx and Lb appendages (Fig. 5D,E).

Like the \( Dfd \) depletion alone, the mandibular appendages are consistently transformed to distal antennae (94% of affected animals; Fig. 5D,E). Like the \( Dfd \) \( Scr \) double depletion, the maxillary appendages are generally transformed to full-length or mid-length antennae (59% and 28% of affected animals respectively; Fig. 5D,E). The phenotype of the labial appendages, however, falls into a range that probably represents the extent of repression of gene activity. The labium is transformed to a pair of appendages ranging from leg to antennal identity: leg-like with claws (31% of affected animals), antenna-like with claws (25%; Fig. 5E), antenna-like (28%), or complete antennal transformation (13%; Fig. 5D). We conclude that, as in the \( pb \) \( Scr \) double, the full-length antenna represents the phenotype of the most complete loss-of-function.

DISCUSSION

RNAi allows functional analysis in the bug

The molecular mechanism of RNAi is still somewhat mysterious, but \( C. \ ella \) researches have characterized several of the necessary cellular components (Hunter, 2000), and embraced the technique as a quick method for producing loss-of-function phenocopies, which can guide subsequent detailed genetic studies (e.g. Boxem et al., 1999; Fay et al., 1999; Hong et al., 1998).

Without the balancers, markers and the short generation time of models like \( Drosophila \), traditional genetics is often impractical in other arthropods. Up to the present, researchers interested in comparative arthropod development have had to be content with inferences based on gene expression patterns, by analogy to what is known in \( Drosophila \). This approach has been fruitful but also frustrating, since expression patterns are often misleading and novel expression can be difficult to interpret. RNAi, however, provides a potential tool for functional analysis in non-model organisms.

Unfortunately, however, the success of RNAi has thus far been limited in the arthropods. It seems to work well in \( Drosophila \), \( Tribolium \) and \( Oncopeltus \), but results in more distant taxa have been discouraging (Kennerdell and Carthew,
This is surprising, since the necessary components for RNAi seem to exist phylogenetically as far away as *C. elegans* and even fungi (Tabara et al., 1999; Catalanotto et al., 2000). We conclude, then, that RNAi may yet be effective in other arthropods, but that for technical reasons it may be more difficult than in flies, beetles and bugs. For instance, the nuclei may be less exposed to the diffusion of dsRNA before cellularization than in the higher insects. Alternatively, there may simply be higher levels of native RNases in some insects that destroy the injected dsRNA. Techniques for targeting or protecting the dsRNA may prove effective in these organisms. It is hoped an improved understanding of the RNAi mechanism will expand the technique’s usefulness into more taxa in the future.

Even when effective, RNAi phenocopies may be somewhat difficult to interpret. When using RNAi, we do know immediately that we have some sort of loss-of-function of the targeted gene, rather than the hypermorphs or neomorphs possible with mutagenesis. Moreover, we tend to get a range of phenotypes, similar to an allelic series. This can be a helpful feature, especially in *Oncopeltus* where the range of phenotypes seems to result from the amount of dsRNA injected, and is not merely a mosaic effect caused by limited diffusion. There is, however, difficulty in knowing if and when

### Table 1. Summary of RNAi depletion experiments

<table>
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<th>NC</th>
<th>Dfd</th>
<th>pb</th>
<th>Scr</th>
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<td>T1 comb wt 100% (n=20 legs)</td>
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<td>Dfd alone 3% Scr alone 1% + Ant undiff 79%</td>
<td>+ T1 leg distorted 20%</td>
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<td>T1 comb wt 100% (n=20 legs)</td>
<td>+ Ant undiff 21%</td>
<td>Dfd alone 3% Scr alone 1% + Ant undiff 79%</td>
<td>+ T1 leg distorted 20%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pe</td>
<td>T1 leg</td>
<td>mixed leg/Ant</td>
<td>T1 leg</td>
<td>mixed leg/Ant</td>
<td>Ant</td>
<td>Ant *</td>
<td></td>
</tr>
</tbody>
</table>

From left to right: negative control injection (NC), single, double and triple gene depletions.

Typical phenotype, the typical resulting phenotypes are illustrated diagrammatically for the mandibular (Mn), maxillary (Mx) and labial (Lb) appendages.

- No. injected, the number of eggs either mock-injected with buffer (NC) or injected with dsRNA.
- No. developed, the number of embryos that completed development.
- Penetrance, the number of embryos exhibiting the typical phenotypes.
- Non-specific, the number of embryos exhibiting undifferentiated-appendage phenotypes due to the injection process itself.
- Other, additional phenotypes seen, either instead of the typical phenotype, or in addition to the typical phenotype (+).
- All percentages refer to the number of embryos with a given phenotype divided by the number of embryos that completed development.
- NA, not applicable.
- *The Lb-to-antenna transformation illustrated here is the extreme phenotype, rather than the typical phenotype for the triple depletion.
- **These embryos were scored from a separate batch of injections.
we have truly mimicked a complete null for the targeted gene. Assuming that there are multiple RNAi complexes present in each cell, it is likely that many cells may be incompletely saturated with dsRNA, and merely have lowered transcript levels of the target gene. Thus presently we can only propose that the most extreme phenotypes are probably closest to representing a complete null phenotype.

**Dfd specifies the mandible**

From the phenotypes produced by the various individual depletions and combinations, we can infer that, of the Hox genes tested, *Dfd* is the sole gene responsible for mandibular identity. This conforms with the expression of *Dfd* in the mandibular segment (Rogers and Kaufman, 1997). The single depletion of *Dfd* transforms the mandibular appendages to distal antennal identity, rather than the long thin styles of the head (Rogers et al., 1997). Because head development is expressed in the mandibular segment, we suspect that this is the sole Hox gene regulating mandibular identity also matches the situation in *Tribolium*, where *Dfd* mutants transform Mn structures, while *maxillipedia* (*mxp*, the *pb* homologue) and *Cephalothorax* (*Cx*, the *Scr* homologue) mutants leave the Mn unaffected (Brown et al., 2000, Shippy et al., 2000; Beeman et al., 1993). The effect of *Hox* mutations on the reduced mandibular structures of *Drosophila*, however, is more difficult to determine. In the embryo, *Dfd* mutations disrupt the dorsolateral papillae of the terminal sense organ, which are thought to derive from the Mn segment (Merrill et al., 1987). In the *Drosophila* adult, *Dfd* mutations disrupt parts of the head capsule (Merrill et al., 1987). Mutations in *pb* and *Scr* do not appear to affect these presumed mandibular structures (Pultz et al., 1988; Pattatucci et al., 1991).

Although both beetles and milkweed bugs use *Dfd* to specify the mandibular segment, the resulting appendages are very different. In contrast to the chewing mandibles of the beetle, the bug mandibles are very thin styles nearly as long as the body (Fig. 1B,D). Again, these are very different from the mandibular appendages in *Drosophila*, which are internal structures in the embryo, and are either missing or incorporated into the head capsule of the adult. Thus we conclude that while *Dfd*’s basic role in the mandible may be conserved, the developmental module driven by *Dfd* is extremely labile.

**Dfd and Scr specify the maxillae**

In the maxillary segment, *Dfd* depletion results in only a partial transformation of the maxilla to antennal identity (Fig. 2; Table 1). The *Dfd Scr* double depletion (Fig. 5; Table 1), however, results in complete transformation of the maxillae to antennae. Thus we can conclude that *Dfd* acts in concert with *Scr* in the maxillary segment. The curled phenotype of the *Dfd* depletion maxillary appendage (Fig. 2; Table 1) suggests that *Scr* may be repressing growth of the transformed limb in its posterior domain; this repression is released in the *Dfd Scr* double (Fig. 5A; Table 1).

The phenotypes in the maxillary segment are somewhat conflicting, however, regarding the role of *pb*. Two results suggest that *pb* is not acting to specify maxillary identity. Depletion of *pb* alone leaves the maxillary segment unaffected, therefore *pb* is not necessary for wild-type maxillary development (Fig. 3; Table 1). Secondly, the phenotype of the *Dfd Scr* double is the same as the triple depletion (i.e., antennae; Fig. 5A,D; Table 1), so we can infer that *pb* is not acting to confer any identity over that of the default (see below). There is, however, a subtle difference between the phenotypes of the *Dfd* single and *Dfd pb* double depletion (compare Fig. 2 with Fig. 5B). While the *Dfd* depletion produces short, curled antenna-like appendages, the *Dfd pb* double produces straight antenna-like appendages, often with pretarsal claws (Fig. 5B). This suggests that in the absence of *Dfd*, *pb* can affect the maxillary appendages. Whether this activity derives from its small dorsal maxillary domain of accumulation, or from an expanded domain of *pb* expression.

![Fig. 6. Differences in expression, regulation and function of the gnathal Hox genes in *Oncopterus*, *Drosophila* and *Tribolium* mouthparts. The three insects control the development of the maxillary appendages using different combinations of Hox genes. In the maxillary and labial appendages, there are different regulatory relationships between the three genes. The functions of these Hox genes also differ between the three species. E. embryonic; A. adult. (Unpublished *Tribolium* data courtesy of S. Brown.)](image-url)
in the Dfd depletion, is not yet clear. Nevertheless, based on the pb depletion, we conclude that, in wild-type embryos, pb is not active in the specification of the maxillary stylet.

This lack of pb function in determining the maxillary stylet matches the prediction made based on the expression pattern of pb in the bug. Thus far this is the only insect known not to use pb to determine maxillary identity (Fig. 6). In Drosophila and Tribolium, pb/mxp mutations cause transformation of the maxillary palps, which become reduced in Drosophila, and transform to legs in Tribolium (Pultz et al., 1988; Beeman et al., 1993; Shippy et al., 2000). In the bug, it would appear that Scr assumes much of that role. This is in contrast to Drosophila or Tribolium, where the Scr/Cx mutation does not affect the maxillary appendage (Pattatucci et al., 1991; Beeman et al., 1993).

While Dfd and Scr work together to specify maxillary identity in the bug, it seems that Dfd has the principal role. Perhaps by activating a similar set of target genes as in the mandible, Dfd may induce a similar stylet identity in the maxillary appendage. This contrasts with mandibulate insects, in which the maxillary morphology is most similar to the labium, probably due to the activity of pb in both of those appendages.

**Scr and pb specify the labium**

By analyzing the depletion phenotypes, we can infer that pb and Scr cooperate to pattern the labium. Dfd depletion leaves the labium unaffected, so we can conclude that Dfd is not necessary for wild-type labial development (Fig. 2; Table 1). Depletion of pb results in a labium that is fused and wild-typebasally, but splits distally into a pair of normal T1 legs (Fig. 3). From this, we infer that pb is necessary for distal, but not proximal, labial development. Scr depletion results in transformation of the entire appendage to a mixed identity between leg and antenna (Fig. 4). From this we infer that Scr is necessary for development of both proximal and distal labium. The pb Scr double depletion, as well as the triple, transforms the labium to a pair of full-length antennae (Fig. 5 C,D; Table 1), which is very different from either single depletion alone. Therefore in the bug as well as the fly and beetle, pb and Scr specify the labium (Fig. 6).

The Scr depletion phenotype in the labium supports the hypothesis that Scr normally functions to fuse the two labial appendages (Rogers et al., 1997). This conclusion is derived from the observation that, in wild-type and the pb depletion animals, the transformed labial appendage is basally fused, while the Scr depletion limbs are often well-separated (Fig. 4).

By comparing the pb depletion to the pb Scr double, we can infer that the function of Scr in the absence of pb is to induce T1 legs. In light of the Drosophila model, this may seem reasonable, since Scr is also expressed in T1 but, in the case of the milkweed bug, it is actually a bit surprising. Based on its ectodermal pattern of accumulation in a single discrete spot on the T1 leg, Scr would not appear to be capable of conferring overall leg identity to this appendage. Rather, in ventral T1 Scr appears to function solely in the specification of the T1 leg comb (see below).

The role of pb in the gnathal appendages is provided by a comparison of the Scr single depletion animals to the pb Scr doubles. The Scr depletion labium is transformed to a pair of appendages with features of both antenna and leg. This suggests that the activity of pb alone is conferring some leg identity over the default antennal state. This contrasts strongly with the function of pb in flies. In two situations in Drosophila, the presence of pb alone appears to induce maxillary identity: first, in Scr hypomorphic mutant adults, the labium is partially transformed to maxillae; second, when pb+ is ectopically expressed in the antennae, they are transformed to maxillary palps (Pattatucci et al., 1991; Cribbs et al., 1995). Since in the bug pb is inducing partial leg identity rather than maxillary identity, this suggests that pb may have a very different function than in the fly.

Since both pb and Scr, when acting alone, each seem to be specifying some leg identity, this suggests that pb and Scr have overlapping functions. We know, however, that the two genes are not entirely redundant, because their depletion phenotypes are different and because when both are present the result is labial identity. Thus the activity of wild-type pb or Scr alone induces leg identity, but the combined synergistic activity of the two together creates a new overriding labium identity. Whether the combinatorial effect of pb and Scr is at the level of transcription of their target genes, or in the activity of their combined target gene products, is not yet clear.

One aspect of the Scr depletion phenotype deserves special mention. Although expression studies have shown that Scr is not expressed in the milkweed bug labium distally (Rogers et al., 1997), the Scr depletion results in a change of identity for the distal labium. Instead of the sensory structures normally formed there (Fig. 3C), pretarsal claws and pulvilli appear (Fig. 4F). This suggests that the presence of Scr elsewhere in the appendage normally acts non-cell autonomously to inducelabial identity. Non-cell-autonomous functions for Scr and other Hox genes have been suggested for other tissues in Drosophila (D. F. B. Miller, S. S. Holtzman, A. Kalkbrenner and T. C. K., unpublished data; Percival-Smith et al., 1997).

**Scr function in the thorax**

Unlike the broad band of Scr expression across the first thoracic segment in fly embryos, in milkweed bugs and other insects, the pattern of Scr is restricted to three thoracic domains: a T1 leg patch, a dorsal T1 patch and the mesoderm of all legs (Rogers et al., 1997). We have been able to address a hypothesis about the function of Scr in one domain, but cannot yet describe the functions of the other thoracic domains in the milkweed bug.

As noted above, Scr is expressed in the T1 limb in an anterior patch midway down the leg. This accumulation correlates with the position of a comb on the distal tibia of this leg and it has been proposed that Scr expression is necessary for the development of that structure (Rogers et al., 1997). The Scr depletions reported here support this hypothesis. Although comb transformation was not invariably associated with labium transformation, of the animals that also had a transformed labium, 80% of the legs had a transformed comb, with bristles resembling those found in a comparable position of the more posterior legs (Fig. 4D bottom). This suggests that, in wild-type, the patch of Scr expression is promoting the formation of the T1 comb from a row of bristles on the distal tibia, as compared to the serially homologous structures found on the second and third legs.

The second domain of Scr expression is a patch on dorsal T1. Tribolium and Drosophila Cx/Scr mutants develop ectopic...
wings on dorsal T1; thus it has been proposed that the dorsal patch of Scr functions to repress wing development in that segment in the other insects as well (Beeman, 1987; Rogers et al., 1997). Unfortunately, this could not be tested with the Scr depletions reported here since, in the bug, the wings do not develop until several instars after hatching. Because depletions of Scr also disrupt the mouthparts, affected animals could not be raised to determine if wing rudiments developed on the first thoracic segment.

The third thoracic domain of Scr is in the mesoderm of all three segments. This expression of Scr has been proposed to non-cell autonomously direct tarsus development in the fly (Percival-Smith et al., 1997). In the Scr depletions, no consistent effect on the tarsi was observed. It would be difficult, however, to ensure that the depletion had completely removed Scr function from all cells of the mesoderm; activity in just a few cells might be sufficient to create enough downstream diffusible signal. Thus determining the generality of this proposed function for Scr requires further experimentation.

Antennae are specified in the absence of Hox

Since the triple depletion animals should lack all Hox activity in the gnathal segments, we can infer the default state of these appendages in the absence of Hox function. Antennae have been thought to represent the default appendage state in the absence of Hox activity, since a large deletion of much of the Hom-C in the beetle was found to produce a larva bearing a series of antennae instead of mouthparts and legs (Stuart et al., 1991). Likewise, in the adult fly, the pb and Scr double mutant combination in the adult labium gives rise to antennae (Percival-Smith et al., 1997).

Our triple depletion indicates that, in the milkweed bug as well, antennae represent the no-Hox state for the gnathal appendages (Fig. 5D; Table 1). In the triple depletion, the maxillary and labial segments form full-length antennae. Since wild-type antennae are the only paired appendages that lack Hox expression, it is reasonable that this morphology is produced in the absence of Hox function. Furthermore, this result indicates that the genetic program that specifies the presence of an appendage is independent of Hox expression, and that the function of the Hox gene products is to confer a specific morphology onto a generic appendage program. In the absence of Hox activity, this generic appendage turns on the antennal differentiation pathway.

In the absence of Hox activity, the mandibular appendages, however, each form only a distal antenna on a short base. This suggests that a portion of the generic appendage is missing. Previous work has suggested that insect mandibles, which lack Distal-less (Dll) expression, are gnathobasic (lack distal portions of the appendage) (Scholtz et al., 1998; Popadic et al., 1998). In Drosophila and Tribolium, Dll is repressed by Dfd in the mandibular segment (O’Hara et al., 1993; Brown et al., 2000). In the milkweed bug, therefore, deletion of Dfd is likely associated with Dll derepression. Consistent with this hypothesis, the morphology of the transformed mandibular appendage is primarily to distal antenna. Interestingly, as noted the basal portions of the appendage are distorted or missing, compared to the maxillary or labial default appendages. This suggests that some aspects of the reduced structure of the insect mandible are independent of the Dfd/Dll pathway.

Novel regulatory relationships

In Drosophila, the expression pattern of pb in the maxillary segment of the embryo is dependent on the activity of Dfd (D. F. B. Miller, B. T. Rogers, A. Kalkbrenner and T. C. K., unpublished data; Rusch and Kaufman, 2000). In Tribolium, however, Dfd is not required to activate mxp in the maxillary palp (S. Brown, personal communication). Therefore, it is interesting to analyze the regulatory relationship between Dfd and pb in the milkweed bug. The lack of pb expression and function in the maxillary appendage suggests that Dfd is not activating pb. It is possible, however, that Dfd represses pb. If this were the case Dfd depletion might be expected to derepress pb in the maxillary segment. Moreover, since in the absence of Scr activity the labium is leg-like, one would expect to see some leg-like identity in the transformed maxillary appendage in Dfd Scr double depletion animals. This treatment, however, produces an antennal appendage on the maxillary segment. Thus it would appear that the expression of pb is largely independent of Dfd (Fig. 6). In the Drosophila and Tribolium embryo, Scr/Cx is necessary to activate the expression of pb/mxp (Fig. 6). In Scr/Cx mutants, the expression of pb/mxp is greatly reduced in the labial segment (D. F. B. Miller, B. T. Rogers, A. Kalkbrenner and T. C. K., unpublished data; S. Brown, personal communication). If Scr were acting similarly in the bug, we would expect that the Scr single depletion would resemble the pb Scr double depletion; i.e., if Scr were necessary for the activity of pb, then in the Scr depletion animals pb would be inactive as well. This is not the case. While the pb Scr double depletion transforms the labium to a pair of antennae, in the Scr depletion, the labium has much more leg-like identity, indicating the activity of pb. Therefore the regulation of pb in the milkweed bug appears to be independent of either Dfd or Scr, and thus is different from both Drosophila and Tribolium (Fig. 6). Likewise, the activity of Dfd and Scr seem to be independent of pb or each others’ activity. There may, however, be subtle regulatory interactions between the three genes that would not be detectable in this analysis; these interactions await further study.

Evolutionary innovation and Hox genes

Hox genes have been studied both as indicators and possible mediators of evolutionary change. In this study, we find evidence of remodeling in the role of Hox genes in head appendage development. This change in the role of the Hox genes is due to three mechanisms: (1) change in expression patterns, (2) change in regulatory relationships and (3) change in function.

Previous work had shown that pb possesses a unique expression pattern in the milkweed bug (Rogers and Kaufman, 1997). The RNAi analysis confirms that the unusual expression pattern does in fact reflect an unusual domain of activity. Unlike Drosophila, Tribolium and probably most other insects, pb is not necessary for development of the maxillary appendages in the milkweed bug.

The pb gene is also regulated differently in the bug, where it appears to be independent of the activity of Dfd and Scr. This contrasts strongly with the regulatory interactions described for the fly and the beetle (D. F. B. Miller, B. T. Rogers, A. Kalkbrenner and T. C. K., unpublished data; Rusch and Kaufman, 2000; S. Brown, personal communication). Studies
of more distant insects are needed, however, before we can confidently infer the ancestral state and the direction of change for these regulatory interactions.

Lastly, we find evidence of novel functions in the bug, which probably reflect differences in the suite of target genes activated by the Hox genes. For example, the function of pb is different in the bug, where it induces a mixed leg-like identity rather than a maxillary palp. Even in the mandibular segment, where Dfd is the conserved controller of identity, the resulting mandibular appendage is strikingly different from the mandibles of other insects, indicating a very different developmental module at work downstream of the Hox signal. We cannot tell, however, whether the repertoire of target genes is different, whether the orthologous target genes themselves have different functions or if both possibilities are correct.

These differences in Hox function are provocative, but we are still some way from describing a likely scenario for the evolution of the Hemipteran mouthparts. The exclusion of pb from the maxillary segment may have caused the loss of the maxillary palp – or the expression of pb may be the result of loss of the palp, which would remove selection to maintain pb in an inactive tissue. Moreover, the evolution of Hemipteran stylet-haustellate mouthparts was clearly a multistep process within the Hemipteroid group: the Psocoptera (booklice) and some Phthiraptera (chewing lice) have pronounced lacinia; the Thyasoptera (thrips), some Phthiraptera (sucking lice) and the Hemiptera have long stylets; and finally, the Hemipterans have lost the maxillary palps entirely (Daly et al., 1998). Understanding the changes that accompanied the evolution of these mouthparts will require additional study of the embryology, homologies and gene expression of these various insect orders.

We wish to thank Paul Z. Liu for innumerable helpful discussions and development of the lateral injection technique. Special thanks to Rudi Turner for preparing the SEMs. We also thank Sue Brown, Teresa Shippy, Kathy Matthews, Paul Liu and two anonymous reviewers for careful reading of the manuscript. C. L. H. is supported by a Howard Hughes Medical Institute predoctoral fellowship. T. C. K. is an investigator of the Howard Hughes Medical Institute.

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