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Crustacean (malacostracan) Hox genes and the evolution of the arthropod trunk

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SUMMARY

Representatives of the Insecta and the Malacostraca (higher crustaceans) have highly derived body plans subdivided into several tagma, groups of segments united by a common function and/or morphology. The tagmatization of segments in the trunk, the part of the body between head and telson, in both lineages is thought to have evolved independently from ancestors with a distinct head but a homonomous, undifferentiated trunk. In the branchiopod crustacean, *Artemia franciscana*, the trunk Hox genes are expressed in broad overlapping domains suggesting a conserved ancestral state (Averof, M. and Akam, M. (1995) *Nature* 376, 420-423). In comparison, in insects, the *Antennapedia*-class genes of the homeotic clusters are more regionally deployed into distinct domains where they serve to control the morphology of the different trunk segments. Thus an originally *Artemia*-like pattern of homeotic gene expression has apparently been modified in the insect lineage associated with and perhaps facilitating the observed pattern of tagmatization. Since insects are the only arthropods with a derived trunk tagmosis tested to date, we examined the expression patterns of the Hox genes *Antp*, *Ubx* and *abd-A* in the malacostracan crustacean *Porcellio scaber* (Oniscidae, Isopoda). We found that, unlike the pattern seen in *Artemia*, these genes are expressed in well-defined discrete domains coinciding with tagmatic boundaries which are distinct from those of the insects. Our observations suggest that, during the independent tagmatization in insects and malacostracan crustaceans, the homologous 'trunk' genes evolved to perform different developmental functions. We also propose that, in each lineage, the changes in Hox gene expression pattern may have been important in trunk tagmatization.

Key words: Crustacean, Insect, Arthropod, Homeotic gene, Tagmatization

INTRODUCTION

Homeotic (HOM-C/Hox) genes function to specify the identity of segments along the anteroposterior axis in insects and vertebrates (Lewis, 1978; Morata and Kerridge, 1981; Lawrence and Morata, 1994). Genetic studies on the fruitfly *Drosophila melanogaster* and other insects have demonstrated that the products of the Hox genes control the transcription of numerous downstream genes within their expression domains and are required to organize the embryo's body plan (Kaufman et al., 1989; Carroll, 1995; Graba et al., 1997). Homologues of the *Antennapedia*-class genes have been found in all arthropod lineages studied and a full complement of the HOM-C genes most likely existed in the last common ancestor of modern arthropods (Beeman et al., 1993; Akam, 1995; Averof and Akam, 1995; Grenier et al., 1997; Peterson et al., 1999). Among insects, the expression patterns and functions of the HOM-C genes appear to be highly conserved, a fact that correlates with their largely invariant body plan (Beeman et al., 1993; Warren and Carroll, 1995; Rogers and Kaufman, 1997; Brown et al., 1999; Peterson et al., 1999). The extension of this comparative analysis into the Crustacea, a sister group of the Insecta (Ballard et al., 1992; Friedrich and Tautz, 1995; Boore et al., 1998; Reviewed in Gilbert and Raunio, 1997), has proven to be useful in understanding the evolution of the Hox genes and their role in arthropod diversity (Averof and Akam, 1995; Averof and Patel, 1997; Abzhanov and Kaufman, 1999b).

Insects and crustaceans have been shown to possess a single set of homologous Hox genes (Akam et al., 1994). In *D. melanogaster*, the Hox genes are split into two clusters, the Antennapedia complex (ANT-C) (Kaufman et al., 1989) and the bithorax complex (BX-C) (Duncan, 1987). The genes of the ANT-C are necessary for the normal development of the gnathal and thoracic segments and appendages whereas the BX-C genes function more posteriorly in the metathorax and abdomen (Kaufman et al., 1989). The genes *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) specify different segment types in the trunk of *Drosophila* (Kaufman et al., 1989; Lawrence and Morata, 1994). *Antp* is expressed in and required for the specification of the three-segmented locomotory thorax in *Drosophila* (Martinez Arias, 1986; Kaufman et al., 1989). Both *Ubx* and *abd-A* are involved in the development of the legless abdomen (Morata and Kerridge, 1991). *Ubx* is also expressed in the posterior thorax where it is

known to be involved in the development of the modified hind wings, the halteres (Lewis, 1978). Some of the developmental and biochemical functions of *Ubx* and *abd-A* are similar, e.g., both are thought to suppress appendage development on the abdomen via downregulation of *Distalless* (*Dll*) expression (Vachon et al., 1992; Casares 1996). The expression patterns of the trunk genes and their function, where studied, have been shown to be very similar in a number of representatives of other insect orders (Beeman et al., 1990, 1993; Stuart et al., 1993; Kelsh et al., 1994; Rogers and Kaufman, 1997; Peterson et al., 1998; Shippy et al., 1998; Zheng et al., 1999). Although most extant insects share the same body plan, the fossil record shows that the thorax and abdomen apparently evolved from a more or less homonomous trunk, i.e., an undifferentiated trunk bearing similar appendages on all segments (Beklemishev, 1964; Kukalova-Peck, 1991). Moreover, similar body plans featuring an almost complete lack of trunk tagmosis can still be found among representatives of some crustacean orders and in the myriapods (Brusca and Brusca, 1990).

The expression of the 'trunk' Hox genes is also known in the branchiopod crustacean *Artemia fransciscana* (Averof and Akam, 1995). Members of the Branchiopoda are characterized by a long undifferentiated trunk followed by several genital and postgenital segments. All three trunk genes, *Antennapedia*, *Ultrabithorax* and *abdominal-A*, are co-expressed in overlapping broad domains throughout the trunk of *Artemia* (Averof and Akam, 1995. Note: 'trunk' in *Artemia* refers to the appendage-bearing postcephalic segments and does not include the postgenital limbless segments). The nested *Artemia* Hox expression pattern is similar to those reported in chelicerates, annelids and chordates and suggests an ancestral condition for the trunk Hox genes in the arthropods (Averof and Akam, 1995; Carroll, 1995; McGinnis and Krumlauf, 1992; Kourakis et al., 1997; Damen et al., 1998; Telford and Thomas, 1998; Abzhanov et al., 1999).

According to most modern arthropod phylogenies the Crustacea is seen as a sister group to the Insecta while the Myriapoda and Chelicerata are viewed as outgroups (Whitington et al., 1993; Friedrich and Tautz, 1995; Dohle, 1997; Wills, 1997; Whitington and Bacon, 1997; Zrzavy et al., 1997; Boore et al., 1998). Almost all studies on crustacean phylogenies position the Branchiopoda and Remipidia, groups with undifferentiated homonomous trunks, at the base of the crustacean tree and place the monophyletic class Malacostraca (higher crustaceans) as a group with the most derived characters (Siewing, 1963; Schram, 1986; Brusca and Brusca, 1990; Wills, 1997; Whitington and Bacon, 1997; Nilsson and Osorio, 1997). Like insects, the higher crustaceans have divided the trunk into morphologically and functionally distinct tagma called the pereon and pleon and contemporary models suggest that the malacostracans are the only crustaceans with a tagmatized trunk (Fig.1; Beklemishev, 1964; Lauterbach, 1975; Schram, 1986; Brusca and Brusca, 1990; Briggs et al., 1992). Furthermore, reminiscent of the derivation of insect trunk tagma, both the pereon and pleon of malacostracans have been proposed to be homologous to and derived from the 'thorax' of branchiopods, cephalocaridans and maxillopods (Lauterbach, 1975; Walossek and Muller, 1997). This implies that, among the mandibulate arthropods, only the malacostracan crustaceans and insects have evolved novel tagmata within their trunks.

Representatives of the order Leptostraca, a basal group within Malacostraca, have eight pairs of paddle-like swimming and feeding appendages on the pereon and six pairs of appendages on the seven-segmented pleon (Brusca and Brusca, 1990). Other malacostracans have modified one or more of their anterior locomotory appendages into maxillipeds and lost the seventh pleonic segment (Beklemishev, 1964; Brusca and Brusca, 1990). *Porcellio scaber* (order Isopoda), the animal in which we have studied the expression patterns of the trunk genes, has one pair of maxillipeds and six segments in the pleon (Fig. 1, and Schram, 1986; Abzhanov and Kaufman, 1999a).

In this work, we used whole-mount in situ hybridization to reveal the expression domains of the homologues of *Antp, Ubx* and *abd-A* in mid-stage embryos of *P. scaber*. We found that, unlike their insect homologues, *PsAntp* and *PsUbx* both are expressed in similar but not identical domains covering the pereon. Interestingly, *abd-A* is detected in the pleon but not the pereon. A comparative analysis of *P. scaber* expression patterns with those of insects and other arthropods supports the view that the last common ancestor of insects and malacostracan crustaceans, although an animal with a number of derived features, likely had an undifferentiated homonomous trunk. In both malacostracan and insect lineages, the trunk has evolved to contain two morphologically distinct parts: a tagma associated with locomotion (thorax/pereon) and another more posterior group the abdomen/pleon. We propose that the Hox genes may have facilitated this process differently in the two lineages by evolving discrete distinctive expression domains and unique developmental roles along the anteroposterior axis.

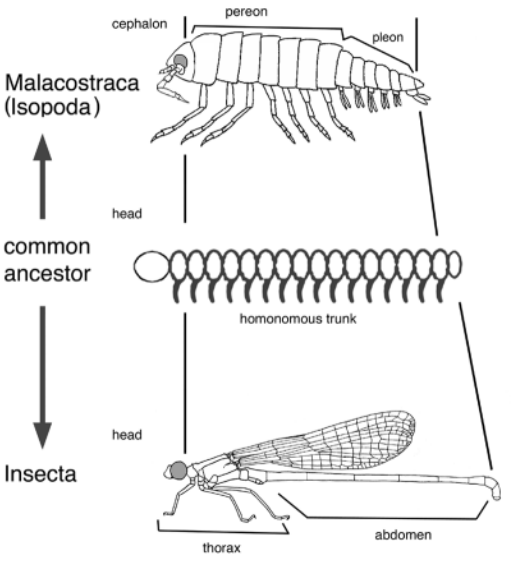

Fig. 1. Trunk tagmosis in mandibulate arthropods. A diagram of malacostracan and insect trunk tagmosis. Isopods (Malacostraca) have a cephalon with antennae, mandibles, 1st and 2nd maxillae (mx1 and mx2) and maxillipeds (T1/Mxp). The trunk is subdivided into pereon and pleon. The last pair of pleonic appendages are modified as uropods. The presumed common ancestor has a head and a uniform homonomous trunk. Insects have a head with posterior mouthparts and their trunk is subdivided into thorax and abdomen.

MATERIALS AND METHODS

Cloning and sequence analysis of the cDNA fragments

Colonies of the common woodlouse *Porcellio scaber* (Isopoda, Oniscidae) were originally established from animals collected around Bloomington, IN, USA. Females of *P. scaber* brood about 50 embryos in a ventral marsupium formed by large oosteogite plates of the walking legs. The transparency of the plates allows developmental staging of the embryos as by Whitington et al. (1993). Embryogenesis lasts about 3 weeks. The embryos were dissected from the marsupium and washed in 0.01 M phosphate-buffered saline (PBS) before fixation for in situ hybridization and antibody staining.

Embryos of *P. scaber* were used for mRNA and genomic DNA extractions using RNA isolation kits (Gentra Systems, Inc) or the TriZol®Reagent (Life Technologies) following the manufacturer's protocols. cDNA fragments of *Ultrabithorax* and *abdominal-A* containing the 'variable' region were cloned using previously published RT-PCR protocols (Peterson et al., 1999; Rogers et al., 1997). A fragment of *Antennapedia* was cloned using reverse PCR from genomic DNA of *P. scaber*. We used the primers 5′ GAT AGG TTT GCC TGC CAC GCT TTC G 3′ and 5′ CTT TGT GTC TCA CGG AAA GGC AAA TC 3′ to clone the genomic *Antp* fragment. The PCR conditions were as follows: 1 cycle at 95°C for 5 minutes and 30 cycles: 95°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute. The *Antennapedia* homeobox fragment used to design the inverse PCR primers was cloned with degenerate primers encoding the YPWMRSQF and WFQNRR conserved motifs. *Ultrabithorax* was cloned using PQIYPWM and ENLEQEK primers (5′ CCR CAR ATH TAY CCR TGG ATG 3′ and 5′ ACY TTY TCY TGY TCR TTI ARY TC 3′, respectively). *abdominal-A* was cloned with PQIYPWM and NIEKVA primers (5′ CCR CAR ATH TAY CCR TGG ATG 3′ and 5′ TCR TTD ATY TCY TTI ACI GCI C 3′, respectively). Multiple independent clones of all cDNA fragments were sequenced with the ABI PRIZM™ Dye Terminator Cycle Ready Reaction Kit (Perkin Elmer). All of the obtained nucleotide sequences were compared against all of the previously cloned fragments available in the laboratory and the NCBI database using MacVector™6.0.1 (Kodak) software. GenBank Accession numbers: Ps-abd-A1, AF241657; Ps-abd-A2, AF241658; Ps-abd-A3, AF241659; Ps-Ubx1, AF241660; Ps-Ubx2, AF241661; Ps-Antp, AF241662.

Fixation of embryos, in situ hybridization and antibody staining

Embryos were dissected and then fixed in 4% formaldehyde in PBT for 2 hours, dehydrated and stored in methanol at −20°C. The in situ hybridization protocol used is similar to the one described in Panganiban et al. (1994) and Rogers et al. (1997), however, embryos were divided into three aliquots that were exposed to the Proteinase K for 10, 20 and 30 minutes, respectively, before being pooled together for in situ hybridization. Antisense and sense riboprobes were labeled with digoxigenin using Riboprobe® Combination System − T3/T7 (Promega) or with T7/T3 polymerases (Boehringer, Mannheim, Germany; Genius RNA labeling kit) from a linearized pBluescript template that included cloned PCR fragments. Incubation was done overnight at 65°C after addition of DIG-labelled probe (~70ng/ml). Embryos were washed at 70°C overnight. An alkaline phosphatase-conjugated antibody was used to detect the signal.

The immunochemical staining procedure is described by Kelsh et al. (1994) and Averof and Patel (1997). The monoclonal antibody FP6.87 against Ubx and Abd-A is described by Kelsh et al. (1994). All mounting, photographing and scanning electron micrograph procedures have been described previously (Gorman and Kaufman, 1995; Rogers et al., 1997).

Microscopy and photography

Stained embryos were mounted on microscope slides in AquaPolymount (PolySciences). Slides were observed on a Zeiss axiophot microscope and photographed at ×50-200 magnification. Images were captured with a ZVS-3C75DE videocamera. Standard Nomarski and dark-field optics were used to observe localization in different cells and tissues. Adobe Photoshop was used on some images for adjusting light and/or contrast.

RESULTS

Cloning of *PsAntp*, *PsUbx* and *Psabd-A*

Orthologs of *Antennapedia*, *Ultrabithorax* and *abdominal-A* from the malacostracan crustacean *Porcellio scaber* were isolated by PCR with codon-degenerate primers. To clone the cDNA fragments, we amplified embryonic cDNA by targeting the YPWM motif upstream of the homeobox and the 3′ end of the homeobox and/or the region immediately downstream of the homeobox. The cDNA fragment initially cloned for *PsAntp* was used to generate a set of specific primers for inverse PCR from genomic DNA. The resulting *PsAntp* genomic DNA fragment included the N-terminal arm of the homeobox, the homeobox itself, the C terminus encoding region and part of the 3′ UTR.

Orthology of the *P. scaber* Hox genes was determined from the alignment of the predicted amino acid sequences (Fig. 2). Only the YPWM motif, the spacer 'variable region', homeodomain and the immediate downstream region are shown. The variable region and the homeodomain contain important gene-specific amino acid residues, which can be used to unambiguously assign genes to specific classes (Peterson et al., 1999). The *Antp* spacer region is well conserved between crustaceans and insects (Fig. 2A). Two apparent splicing variants were recovered for *PsUbx*, which differ in their variable region. *PsUbx1* has a spacer region identical to that of *Artemia* whereas *PsUbx2* encodes five additional residues in that region (Fig. 2B). Three apparent splice variants were cloned for *Psabd-A* (Fig. 2C). The shortest transcript, *Psabd-A1*, encodes only three residues in its variable region in addition to the five amino acids of a conserved region flanking the N-terminal arm of the homeodomain. *Psabd-A2* encodes 17 additional residues, which are also present in *Psabd-A3*. The longest apparent splice variant, *Psabd-A3*, encodes another 25 residues added just downstream of the YPWM motif (Fig. 2C). In Fig. 2B,C, arrows indicate the amino acids recognized by the FP6.87 antibody, which identifies both Ubx and Abd-A proteins in insects and crustaceans (Kelsh et al., 1994; Averof and Akam, 1995; Averof and Patel, 1998). The residues are present in all forms of both in *PsUbx* and *Psabd-A* (Fig. 2B,C) which is consistent with the expression pattern data presented below.

Multiple copies of each cDNA fragment were cloned and sequenced. We found no evidence for paralogous Hox groups suggesting that only one Hox cluster is present. Overall, the amino acid sequences of the homeodomains in *P. scaber* Hox genes are well conserved with their insect homologues.

Embryonic expression patterns

Members of the Isopoda and related malacostracan orders have yolky eggs and early cleavage is superficial (meroblastic) as in

most other crustaceans. However, *P. scaber* develops directly and unlike many other crustaceans does not have nauplius and postnauplius larval stages. Embryogenesis terminates in hatching of the manca larva, which is morphologically similar to the adult but lacks a seventh pair of pereonic appendages (Schram 1986; Abzhanov and Kaufman 1999a). The early-to-mid-stage embryos (30-60% stage) are the most suitable for in situ analysis as embryos of later stages deposit cuticle on the

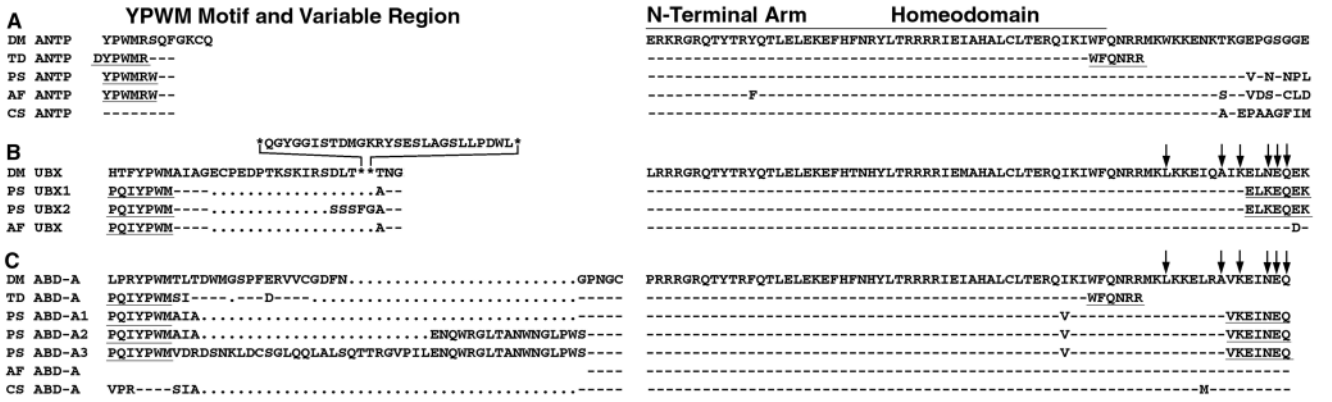

Fig. 2. (A-C) Alignment of the *Porcellio* (PS) Hox genes with the insects fruitfly *Drosophila melanogaster* (DM) and firebrat *Thermobia domestica* (TD), the branchiopod crustacean *Artemia fransciscana* (AF) and the spider *Cupiennius salei* (CS). All non-*P. scaber* sequences were obtained from the GenBank. Dashes indicate identical residues, dots indicate gaps. Regions targeted with degenerate primers during PCR from embryonic cDNA are underlined. Three regions are shown: YPWM motif, 'variable region' and the homeodomain with its N-terminal arm. (A) *Antennapedia* homeodomain is almost perfectly conserved between apterygote insect, crustaceans and the spider. The N-terminal arm and the homeodomain are marked with thick black line above. ANTP variable regions of TD, PS, AF and CS are only 2 amino acids long while DM ANTP is slightly longer. (B) DM UBX has a very long (50 aa) variable region. Both AF and PS variable regions are short. PS UBX2 has 5 amino acid residues inserted in the same relative position within the variable region as in *D. melanogaster Ubx*. (C) Although the homeodomains of *abd-A* homologues are highly conserved throughout arthropods, the variable regions are very divergent in length and amino-acid composition.

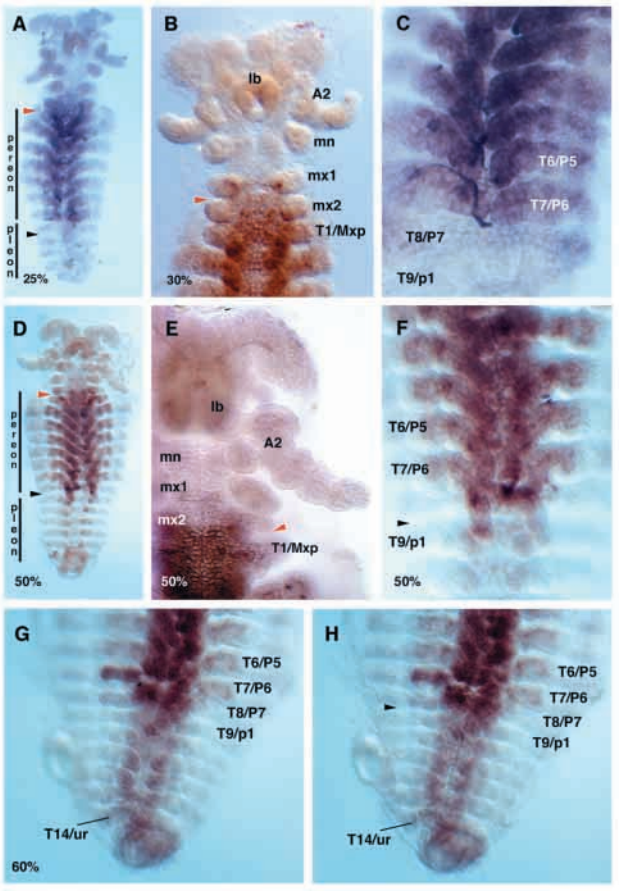

Fig. 3. (A-H) Expression of *Antennapedia* in *P. scaber.* All embryos are oriented anterior to the top of the page. The red arrowhead and the black arrowhead mark the tagmatic boundaries. The red arrowhead marks embryonic head-trunk boundary (between 2nd maxillary and T1/Mxp), which is located within the cephalon of the adult. The black arrowhead indicates the adult pereonic/pleonic boundary between T8/P7 and T9/p1. Abbreviations used to label segments: lb, labrum; A1, 1st antennal; A2, 2nd antennal; mn, mandibular; mx1, 1st maxillary; mx2, 2nd maxillary; T1/Mxp, embryonic first trunk/adult maxilliped segment; T2/P1 to T8/P7, embryonic second to eighth trunk/adult first to seventh pereonic segments; T9/p1 to T13/p5, embryonic ninth to thirteenth trunk/adult first to fifth pleonic segments; T14/ur, embryonic fourteenth/adult uropod segment. Embryonic stages are indicated in the bottom left corner of each panel. (A-C) *Antp* is strongly expressed in the posterior head and pereon of young 30-40% stage *Porcellio* embryos. (B) The anterior boundary is in the mx2. Expression is relatively uniform throughout the pereonic segments. (C) The posteriormost expression is in the T7/P6 with some weak lateral staining in T8/P7. The posterior pereonic appendages are limb buds at this stage. There are no discernable limb buds on the pleon at this point. (D,E) The main expression domain is maintained in the 50-60% stage embryos. No expression is seen in the anterior head or pleon. (E) The anterior boundary has retracted to the posterior mx2 segment but is readily seen in the mx2 appendages. Ventral staining becomes stronger than lateral. (F) There is strong expression in the mesoderm of the pereonic appendages but, at least in the coxa and basis, no ectodermal staining is seen. No expression is detected in the ventral pleon at this stage. (G,H) 60-70% stage embryos that have completed dorsal closure. In G, the appendages are in focus whereas, in H, the ventral body surface is in focus. Weak staining in the ventral pleon with the anterior boundary between T8/P7 and T9/p1 is observed. No expression is detected in the pleonic appendages.

ventral portion of the posterior head, the pereon and most of the pleon impeding detection of the Hox mRNA (stages in Materials and Methods). Additionally, embryos of earlier stages often fragment during dissection but compilation of data from the stages before 30% are presented in the text where available.

PsAntp marks the embryonic tagmata boundaries

Using in situ hybridization, we found that *Antp* is principally expressed in the pereon (Fig. 3A-H); although at later stages (after ~60% stage) weak expression is also observed in the ventral pleon (Fig. 3G,H). In early embryos, the anterior boundary often appears to coincide with the mx1/mx2 segmental boundary, perhaps even extending into the posterior mx1 segment, although expression in the anterior mx2 is weaker than in the rest of the expression domain (Fig. 3A,B). In later stages the anterior boundary of expression is in the posterior mx2 segment and includes the posterior portion of the mx2 appendages (Fig. 3A,B,D,E). The posterior boundary of accumulation is in the T7/P6 segment, which corresponds to the posterior thoracic border in early 30-40% stage embryos (Fig. 3A-C) and later mid-stage embryos (Fig. 3D-F). Note that the T8 segment does not have any appendages until after hatching when a pair of walking legs similar to the other pereopods develop (Schram, 1986; Abzhanov and Kaufman, 1999a).

The main domain of expression in the pereon is initiated early in development, prior to and during the morphological establishment of the tagmata (Fig. 3A-C; not shown). The expression in the pereonic appendages is both ectodermal and mesodermal (Fig. 3E-G). However, there is a clear gradient along the proximodistal body axis in the distribution of *PsAntp* transcripts within these appendages (Fig. 3F). Notably, in the coxa (coxapod) and, to some extent, in the basis (basipod) of the more posterior pereopods, there appears to be a substantial decrease in the ectodermal expression whereas mesodermal expression remains at levels comparable to the more anterior limbs (Fig. 3F). During development *PsAntp* transcripts accumulate at an apparently higher level along the ventral midline (between the pereopods) in the pereon as compared to the lateral plates. This expression may represent cells of the developing central nervous system (CNS; Fig. 3A,D,G,H). Also, there is late expression in the ventral pleonic ectoderm. This expression is markedly lower than in the ventral pereon

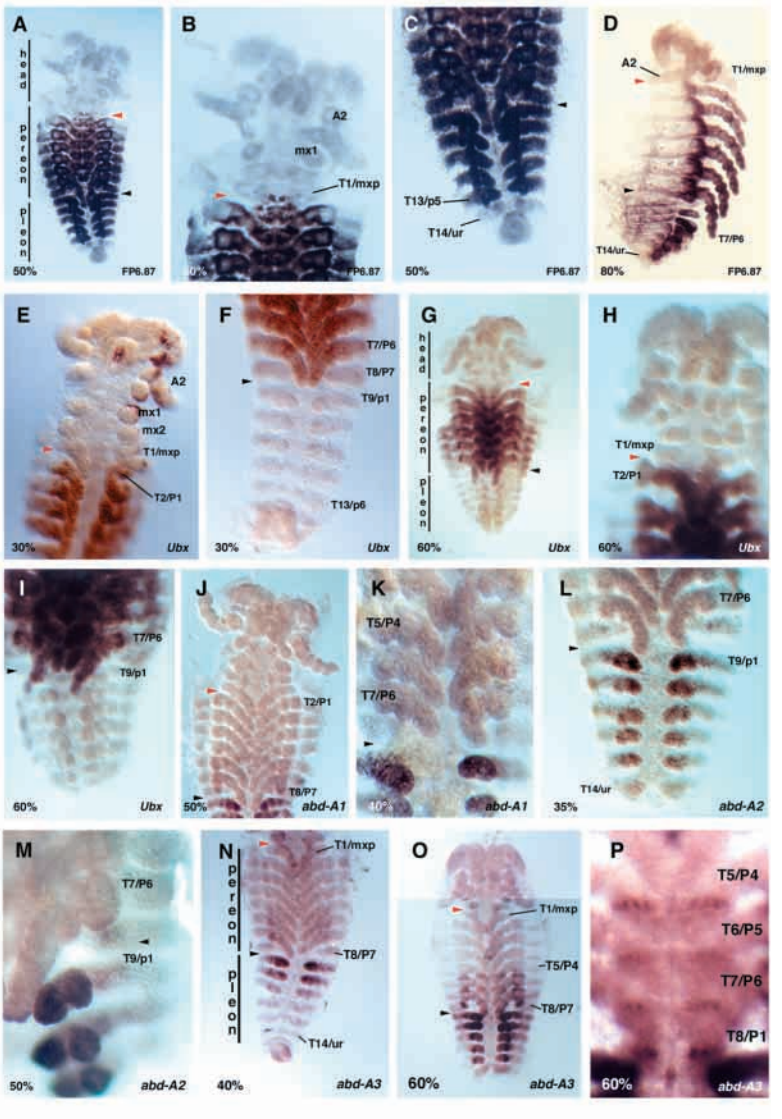

Fig. 4. (A-J) Expression of *Ubx* and *abd-A* in *P. scaber* as revealed with whole-mount in situ hybridizations. The red arrowhead and the black arrowhead are used as references to mark the tagmatic boundaries. The red arrowhead marks embryonic head/trunk boundary (between 2nd maxillary and T1/Mxp), which is located within the cephalon of the adult. The black arrowhead indicates adult pereonic/pleonic boundary between T8/P7 and T9/p1. Abbreviations are as in Fig. 3. Embryonic stages are indicated in the bottom left corner of each panel. (A-D) Ubx/Abd-A accumulation detected with the FP6.87 antibody and strongly enhanced with NiCl. Signal can be seen in both the pereon and pleon. (B) The anterior boundary of Ubx/Abd-A expression is in the posterior ventral T1/Mxp but not in the appendages. (C) The posterior boundary of Ubx/Abd-A is in T13/p5 segment. Note the absence of signal in the ventral pleon and uropods. (D) Ubx/Abd-A expression in 80% stage embryos. Note the gradients in protein accumulation both in the pereon and pleon. (E-I) *Ubx* mRNA is detected in the pereon. (E,F) In early 30% stage embryos the anterior and posterior boundaries are already established. No signal is observed in the cephalon (including the T1/mxp appendages) and pleon. Note (weaker) expression in T8/P7 segment, the future last segment of the adult pereon. (G-I) The *Ubx* expression domain remains the same throughout the embryonic stages assayed. Note the gradient of *Ubx*mRNA distribution in the lateral pereonic plates. (J-L) The expression of *abd-A* is in the pleon only. (J) No *abd-A* expression is seen in the cephalon or pereon. (K) In addition to the strong expression in the pleonic appendages, a spot of weak expression is also seen in cells which will likely give rise to the T8/P7 leg. (L) Note the gradient of *abd-A* accumulation in the pleonic appendages – the anterior pleopods show an apparently higher signal. (M) Higher magnification of the 50% stage embryo stained with a probe recognizing the *abd-A2* transcript. (N-P) Expression pattern of the *Psabd-A3* splicing version. Note that accumulation is restricted to the pleon of early embryos (N) but expands into the pereon at later stages (O). Pereonic expression of *abd-A3* is reiterated in rows of cells in several posterior pereonic segments (P).

and is also likely associated with the CNS (Fig. 3G,H). Note that, in late development, a strong ventral band of expression extends to the T8 segment, the future last pereonic segment (Fig. 3H).

As noted the late ventral expression of *PsAntp* is most likely associated with the developing CNS. The differences in the levels of expression between pereon and pleon might be indicative of a role in specification of very different types of ganglia in these two tagma found in adult isopods (Walossek and Muller, 1997). The T8 segment also exhibits some very weak lateral expression of *PsAntp*, particularly earlier in embryogenesis (Fig. 3C,F). We could not detect *PsAntp* mRNA in the cephalon, lateral pleonic ectoderm, pleonic appendages or uropods. The distribution of PsAntp protein products is currently unknown but merits further investigation, especially in the 70% and older embryos (see Discussion and Abzhanov and Kaufman, 1999a).

In summary, we observe that the distribution boundaries of *PsAntp* mRNA coincide with the morphological boundaries of the embryonic tagma during midstage development. The anterior boundary marks the border between head and pereon when the T1 appendage is still leg-like. The posterior boundary is between T7/P6 segment, which has a walking leg, and T8/P7 segment, which is legless at this stage.

FP6.87 antibody detects UBX and ABD-A proteins in the trunk

The monoclonal antibody FP6.87, which recognizes a conserved epitope in both Ubx and Abd-A, has been used in a variety of different arthropods (Kelsh et al., 1994; Averof and Patel, 1997; Abzhanov and Kaufman, 1999a). This epitope is present and conserved in both *PsUbx* and *Psabd-A* (Kelsh et al., 1994; Averof and Akam, 1995; see Fig. 2B,C). In crustaceans, the anterior border of the FP6.87-detected signal correlates with the cephalon/trunk boundary (Averof and Akam, 1995; Averof and Patel, 1997). In many malacostracan crustaceans, including the Isopoda, the anterior trunk appendages evolved into mouthpart maxillipeds (Schram, 1986; Brusca and Brusca, 1990). In *P. scaber*, the Ubx/Abd-A anterior boundary, as detected by the FP6.87 antibody, is in the second trunk segment (the first adult pereonic segment or T2/P1; Fig. 4A-D). The legs of the first trunk segment (T1) develop into maxillipeds (mxp). Close examination of the 50-60% stage embryos reveals that the Ubx/Abd-A domain extends anteriorly and parasegmentally into the ventral T1 segment but that no Ubx/Abd-A accumulation is observed in the head, most of the T1/mxp segment, the ventral portion of the pleon or uropods (Fig. 4A-D).

Overall, Ubx and Abd-A are detected in the developing trunk. This is similar to the collective distribution domain of Ubx and Abd-A in *Artemia* and other crustaceans (Averof and Akam, 1995; Averof and Patel, 1998).

PsUbx expression domain has a novel posterior boundary

Expression of *PsUbx* can be detected shortly after the formation of the future trunk region but before and during the pereon and pleon morphological differentiation (Fig. 4E,F; not shown). We used specific probes to examine the expression patterns of the two splicing versions which differ in the 'variable region' upstream of the homeobox but are otherwise identical (Fig. 2B). Both *Ubx* transcripts are expressed exclusively in the locomotory pereon and completely overlap (Fig. 4E-H, not shown). Consistent with the FP6.87 antibody results, the anterior boundary of *PsUbx* expression is in the posterior ventral T1/Mxp segment (Fig. 4E,H). The posterior boundary of expression is in the T8/P7 segment (the last pereonic leg-bearing segment in the adult) and no expression is detected in the pleon (Fig. 4F,G). These boundaries are established very early in development and pereonic expression is clearly seen when only small limb buds (20% stage) are present on the germ band (not shown). Therefore, beginning in early development, the *PsUbx* expression domain overlaps extensively with that of *PsAntp* but its anterior boundary lies posteriorly by one segment. Note, however, that in early development only low levels of *PsUbx* expression are detected in the ventral portion of the embryo (Fig. 4E). However, embryos at similar stages show high levels of *PsAntp* in this domain suggesting that different Hox codes may be active in the ventral as compared to the lateral regions of the pereon (Fig. 3A-C).

It should be noted that both in situ hybridization and FP6.87 antibody results reveal a gradient in *Ubx* accumulation in the mesoderm of the proximal podomeres (leg segments) of the pereonic legs – from no mesodermal expression in T2/P1 to high expression in T7/P6 (Fig. 4B,C,F,G). The ectodermal accumulation shows a similar gradient in the plates of the lateral pereon but not in the appendages (Fig. 4A,B,E,F). In addition to the distinct distributions of *PsAntp* and *PsUbx* in the mesoderm and ectoderm of the appendages, in 50% stage and later embryos, we also noted that *PsUbx* accumulates at higher levels in the lateral plates whereas *PsAntp* is more abundant in the ventral part of pereon (Figs 3, 4A-H). The differences in *PsUbx* and *PsAntp* distribution may reflect distinct developmental functions for these genes in these portions of the germ band (see Discussion).

Psabd-A is restricted to the developing pleon

We studied the distribution of all three putative splicing variants of *Psabd-A* (Fig. 4J-P). The expression patterns detected with the three probes are very similar (but not identical) and restricted primarily to the pleon (Fig. 4J-O). Probes to *Psabd-A1* and *Psabd-A2* detect a signal in the developing pleon with an anterior boundary in the first and a posterior boundary in the last pleopod-bearing segments (T9/p1 and T13/p5, respectively; Fig. 4J,K). The expression of these variants is strongest in the first two pairs of the pleonic appendages (T9/p1 and T10/p2, Fig. 4J,K). The signal in the three remaining pairs of pleopods is uniform but weaker than in p1 and p2 (Fig. 4J-L). This pattern is established early in development and continues throughout embryogenesis. The receding anteroposterior gradient in the pleon of the FP6.87-stained embryos is matched by *abd-A1* and *A2* transcript accumulation and no expression is detected in the cephalon, pereon, uropods or in the ventral part of pleon (Fig. 4K,L). The anterior boundary of expression detected with *Psabd-A1* and *Psabd-A2* probes is similar to that of *Psabd-A3* in early embryos (Fig. 4L).

Probe to the third and the largest variant, *Psabd-A3*, produces signal most strongly in the first two pairs of pleopods (Fig. 4N,O). However, unlike *Psabd-A1* or *Psabd-A2* transcripts, *Psabd-A3* domain extends into the posterior pereon

after the 50% stage (Fig. 4N,O). At these later stages, a spot of strong signal is detected in ventrolateral portion of the T8/P7 segment and a weaker signal is observed on the periphery of the coxa and mesoderm of the developing P6 and P7 pereopods and possibly on even more anterior pereonic appendages (Fig. 4O). This late expansion into already morphologically distinct tagma is reminiscent of expression domain expansion of *Ubx* into the posterior thorax of the apterygote insect *Thermobia domestica* (Peterson et al., 1999). Another novel expression detected with the *Psabd-A3* probe in late embryos is in the single row of cells in the anterior part of the T6/P5, T7/P6 and T8/P7 segments (Fig. 4P). We interpret this expression as specific to some neuronal cells. Interestingly, none of the *Psabd-A* variants is accumulated in the pleonic CNS.

The lack of *Psabd-A* expression in the ventral pleonic ectoderm might be due to the expression of *PsAntp* in this domain. This 'gap' is also observed in the pleon of embryos stained with the FP6.87 antibody (Fig. 4C). In summary, the expression domain of *Psabd-A* is principally restricted to the developing pleon with an anterior boundary that borders the posterior boundary of the *PsUbx* transcripts. Taken together, the combined *PsUbx* and *Psabd-A* mRNA expression domains closely match the distribution of their combined protein products, as detected with the FP6.87 antibody (Fig. 4A-L). Their individual domains while both restricted to the trunk are separate and largely non-overlapping beginning in early development. This pattern is novel for any arthropod studied thus far and correlates with the morphological border between pleon and pereon.

DISCUSSION

Trunk Hox gene expression patterns have been determined most extensively in representatives of various orders of the Insecta (Kelsh et al., 1994; Hayward et al., 1995; Peterson et al., 1999). Outside of this group, arthropod Hox genes have been studied only in a branchiopod crustacean *Artemia franciscana* and, more recently, in the Chelicerata (Averof and Akam, 1995; Damen et al., 1998; Telford and Thomas, 1998; Abzhanov et al., 1999). A comparison of the expression patterns seen in *A. franciscana* and insects generated an hypothesis as to the origin of the tagmata in the insect body plan (Averof and Akam, 1995). It was proposed that the appendage-bearing 'thorax' of *A. franciscana*, which displays overlapping domains of trunk Hox gene expression, is homologous to both the thorax and abdomen of insects (Averof and Akam, 1995). According to this model, the evolution of distinct trunk tagmata is associated with and facilitated by the evolution of distinct trunk Hox gene expression domains and functions. Since the validity of the argument was based on only one tagmatized mandibulate trunk, that of the Insecta, we thought it judicious to analyze the Hox expression patterns in the tagmatized trunk of the higher crustacean *Porcellio scaber* (Oniscidae, Isopoda, class Malacostraca). This species belongs to a lineage that evolved a tagmatized trunk, yielding a morphologically distinctive pereon and pleon, independently from insects (Lauterbach, 1975; Schram, 1986; Brusca and Brusca, 1990; Walossek and Muller, 1997). Our observations indicate that both the anterior and posterior boundaries and domains of all the trunk Hox genes investigated differ from their homologues in insects. Nevertheless, these expression domains do coincide extremely well with the tagmatic boundaries of the developing malacostracan trunk (Fig. 5A,B).

Possible new regulatory interactions

The coincidence of the anterior and posterior expression boundaries of the *Porcellio* Hox genes with the morphological limits of the tagma, both in the embryonic and adult body plans can be used to imply possible functions for these genes. For example, *PsAntp* is likely to play a role in the specification of the embryonic thorax and particularly in imparting identity to the legs as it is the only Hox gene expressed in all of these appendages, including T1/mxp, in early development. However, after the midpoint of embryogenesis, its function appears to be overridden by *PsScr* in the first trunk legs (T1/mxp), which then transform into mouthparts (Abzhanov and Kaufman, 1999a). It is at this point that Scr protein first appears in the T1/mxp appendages. Unfortunately, we do not know whether Antp protein is also present and data on the Antp distribution pattern might help us to understand more about the post-transcriptional regulation of *PsScr* in this segment (Abzhanov and Kaufman, 1999a). One intriguing possibility is that, while the transcripts of both *PsAntp* and *PsScr* are present in the T1/mxp appendage throughout its development, the two proteins may be reciprocally accumulated; i.e., Antp is present early when the appendage is leg-like but that later in development Scr protein appears while Antp diminishes. The validity of this proposition awaits the development of an antibody that will recognize PsAntp.

A second novel functionality concerns *PsUbx* which could be involved in the later development of the pereonic appendages, e.g., via suppression of the head genes and/or head identities. This possibility is strengthened by a survey of various crustaceans using the FP6.87 antibody, which recognizes Ubx and Abd-A. This analysis showed a good correlation between the Ubx/Abd-A anterior expression boundary and the cephalon/pereon border (Averof and Patel, 1997). We have shown that, in *P. scaber*, this boundary belongs to *Ubx* alone and, by extension, may be characteristic of the higher crustaceans in general. The novel posterior boundary of *Ubx* expression is also interesting and might be indicative of repression of *Ubx* (and for that matter *Antp*) by *abd-A*. In *Drosophila*, *Antp* expression and function are known to be suppressed by the more posteriorly expressed BX-C genes (Gonzales-Reyes and Morata, 1990; Hafen et al., 1984; Krasnow et al., 1989). It is interesting to note that the last pereonic segment (T8/P7) accumulates *Ubx* whilst there is no *Antp* expression at the early- to mid-embryonic stage. This segment is limbless throughout embryogenesis and grows a full-size pereonic leg during one of the subsequent postembryonic molts (Schram, 1986). It is not known whether *Antp* is expressed in T8/P7 after hatching but our hypothesis that *Antp* and *Ubx* co-expression specifies the adult pereon predicts that it should be. Here again antibody to PsAntp will be a valuable reagent for testing this hypothesis.

It is interesting to note that *abd-A* is the only Hox gene expressed in the pleon and, unlike in *Drosophila*, this gene apparently produces three splicing variants. We propose that all *abd-A* splicing variants play a role in the developing pleon and that the observed modulation of expression is important to producing the morphological diversity seen within this tagma. For example the higher *abd-A* expression in T9/p1 and T10/p2

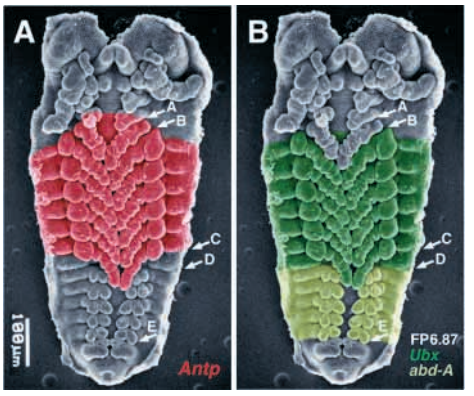

Fig. 5. (A,B) A diagram summarizing the Hox expression domains in the 50-60% stage *Porcellio* embryo. Some important morphological landmarks: A, the posterior mx2 segment; B, the T1/Mxp segment; C, T7/P6, the last leg-bearing embryonic segment; D, T8/P7, the last leg-bearing adult segment; E, the border between T13/p5 and T14/uropod segments. (A) *Antp* is color-coded in red. (B) Both green color-coded domains represent a signal detected with the FP6.87 antibody; dark green is *Ubx* and light-green is *abd-A*.

(as detected with *abd-A1* and *abd-A3* specific probes) correlates with the modification of these appendages into lung-bearing structures and this accumulation may be significant to the specification of these structures.

Comparative analysis of trunk Hox genes in the Branchiopoda and Insecta

Contemporary molecular and morphological phylogenies of the Crustacea indicate that this group comprises a monophyletic assembly with some classes such as the Remipidia and Branchiopoda at a basal position and the Malacostraca as a crown group (Brusca and Brusca, 1990; Wills, 1997; Whitington and Bacon, 1997; Nilsson and Osorio, 1997). Additionally, according to recent phylogenies, the Crustacea are placed as the sister group to the Insecta in the subphylum Mandibulata. Alternatively, some studies suggest that crustaceans may be paraphyletic with regard to the Insecta with the Malacostraca as the closest sister group to insects (Nilsson and Osorio, 1997; Whitington and Bacon, 1997). The Mandibulata also includes the more distantly related Myriapoda. The Chelicerates are generally regarded as a sister group to the Mandibulata (Ballard et al., 1992; Boore et al., 1995, 1998; reviewed in Gilbert and Raunio, 1997).

In the insects, epidermal *Antp* expression is confined primarily to the thorax although minor but detectable accumulation is also observed in the abdomen (Martinez Arias, 1986; Akam, 1987; Carroll et al., 1988; Ingham, 1988; Kaufman et al., 1989). This expression domain is highly conserved in all studied members of the Insecta (Beeman et al., 1990, 1993; Hayward et al., 1995; Denell et al., 1996; Peterson et al., 1999). For example, in the apterygote insect *Thermobia domestica*, *Antp* is detected in the three thoracic segments and at lower levels in the abdominal segments, except the most terminal segment bearing cerci (Peterson et al., 1999). It has been postulated that the strong uniform expression of *Antp* in the thorax with clear segmental boundaries and weak expression in the abdomen represents a pattern basal to all insects (Peterson et al., 1999). The anterior boundary of insect *Antp* resides in the posterior labial segment ventrally and in the anterior prothoracic segment and appendages laterally (Peterson et al., 1999).

In the branchiopod crustacean, *A. franciscana*, *Antp* is expressed throughout the trunk in the eleven-segmented,

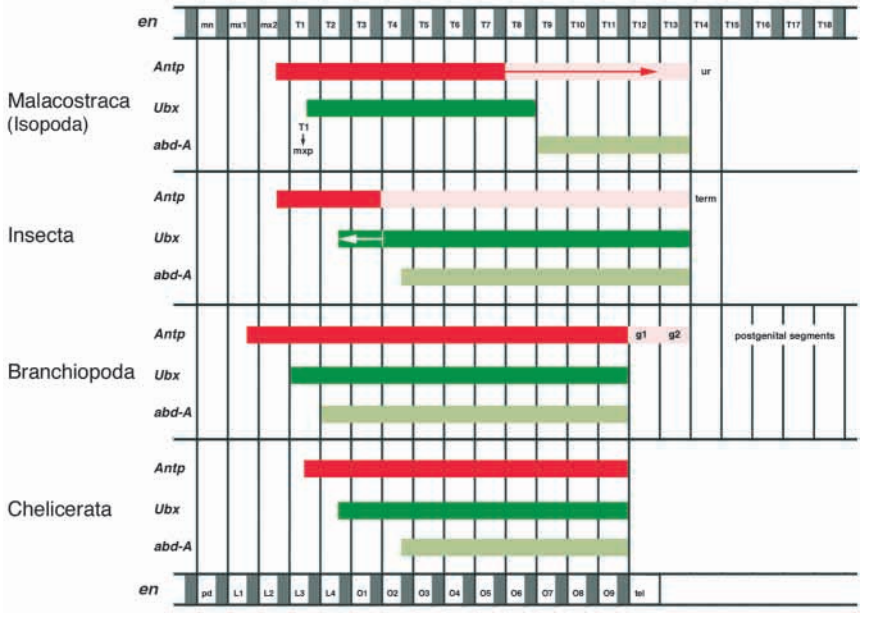

Fig. 6. A schematized comparison of the expression domains of the trunk Hox genes in the Malacostraca, Insecta, Branchiopoda and Chelicerata. No attempt was made to homologize the segments and straightforward alignment of the *engrailed* (*en)* stripes was used as a reference. This alignment is comparable with most traditional interpretations of the corresponding segments in arthropods. The most anterior mandibulate head segments and their homologues in chelicerates are not shown. A number of simplifications were introduced into the scheme so it should be used as a general reference only. Bars indicate the expression domains. The darker color indicates the main domain of expression whereas lighter colors mean weak, transient or restricted to ventral side only modulated expression. Arrows indicate a shift in expression pattern boundaries during development. The generalized sequence of head and trunk segments common to all mandibulates is indicated at the top. The chelicerate segments are shown on the bottom. Note that the number of segments varies among the classes. The malacostracan pattern is from *P. scaber* (this work). The insect pattern is a compilation of data from various sources, mostly on more basal insects (Beeman et al., 1990; Tear et al., 1990; Nagy et al., 1991; Beeman et al., 1993; Kelsh et al., 1994; Hayward et al., 1995; Shippy et al., 1998; Peterson et al., 1999). The branchiopod data is from study of *A. franciscana* (Averof and Akam, 1995). The chelicerate patterns have been published recently for several species of spiders and an oribatid mite (Damen et al., 1998; Telford and Thomas, 1998; Abzhanov et al., 1999). New abbreviations: *en*, *engrailed*; term, terminus bearing cerci in lower insects; g1 and g2, genital segments in *Artemia franciscana*; pd, pedipalp; L1 to L4, walking legs; O1 to O9, opisthosomal (‘abdominal’) segments; tel, telson.

uniform thorax-like tagma anterior to the genital segments (Averof and Akam, 1995). The anterior boundary extends into the gnathal region and is observed in posterior mx1 (Averof and Akam, 1995). *Antp* expression is not uniform in the trunk but is restricted chiefly to the legs (Averof and Akam, 1995). As branchiopods are believed to be phylogenetically basal with respect to malacostracan crustaceans, we conclude that the discrete pereonic expression domain of *Antp* reported here for *P. scaber* is derived. However, it must be emphasized that the *PsAntp* domain is different from that seen in insects and *Artemia*. Firstly, the anterior boundary is roughly one segment more anterior than what is observed in *Artemia* and, in this respect, appears to be similar to insect *Antp* (Figs 5A, 6). Secondly, the posterior boundary lies in the seventh trunk segment rather than in the third trunk segment as in insects or in the last, eleventh trunk segment as in *Artemia* (Fig. 6). Thirdly, unlike in insects *PsAntp* expression is highly compartmentalized within the segments suggesting that this gene may be playing a unique role in the specification of the pereon in spite of a virtually complete overlap with the *Ubx* domain. Importantly, however, both in insects and crustaceans the anterior and posterior boundaries correlate well with the morphological tagmatic borders (Peterson et al., 1999; Fig. 5A).

In insects, *Ubx* possesses a well-conserved expression domain in the abdomen (Akam and Martinez-Arias, 1985; Akam, 1987; Carroll et al., 1988; Ingham, 1988; Kelsh et al., 1994). In *Drosophila* the earliest expression is seen first in A1 and more posterior abdominal segments (Irvine et al., 1991). This early domain of *Ubx* expression is invariant amongst all the classes studied (Kaufman et al., 1989; Beeman et al., 1993; Kelsh et al., 1994; Denell et al., 1996; Peterson et al., 1999). In these insects, later in development *Ubx* expands into the posterior thorax. The exact extent of this shift is different from order to order, e.g., in *Drosophila*, *Ubx* moves into the hind wing and hind leg primordia, whereas, in the firebrat, it is restricted the posterior portion of the segments at the periphery of the T2 and T3 legs (Peterson et al., 1999). The two distinct temporal domains likely reflect the two different developmental functions of *Ubx*: the early function of *Ubx* is to specify abdominal identity whereas later it plays a modifying role in specialization and differentiation of existing thoracic structures (Vachon et al., 1992; Casares et al., 1996). In *Artemia Ubx* is expressed throughout the trunk with the anterior boundary in the first trunk segment (Fig. 6; Averof and Akam, 1995). Interestingly, the anterior boundary of *Ubx*, as detected with the FP6.87 antibody, has been modified several times during crustacean evolution. This change is correlated with the transformation of the anterior legs into maxillipeds which have lost *Ubx* expression (Averof and Akam, 1997). In isopod malacostracans, including *P. scaber*, this is seen in the first pair of trunk legs which are transformed into maxillipeds during development and never express *Ubx*. The posterior boundary of *PsUbx* expression falls at the pereon/pleon border. Note, that unlike *PsAntp* whose domain reflects embryonic morphological junctions, *PsUbx* demarcates the future adult cephalon/pereon and pereon/pleon borders. A comparison with the domain of *Ubx* in insects reveals that *PsUbx* exhibits a surprising dissimilarity as this gene is expressed in the abdomen of insects but in the pereon (analogous to the insect thorax) of *Porcellio*.

The developmental role of *abd-A* in insects is similar to *Ubx* (Casares et al., 1996). The products of both genes are expressed in similar, largely overlapping domains and are used to specify abdominal identity (Karch et al., 1990; Macias et al., 1990). The *Drosophila abd-A* expression domain covering A1-A7 (out of 10 segments) is similar in *Thermobia*, *Schistocerca*, *Tribolium* and *Manduca* (Tear et al., 1990; Nagy et al., 1991; Shippy et al., 1998; Peterson et al., 1999). The anterior boundary in the posterior A1 segment is conserved amongst all insects studied. The more basal insects, however, have a posterior boundary that extends to the end of the abdomen into the A10 segment suggesting retraction of this boundary during insect evolution, perhaps via changes in expression and function of the *Abd-B* gene (Peterson et al., 1999). In *Artemia*, unlike insects, *abd-A* is expressed in the trunk region anterior to the genital and postgenital segments (Averof and Akam, 1995). The anterior boundary lies in the second trunk segment and expression is strongest in the neuromeres. The resulting overlap with the other trunk genes, *Antp* and *Ubx*, suggests redundant, fractional and/or mosaic control over trunk identity (Fig. 6; Averof and Akam, 1995). As noted above, recent studies on chelicerates have revealed broadly overlapping expression domains of these trunk Hox genes in the opisthosoma, the posterior chelicerate tagma (Damen et al., 1998; Telford and Thomas, 1998; Abzhanov et al., 1999). These observations from a recognized non-mandibulate outgroup confirm the proposed ancestral condition concluded from studies on *Artemia* (Averof and Akam, 1995). Thus it would appear that the *Psabd-A* pleonically restricted domain of expression (Figs 4J-L, 5B, 6) is a derived condition. The resemblance to the insect *abd-A* domain in the abdomen, which is analogous to the pleon, is intriguing and implies the deployment of *abd-A* to the posterior tagmata has occurred separately in the insect and crustacean lineages. The salient difference being that sequestration of *abd-A* was accompanied by *Ubx* in the insects but was accomplished singly in the case of higher crustaceans.

In summary, the expression domains of the trunk genes in *P. scaber* are distinct from both insects and branchiopod crustaceans. They are better defined than the broadly overlapping domains in *A. franciscana* and despite a superficial resemblance to the discrete domains of their insect homologues, the anterior and posterior expression domain boundaries are quite different from those in insects. Our observations suggest that the trunk genes were co-expressed and performed redundant roles in the homonomous trunk in the last ancestor of insects and higher crustaceans, and that the trunk of the ancestor has independently differentiated into the thorax + abdomen of insects and pereon + pleon of malacostracans via specialization in the deployment and function of the Hox genes. This being the case, it is likely that the homologous Hox trunk genes have evolved to acquire different developmental functions in the closely related classes Insecta and Malacostraca (Ballard et al., 1995; Boore et al., 1995, 1998; Friedrich and Tautz, 1995; Osorio et al., 1995; Whitington and Bacon, 1998; Nilsson and Osorio, 1997; Dohle, 1997; Zrzavy et al., 1997; reviewed in Gilbert and Raunio, 1997).

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