Hox Genes and the Regulation of Movement in *Drosophila*

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ABSTRACT: Many animals show regionally specialized patterns of movement along the body axis. In vertebrates, spinal networks regulate locomotion, while the brainstem controls movements of respiration and feeding. Similarly, amongst invertebrates diversification of appendages along the body axis is tied to the performance of characteristically different movements such as those required for feeding, locomotion, and respiration. Such movements require locally specialized networks of nerves and muscles. Here we use the regionally differentiated movements of larval crawling in *Drosophila* to investigate how the formation of a locally specialized locomotor network is genetically determined. By loss and gain of function experiments we show that particular Hox gene functions are necessary and sufficient to dictate the formation of a neuromuscular network that orchestrates the movements of peristaltic locomotion. © 2007 Wiley Periodicals, Inc. Develop Neurobiol 68: 309–316, 2008

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INTRODUCTION

Innate patterns of movement and behavior are as much an inherited characteristic of a species as its morphology. Despite the prevalence of such inherited behaviors, which range from the complex and highly stereotyped sequences of courtship in *Drosophila* and other species to the much simpler rhythmic movements required for essential activities such as feeding, locomotion, and respiration, the way in which the underlying neuromuscular networks are genetically specified is largely unknown.

In a recent discussion (Baker et al., 2001) it has been suggested that there might be dedicated regulatory genes whose function would be to orchestrate the activities of the many other genes that would be necessary to construct the circuitry underlying some particular behavior. Such a gene would be demonstrably necessary and sufficient for the behavior concerned, much as the gene *eyeless* can be shown to be necessary and sufficient to orchestrate the formation of the complex network of cells that forms the compound eye of the fly (Halder et al., 1995).

However, this, for a dedicated behavioral regulatory gene, would be a difficult criterion to fulfill, since the test of sufficiency carries with it the notion of a neuromuscular network and behavior that are generated at an ectopic location. Just as *eyeless* requires imaginal ectoderm in which to be expressed if it is to provoke the formation of an eye

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(Halder et al., 1995), so a behavioral regulatory gene would have to be expressed in cells that were capable of differentiating into the neuromuscular components that were required for the behavior concerned. Not surprisingly therefore, the candidate behavioral gene that comes closest to fulfilling the criterion of sufficiency, *fruitless*, is one that in normal flies is differentially expressed in an apparently equivalent set of cells in males and females. A transcript that is normally expressed exclusively in males can reconfigure female cells to form circuitry that generates male-specific courtship behavior (Demir and Dickson, 2005). Here we provide evidence for a more generalized form of genetic control that assigns equivalent cells at different levels in the anterior–posterior axis to form the different networks that underlie regionally specialized patterns of motor behavior.

In many animals there is a regional differentiation of patterns of movement along the body. The motor circuits that underlie these movements are distributed along the axis of the nervous system and have been described as a "neuronal toolbox", elements of which can be activated according to behavioral needs (Grillner et al., 2005). For example, in vertebrates locomotion is controlled by central pattern generating circuits embedded in the spinal cord, whereas the networks that produce chewing, swallowing, and respiratory movements are located in the brainstem (Grillner, 2003). Nowhere is the existence of this toolbox of motor programs more apparent than in the arthropods. In these animals there is a striking diversification of body segments to form structures such as swimmerets, legs, wings, and mouthparts and this is matched by the formation centrally of locally specialized neuronal networks that drive the characteristically different movements of each type of appendage [see, for example: (Wilson, 1968; Murchison et al., 1993; Rast and Bräunig, 2001)]. In vertebrates there is experimental evidence that motor networks are "hardwired" into the neural tube as it develops, so that specific motor programs are an autonomous property of particular levels in the spinal cord (Narayanan and Hamburger, 1971).

In developing arthropods a segmental groundplan of neural progenitors (neuroblasts) and early differentiating nerve cells is repeated along the body axis, so that specialized neural networks, characteristic of particular units of the body, are generated from fundamentally equivalent cell sets in different segments of the embryo (Thomas et al., 1984). Hox genes regulate the division patterns of the neuroblasts and the number and types of neurons and glial cells that they generate in different segments (Technau et al., 2006). The Hox genes continue to be expressed in the cells of the nervous system as they begin to differentiate, put out axons and dendrites, and form synaptic connections (Hirth et al., 1998), so that these genes have the potential to act as regulators, orchestrating the formation and differentiation of segment specific networks for specialized patterns of movement.

Here we use the regionally differentiated movements of larval crawling in *Drosophila* to test this hypothesis. By loss and gain of function experiments we show that particular Hox gene functions are necessary and sufficient to dictate the formation of a functional neuromuscular network that generates the characteristic movements of peristaltic locomotion.

METHODS

Genotypes

Wild type flies are Canton-S. For genotypes of loss- and gain- of function Hox mutants please see Table in Supplementary information.

Behavior

To observe crawling behavior in mutant (nonhatching) larvae, late stage embryos were dechorionated with bleach, rinsed and placed ventral side down on a layer of transparent agar in a petri dish. The vitelline membrane was then broken with a fine glass needle so that the larvae emerged onto the agar substrate. Hatching larvae (including wild type) emerged onto a similar substrate. The dish was inverted and placed on a white disc on the stage of a Leica M420 or Olympus SZX12 microscope for filming. Frames were captured at 25 per sec using a JVC CCD camera and a Sony DSR-30P digital videocassette recorder, and downloaded as Quicktime movies to a Macintosh G5 computer for further analysis. To facilitate the analysis of dorso/ventral movements during peristalsis, embryos were filmed from the side at stages when spontaneous waves of peristaltic contractions are generated prior to hatching. Under these conditions, the raising and lowering of segments is readily visualized as waves pass from posterior to anterior. Late stage embryos were dechorionated and then placed on their sides on sticky tape in a drop of halocarbon oil. Frames were captured as before using a Leica M420 microscope and CCD camera.

RESULTS

Thoracic and Abdominal Segments Show Distinct Patterns of Movement During Peristalsis

Drosophila larvae crawl by means of peristaltic contractions that pass forwards along the body axis. There are three, region-specific phases to the movement (Fig. 1 and Suppl. movie 1). Crawling is initi-

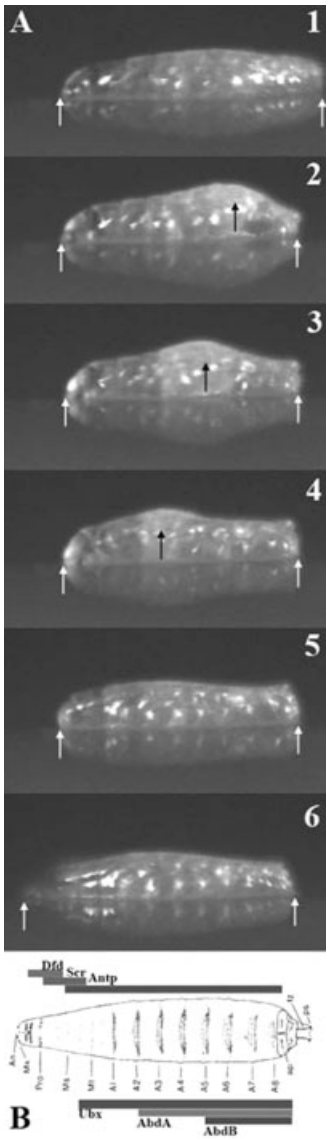

Figure 1 Peristaltic crawling and domains of Hox gene expression. (A) One cycle of peristaltic crawling in a first instar *Drosophila* larva viewed from the side as it moves over an agar substrate. The positions of the anterior (left) and posterior end (right) are indicated by white arrows. At the start of the cycle (1) the most posterior segments are drawn forward and anchored to the substrate (2). Each abdominal segment in turn is then raised from the substrate (black arrows, 2,3,4,), drawn forward, lowered, and anchored to the substrate by a band of cuticular denticles. The thorax and head (5,6) extend by a telescoping movement as the mouth hooks are released from the substrate (5) and the anterior segments move forwards, either exploring the substrate or initiating a further cycle of contractions (6) as the mouth hooks are reinserted and the posterior segments are drawn forwards. (B) Pattern of denticles on the ventral surface of the *Drosophila* larva and corresponding domains of Hox gene expression.

ated by the simultaneous contraction of posterior segments (A8/9). Thereafter each of the more anterior abdominal segments (A1–A7) is transiently lifted from the substrate, pulled forwards, and then lowered. Segments anterior to the abdomen (head and thorax) move differently. At the start of a peristaltic wave, they are extended forwards and anchored by the mouth hooks. At varying times during the wave, but often at its culmination, they contract, before extending further and making lateral explorations of the substrate. Each abdominal segment engages with the substrate through an anterior belt of cuticular denticles that act as anchorage points as neighboring segments move forwards in the peristaltic wave. The characteristically different roles of abdomen, thorax, and head in crawling locomotion are reflected in the distribution of these peristaltic effectors. The prominent denticle bands that are present in the abdomen are much reduced in the head and thorax (Lohs-Schardin et al., 1979) (Fig. 1).

The Bithorax Complex is Essential for the Development of Abdominal Peristaltic Movement

It is already well established that the regional differentiation of segmental patterns of ectodermal structures (including denticle bands) depends on segment specific patterns of Hox gene expression (Akam, 1987). To study the role of these genes in the regulation of segmental patterns of movement we analyzed the crawling behavior of larvae that were deficient for one or more elements of the two Hox gene clusters, the Bithorax (BX-C) and Antennapedia (ANTP-C) complexes. Although loss of function in elements of BX-C or ANTP-C causes embryonic lethality in most cases, the embryos survive until the point at which they would normally hatch. At this time we released such animals from the vitelline membrane by pricking it anteriorly and allowed the hatched larvae to crawl out over a substrate of transparent agar, through which the movements of the denticle bands could be clearly recorded (Fig. 2 and Suppl. movie 2). To begin our analysis of the role of Hox genes in specifying region specific patterns of movement, we looked first at larvae deficient for the entire BX-C. Morphologically, all abdominal segments in such animals are transformed towards mesothorax (MS) (Lewis, 1978). Larvae deficient for BX-C show no sign of coordinated peristalsis (Figs. 2 and 3 and Suppl. movie 3). In some cases they succeed in moving over the substrate by dragging with their anterior segments. We conclude that local specializations under the control of BX-C are essential for peristaltic movement.

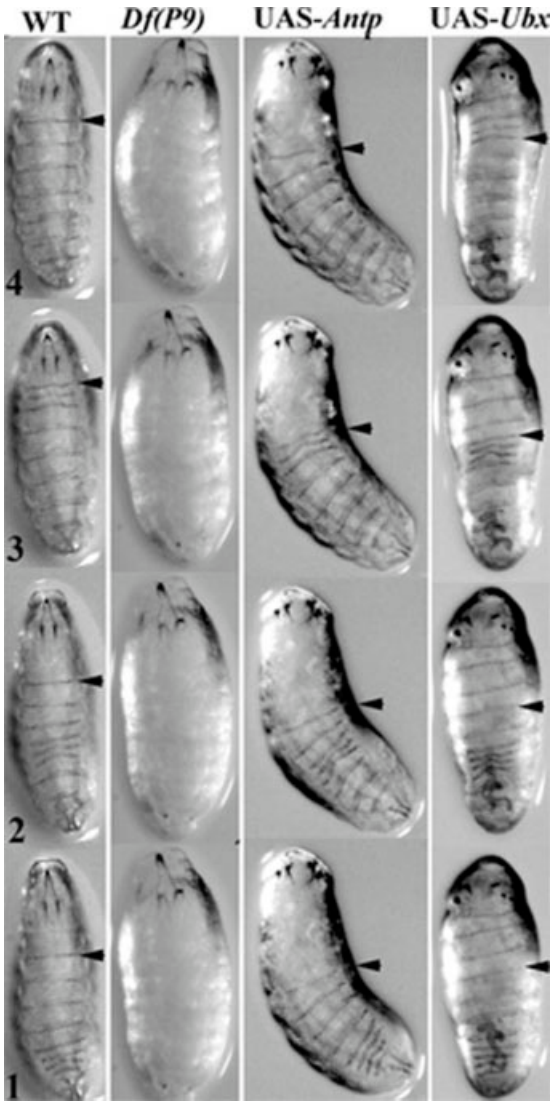

Figure 2 Cycles of peristaltic crawling in hatched larvae. Larvae are wild type (WT); deficient for BX-C [*Df (P9)*]; or with ectopic expression of *Antp* or *Ubx*, driven by *arm*-Gal4 (UAS-*Antp*; UAS-*Ubx* respectively). Larvae are filmed through transparent agar and captured video frames show (1–4) the progress of a single peristaltic wave from posterior (1) to anterior (4). In *Df(P9)*, MT and all abdominal segments are transformed to MS and there is no peristalsis. Ectopic expression of *Antp* transforms segments anterior to MS towards MS, but abdominal peristaltic contractions proceed normally. Ectopic expression of *Ubx* transforms segments anterior to A1 towards A1. Here the wave of peristaltic contractions continues from the abdomen through the transformed anterior segments. Black arrowheads indicate position of A1 denticle band. For details of genotypes see Suppl. Table 1.

Disruption of Anterior Segments by *Antp* mis- Expression Does Not Affect the Development of Abdominal Peristalsis

To show whether an abdominal network is sufficient for peristalsis, or, alternatively, requires additional input from the brain or segments anterior to MS, we used a gain of function approach (Brand and Perrimon, 1993), misexpressing the *Antennapedia* (*Antp*) gene under UAS control using a ubiquitous early embryonic driver, *armadillo*-Gal4 (*arm*-Gal4). In such embryos, the normal structure of the brain is grossly deranged and segments anterior to MS are transformed towards MS (Li and McGinnis, 1999). Despite these obvious abnormalities, hatched larvae showed sustained peristaltic crawling with their abdominal segments (Fig. 2 and Suppl. movie 4). To confirm that normally differentiated anterior segments are not required for peristalsis, we studied the locomotion of larvae that were separately deficient for each of the elements of the ANTP-C. Such larvae were fully capable of peristaltic crawling with their abdominal segments (Fig. 3 and Suppl. movie 5). While these results suggest that machinery adequate for peristaltic movements is located in segments posterior to MS a recent report (Pereanu et al., 2007) has suggested that there may be a role for the protocerebrum in triggering crawling movements.

Either *Ubx* or *abdA* is Required for Peristaltic Crawling

Next we investigated which elements of BX-C are essential for the characteristic pattern of abdominal movement during peristalsis (Fig. 3). Larvae lacking either *Ubx*, *abdA*, or *AbdB* perform peristaltic crawling, as do larvae lacking both *Ubx* and *AbdB* (Suppl. movie 6) and larvae deficient for *abdA* and *AbdB* (Suppl. movie 7). Uniquely, larvae that lack both *Ubx* and *abdA* show no peristaltic movements. Instead, like those deficient for the entire BX-C, such larvae tend to move across the agar substrate by dragging with their anterior segments (Suppl. movie 8). Thus *abdA* and *Ubx* appear to be required for peristaltic crawling. Nonetheless, since either gene may be removed separately without loss of peristalsis, we conclude that the two genes in this instance, as in others (Azpiazu and Morata, 1998; Chauvet et al., 2000), can substitute for each other and this is consistent with their overlapping domains of expression in the abdomen.

However, larvae that are mutant for *Ubx* lack both Ubx and abdA in segments anterior to the normal domain of *abdA* expression. If there is an essential

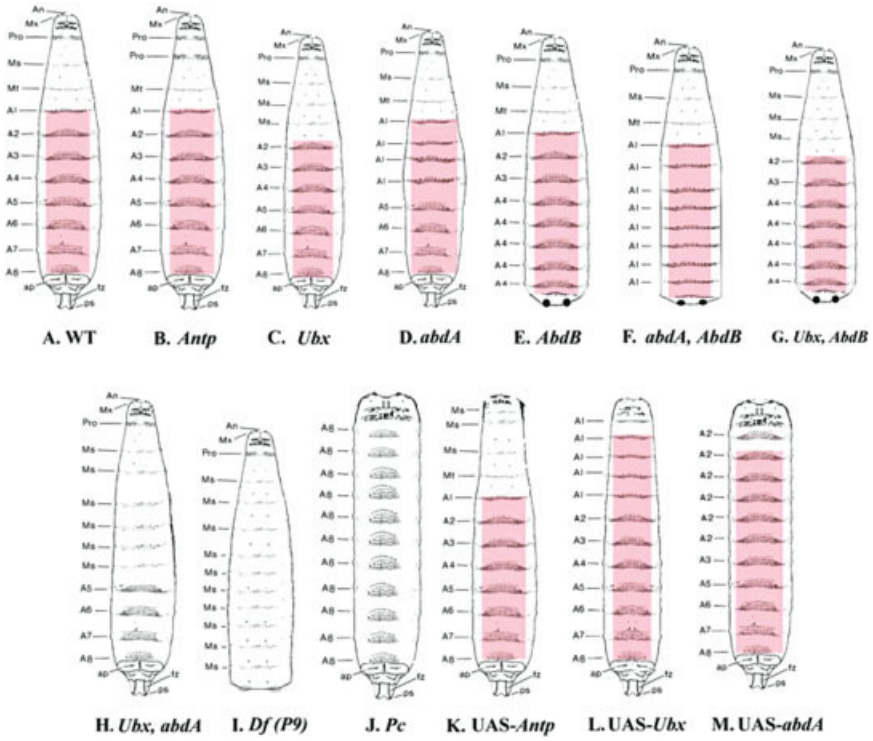

Figure 3 Peristaltic movement in loss- and gain- of- function mutants of the ANTP-C and BX-C. In wildtype (A) characteristically abdominal peristaltic movement begins posteriorly and extends to the first abdominal segment, A1. The domain where this movement is seen is shaded in this and other panels. The segmental transformations are shown in each case. An, antennal; Mx, maxillary; Pro, prothorax; Ms, mesothorax; Mt, metathorax. A1–A8 represent abdominal segments 1 to 8. ap, anal plates; fz, filzkörper; and ps, posterior spiracles. The phenotypes of mutations in *pb*, *lab*, *Dfd*, and *Scr* resemble that shown in B for *Antp*. Loss-of-function phenotypes were examined, where possible, in multiple heteroallelic combinations of mutants of the ANTP-C and BX-C. BX-C mutants were examined, in addition in trans combination with *Df* (*P9*), a chromosomal deficiency that removes the entire BX-C. Penetrance is essentially complete under these conditions and was also similarly high for gain-of-function contexts as seen by cuticular transformations. At least 50 animals were examined in locomotion assays for each genotype. Penetrance of behavioral phenotypes was high and similar to the that seen for cuticular transformations. See Supplementary Table 1 for details. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

requirement for either *Ubx* or *abdA*, such segments should fail to perform normal peristaltic movements. We reviewed this possibility by monitoring the cyclical raising and lowering of each segment that occurs during normal peristaltic movement. These characteristic movements are indeed lost from segment A1 and partially from A2 and 3 in *Ubx* mutant embryos and larvae (Fig. 4).

Either *Ubx* or *abdA* is Sufficient to Allow the Development of Peristaltic Behavior

A further corollary of our finding that functions encoded by *Ubx* or *abdA* are required for peristaltic movement is that ectopic expression of these genes in segments anterior to the abdomen might be sufficient to transform the movements of these segments to a peristaltic phenotype. To test this notion, we expressed either *Ubx* or *abdA* under UAS control using the *arm*-Gal4 driver. Morphologically, anterior segments in such animals are transformed to an abdominal phenotype (UAS-*Ubx*: A1; UAS-*abdA*: A2) (Sanchez-Herrero et al., 1994; Li and McGinnis, 1999; Chauvet et al., 2000). Behaviorally, and quite uniquely, such segments now show the same peristaltic cycle of movements as their more posterior neighbors (Figs. 2, 3, and 4 and Suppl. movie 9). We conclude that either *Ubx* or *abdA* is necessary and sufficient to specify a neuromuscular network that can coordinate the normal movements of peristalsis in

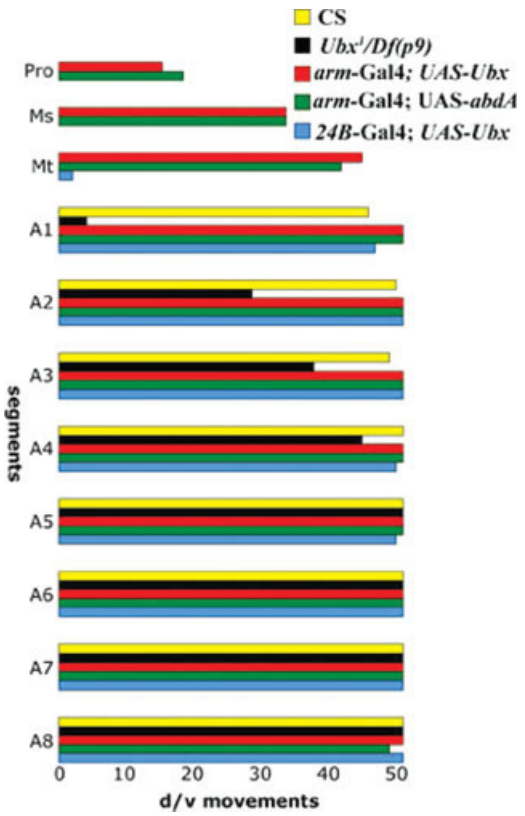

Figure 4 Chart showing the distribution in different genotypes of dorsoventral movements that are unique to abdominal segments during peristaltic crawling. Vertical axis shows segments; horizontal axis shows number of recorded movements. Genotypes as indicated by color code, see text for details. Late embryos were filmed from the side during cycles of peristalsis (see Methods). Ten embryos were monitored for each genotype and movements were recorded for segments shown during each of five peristaltic cycles. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

those body segments in which these genes are expressed. By contrast when all segments are transformed towards A8 (in embryos deficient for the gene *Polycomb* (Lewis, 1978) there is no peristaltic movement (Fig. 3).

Transforming a Thoracic Muscle Pattern to an Abdominal One is Not Sufficient for the Development of Ectopic Abdominal Movement

We assume that coordinated movement requires the locally integrated differentiation of all three network elements: nerves, muscles, and effectors (Dickinson et al., 2000). An alternative view might be that uniform central pattern generating circuits would act on locally different muscle patterns to produce regionally specialized patterns of movement (Katz and Harris-Warrick, 1999). We therefore tested the idea that segmental specialization of the muscles alone might be sufficient to cause the altered patterns of movement that we observe in our experiments. We repeated our gain of function studies using the pan mesodermal driver *24B*-Gal4 to promote ectopic expression of *Ubx* or *abdA* in the mesoderm, with the result that thoracic muscle patterns were transformed to abdominal (Michelson, 1994). Despite this altered pattern of muscles, we could not detect a transformation of movement such as we had previously found with ubiquitous early expression of the same genes (Fig. 4). We conclude that a shift of muscle pattern from thoracic to abdominal is not, by itself, sufficient to produce abdominal patterns of movement. Misexpression of *Ubx* or *abdA* in neurons alone also fails to transform movement (data not shown). However this result is not surprising since the neuroblasts and their lineages are not transformed in this experiment. Thus, while some aspects of postmitotic neuronal differentiation are likely to be altered, fundamental differences in the pattern of neurons contributing to particular segments will remain unchanged.

DISCUSSION

The results that we report here show that homeotic transformations of locomotor behavior can accompany the morphological transformations originally described by Lewis for loss and gain of function of elements of the BX-C complex in *Drosophila* (Lewis, 1978). We believe, although we have not yet demonstrated, that this depends on the reorganization of neural circuitry to match the transformed pattern of muscles on which it operates. If this is to be a coordinate transformation then there must be a match between the domains of Hox gene expression in these tissues. For the muscles, the boundaries of Hox gene expression are segmental (Bate, 1993). In the nervous system the boundaries are parasegmental (Hirth et al., 1998). However a recent study reveals that the muscles are innervated by motorneurons whose dendrites are organized to form a myotopic map, the boundaries of which are parasegmental (Landgraf et al., 2003). In the larva of the fly, the domain of *Ubx* and *abdA* expression in the muscles encompasses A1 to A7, the segments that perform the characteristic movements of peristalsis (Michelson, 1994). In these segments, the muscle pattern is identical, except for small variations in A1, while the muscle patterns anterior to A1 and posterior to A7 are characteristically

different (Bate, 1993). In the nervous system on the other hand *Ubx* is expressed at high levels in parasegment 6, that is the posterior compartment of T3 and the anterior compartment of A1 while *Ubx* and *abdA* are coexpressed from parasegment 7 posteriorly to parasegment 12, ending with the anterior compartment of A7 (Hirth et al., 1998). Thus the combined expression of *Ubx* and *abdA* precisely encompasses domains within which muscles required for peristaltic movement and the neurons that innervate them are organized into a characteristic matched pattern.

Hox gene expression is initiated early in embryogenesis and dictates segment specific patterns of myogenesis and neurogenesis (Michelson, 1994; Technau et al., 2006). However Hox proteins are also present in neurons as they begin to differentiate. This pattern of expression in the cns is vividly maintained throughout the later phases of embryogenesis (Hirth et al., 1998), but its functions remain largely unexplored. Nonetheless, there is accumulating evidence for an essential role for Hox gene expression during neuronal differentiation in the fly. Thus in both *labial* and *deformed* mutants, cns neurons are formed but fail to differentiate in the domains in which these genes are normally expressed (Hirth et al., 1998). In other regions of the nervous system post mitotic expression of Hox genes in differentiating neurons determines segment specific patterns of death and survival (Miguel-Aliaga and Thor, 2004). The findings reported here suggest that the Hox proteins continue to act in the later stages of nervous system development, providing neurons with the positional distinctions that allow them to assemble to form the elements of region-specific neural circuitry.

We believe that this action of the Hox genes is a general one, both in the *Drosophila* larva, where, for example anterior and posterior segments have specialized neuromuscular systems associated with the movements of feeding, digging, exploration, and defecation, and in other organisms. The clearest analogy is with the vertebrate nervous system, where the diversification of neuromuscular systems linked to the hindbrain is prefigured in the embryonic neural plate by the formation of a series of units, the rhombomeres. The boundaries of rhombomeres coincide with the boundaries of Hox gene expression in the developing nervous system (Lumsden and Krumlauf, 1996). From these domains stem both the neural crest derived elements of the branchial arches and the motorneurons that will innervate them. An elegant series of transplant and ectopic expression experiments shows that the matched expression of Hox genes in these two derivatives is an essential determinant of their connectivity (Bell et al., 1999).

More posteriorly, Hox proteins are required for the axial patterning of motor neurons to form region specific columns that match, for example, the local formation of limbs (Dasen et al., 2003). Interestingly the interacting network of Hox genes and their products also allows for the diversification of motor columns into motor pools (Dasen et al., 2005). Here the two aspects of Hox function are very apparent: a repressive function that allows for the mutual exclusion of Hox proteins from inappropriate domains and an activating function that allows for the local initiation of transcriptional programs that lead to specific patterns of differentiation. A similar distinction has been made in the fly between identity-determining functions and morphogenetic functions of Hox proteins in the domains where they are expressed (Hombria and Lovegrove, 2003). In our view, the formation of a neuromuscular network is a morphogenetic process in which groups of cells are marshaled together to form the structures that underlie movement. We suggest that in this process, Hox proteins act as essential cofactors that confer positional specificities to the common transcriptional programs required to generate neuromuscular networks at different levels of the body axis.

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