Posterior patterning genes and the identification of a unique body region in the brine shrimp *Artemia franciscana*

Tijana Copf[1], Nicolas Rabet[2], Susan E. Celniker[3] and Michalis Averof[1,*]

[1]Institute of Molecular Biology and Biotechnology (IMBB-FORTH), Vassilika Vouton, 71110 Iraklio Crete, Greece
[2]Laboratoire de Biologie du Développement, Université Paris 7, 7 quai St Bernard, 75005 Paris, France
[3]Berkeley Drosophila Genome Project, Lawrence Berkeley National Laboratory, One Cyclotron Road, Berkeley, CA 94720, USA
*Author for correspondence (e-mail: averof@imbb.forth.gr)

Accepted 28 August 2003

Development 130, 5915-5927
© 2003 The Company of Biologists Ltd
doi:10.1242/dev.00835

Summary

All arthropods share the same basic set of Hox genes, although the expression of these genes differs among divergent groups. In the brine shrimp *Artemia franciscana*, their expression is limited to the head, thoracic/trunk and genital segments, but is excluded from more posterior parts of the body which consist of six post-genital segments and the telson (bearing the anus). Nothing is currently known about the genes that specify the identity of these posterior structures. We examine the expression patterns of four candidate genes, *Abdominal-B*, *caudal/Cdx*, *even-skipped/Evx* and *spalt*, the homologues of which are known to play an important role in the specification of posterior structures in other animals. *Abdominal-B* is expressed in the genital segments of *Artemia*, but not in the post-genital segments at any developmental stage. The expression of *caudal*, *even-skipped* and *spalt* in the larval growth-zone suggests they may play a role in the generation of body segments (perhaps comparable with the role of gap and segmentation genes in insects), but not a direct role in defining the identity of post-genital segments. The expression of *caudal* at later stages suggests a role in the specification of anal structures. A PCR screen designed to isolate Hox genes expressed specifically in the posterior part of the body failed to identify any new Hox genes. We conclude that the post-genital segments of *Artemia* are not defined by any of the genes known to play a role in the specification of posterior segments in other arthropods. We argue that these segments constitute a unique body region that bears no obvious homology to previously characterised domains of Hox gene activity.

Key words: Hox genes, Crustaceans, Body plans, Evolution

Introduction

The differential expression of Hox genes along the anteroposterior axis of the body plays a central role in defining the identity of different body regions in diverse animals (McGinnis and Krumlauf, 1992). Comparing the expression of Hox genes in different species has been used to study the origin and relationships of individual body regions and to understand the genetic changes that lie behind the evolution of body plans. In arthropods, where the action of Hox genes is associated with the specification of distinct segmental identities, there are now several examples where the expression of Hox genes has helped to identify homologous segments among diverse groups (Averof and Akam, 1995; Damen et al., 1998; Telford and Thomas, 1998) and to explain changes in patterns of segmental specialisation (Averof and Patel, 1997; Abzhanov and Kaufman, 1999). In spite of this progress, however, very little is known about how new body regions can arise during evolution, about how these regions can acquire a new segmental identity and about the possible role of Hox genes in this process. We focus on the post-genital region of the crustacean *Artemia franciscana*, a unique region that bears no obvious relationship to previously characterised body regions of other arthropods.

The adult body of *Artemia* consists of a head, eleven 'thoracic' segments, two genital segments, six post-genital segments and a telson (Fig. 1B). Like many other crustaceans, *Artemia* hatches as a nauplius larva, which consists only of the anterior head segments, a growth-zone and the telson. Most body segments, including the thoracic, genital and post-genital segments, are generated sequentially from the growth zone during the course of larval development (Fig. 1A-D).

In order to understand how these distinct types of segments are specified, we previously cloned a set of Hox genes from *Artemia* and analysed their expression patterns (Averof and Akam, 1993; Averof and Akam, 1995). *Artemia* appears to have the same set of Hox genes that have been found in most arthropods, including distinct homologues of *Antennapedia* (*Antp*), *Ultrabithorax* (*Ubx*), *Abdominal-A* (*AbdA*) and *Abdominal-B* (*AbdB*) (Averof and Akam, 1993; Grenier et al., 1997; Hughes and Kaufman, 2002b). The homologues of *Antp*, *Ubx* and *AbdA* are expressed in the eleven 'thoracic' segments, while the homologue of *AbdB* is expressed in the two genital segments, suggesting that the 'thorax' of *Artemia* may be homologous to the entire trunk of other arthropods – to the thoracic and abdominal segments of insects, to the pereon and pleon of malacostracan crustaceans, and to all the trunk segments of myriapods, which are all marked by the expression of *Antp*, *Ubx* and *AbdA* (Averof and Akam, 1995; Abzhanov and Kaufman, 2000a; Hughes and Kaufman, 2002c). For clarity, these segments will be referred to as the thoracic/trunk segments of *Artemia*. The genital segments of *Artemia* and

insects (which lie just posterior to these thoracic/trunk segments) also appear to be related, and are characterised by the expression of *AbdB* (Averof and Akam, 1995).

In all arthropods that have been studied to date, with the exception of *Artemia* (including insects, myriapods and spider) (Delorenzi and Bienz, 1990; Kelsh et al., 1993; Peterson et al., 1999; Hughes and Kaufman, 2002c; Damen and Tautz, 1999), the *AbdB* expression domain marks the most posterior segments of the body. Posterior to this domain lie only the anal structures (thought to derive from the non-segmental telson) and the ectodermal cells that invaginate to give rise to the hindgut. In *Artemia*, the post-genital region lies between the *AbdB*-expressing genital segments and the anal structures, and consists of six well-formed segments with a characteristic morphology that distinguishes them clearly from the other segments. The post-genital segments are morphologically similar to each other; they have a relatively elongated cylindrical shape, characteristic musculature, lack all trace of appendages and do not contain any ganglia of the central nervous system (see Schrehardt, 1987; Criel, 1991) (Fig. 1F). Engrailed is expressed in the posterior part of these segments (Fig. 1E). The observation that all the known Hox genes are expressed anterior to these segments poses interesting questions concerning their origin and identity.

Nothing is currently known about the genes that specify the identity of the post-genital segments in *Artemia*. Already known developmental genes could play a role, or new genes (perhaps new Hox genes) may have evolved to fulfil this function. Likely candidates are the homologues of the homeobox-containing genes *caudal* (*Cad/Cdx*) and *even-skipped* (*Eve/Evx*), and of the zinc-finger transcription factor *spalt* (*Sal*), that are known to play an important role in the specification of posterior body regions in *Drosophila*, in *C. elegans* and/or in vertebrates. Cad/Cdx genes are closely related to Hox genes and are involved in posterior patterning in diverse animals like *Drosophila*, *C. elegans* and chordates (Macdonald and Struhl, 1986; Moreno and Morata, 1999; Hunter and Kenyon, 1996; Edgar et al., 2001; Brooke et al., 1998; Meyer and Gruss, 1993; Subramanian et al., 1995; Chawengsaksophak et al., 1997; Marom et al., 1997; Epstein et al., 1997; van den Akker et al., 2002). In *Drosophila*, *Cad* has been shown to act like a homeotic gene to specify the identity of the anal structures and hindgut of the adult (Moreno and Morata, 1999). Similarly, *Eve/Evx* genes are closely related to the Hox genes and have been implicated in the development of posterior structures in *C. elegans* and in chordates (Ahringer, 1996; Ferrier et al., 2001; Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; Beck and Slack, 1999). In *Drosophila*, no clear role in posterior patterning has been found for *Eve*, but the gene is expressed specifically in posterior parts of the body and this expression is conserved among divergent arthropods (Frasch et al., 1987; Moreno and Morata, 1999; Patel et al., 1994; Hughes and Kaufman, 2002a). Finally, *Sal* is a conserved zinc-finger transcription factor that is required for the specification of anterior and posterior structures during early embryogenesis in *Drosophila*; in particular, *Sal* is thought

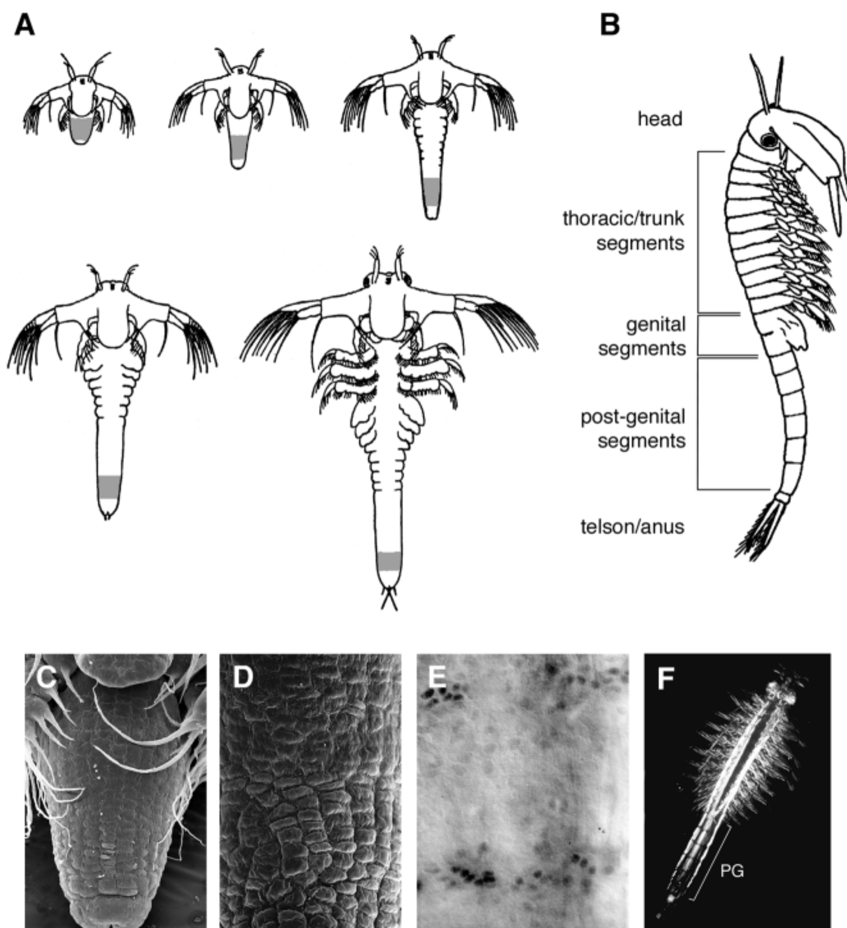

Fig. 1. Larval development and establishment of the body plan in *Artemia*. (A) Schematic representation of the early stages of larval development in an anostracan crustacean, corresponding roughly to stages L1, L2, L4, L6 and L8 of *Artemia* development. Body segments are generated sequentially from the growth zone (in grey) during larval development. (B) The adult body plan of *Artemia* consists of the head, 11 thoracic/trunk segments, two genital segments, six post-genital segments and the telson. (C,D) Scanning electron micrographs of the *Artemia* growth zone showing the outline of individual ectodermal cells, shortly before hatching (C) and during stage L3 (D). The growth zone is characterised by the regular arrangement of these undifferentiated ectodermal cells into columns. (E) Engrailed expression in the post-genital segments; Engrailed protein can be detected in a narrow stripe at the posterior of each segment. (F) The musculature of a fully segmented *Artemia*, seen using polarised light microscopy. The post-genital segments (labelled PG) have a characteristic pattern of muscles that is distinguishable from that of other trunk segments.

to cooperate with Hox genes to define the identity of the posterior genital and anal regions (Jurgens, 1988; Kuhnlein et al., 1994). *Cad*, *Eve* and *Sal* are the only genes with a well-described role in defining the identity of posterior structures, besides Hox genes.

We take the first steps towards characterising the post-genital region of *Artemia*, by examining the expression of genes that could play an important role in specifying the identity of posterior parts of the body. First, we examine in detail the expression of *AbdB*, the most posterior-acting of known Hox genes, to see whether its expression extends into the post-genital region at any developmental stage. Second, we ask whether the post-genital segments could be related to the posterior genital or anal structures that express *Cad*, *Eve* and *Sal* in *Drosophila* and in other species. We describe the isolation of homologues of these genes from *Artemia* and examine their expression. Finally, we describe a screen to isolate previously unidentified Hox genes that could be expressed in these segments.

Materials and methods

Artemia cultures and staging

Artemia franciscana diapause cysts from the Great Salt Lake were hydrated, and larvae were raised in well-aerated 3% artificial seawater, supplemented with brine shrimp food from NT Laboratories. Larval stages were determined according to existing schemes (Schrehardt, 1987).

Preparation of *Artemia* genomic DNA and first strand cDNA

Genomic DNA was isolated as described previously (Averof and Akam, 1993). For the production of first strand cDNA, ~100 μg of material were homogenised and poly-A mRNA was purified using Dynabeads (Dynal). The eluted mRNA was treated with DNase I (DNA free kit, Ambion) to remove traces of genomic DNA. The RACE-polyT primer (GACTCGAGTCGACATCGATTTTTTTTT-TTTTTTTT) was used for first strand cDNA synthesis using the Superscript II kit (GibcoBRL), following the manufacturer's instructions. First strand cDNAs were then treated with 2 units RNaseH for 30 minutes at 37°C, followed by inactivation of the enzyme for 15 minutes at 75°C.

Cloning of *Artemia Cad*, *Eve* and *Sal* homologues

AfCad

Specific primers were designed based on a short fragment of the *AfCad* homeobox isolated by PCR (details available on request) and on a similar short sequence kindly provided by G. Balavoine and M. Akam. These primers were used to recover large fragments of AfCad cDNAs by nested 3′ and 5′ RACE, and by PCR on a phage cDNA library. A radioactive probe prepared from one of these larger fragments was used to screen a cDNA library prepared from unhatched cysts (Escalante and Sastre, 1993). Three independent phage clones were recovered, containing full-length cDNAs of *AfCad*.

AfEve

Degenerate primers were designed to target conserved parts of the Eve homeodomain:

EveF1(TAFTREQ), CGGGATCCACIGCITT(T/C)ACI(A/C)GIG-A(A/G)CA; EveR1(MKDKRQR), GGAATTCC(T/G)(T/C)TGIC-(T/G)(T/C)TT(A/G)TC(T/C)TTCAT.

These primers were used for PCR on first strand cDNA prepared from posterior regions of *Artemia* larvae, and a short fragment of *AfEve* was recovered. Based on the sequence of this fragment, specific forward primers were designed and used for nested 3′ RACE carried out on the same cDNA pool. A ~710 bp fragment was recovered, corresponding to the 3′ part of the *AfEve* cDNA.

AfSal

Degenerate primers were designed to target conserved parts of the zinc-finger 2 region of Sal:

SalF3(HTGERPF), GGAATTCA(T/C)ACIGGIGA(A/G)(C/A)G-ICCITT; SalR3(CPICQKK), GCTCTAGATT(T/C)T(G/T)ITG(A/G)-CAIA(T/C)IGG(A/G)CA.

These primers were used for PCR on *Artemia* genomic DNA and a short fragment of *AfSal* was recovered. Based on the sequence of this fragment, specific primers were designed for nested inverse PCR. A ~390 bp fragment was obtained corresponding to the zinc-finger 2 region of *AfSal*.

In situ hybridisation

DIG-labeled antisense RNA probes were prepared using the Megascript T3 or T7 kits (Ambion). In situ hybridisation was carried out on stage L1-L3 *Artemia* larvae, as described previously (Gibert et al., 2000; Mitchell and Crews, 2002).

Production of cross-reacting antibody against AbdB

A polypeptide containing 61 amino acids of the *Drosophila* AbdB homeodomain and 10 additional C-terminal residues, was expressed and purified from *E. coli*, using the expression vector pABD-B HD72 (Ekker et al., 1994). A mouse was immunised intraperitoneally with 20-60 μg of protein in complete Ribi adjuvant, six times over a period of 10 weeks. The serum was tested for crossreactivity in a number of species, including *Drosophila virilis*, *Schistocerca americana* and *Artemia franciscana*, and was found to recognise AbdB proteins in these species. The serum was used for immunochemical stainings at 1:1000 dilution.

Production of antibodies against AfCad and AfEve

The full length of the available *AfCad* and *AfEve* cDNA fragments was cloned into the *Bam*HI/*Eco*RI and *Bam*HI/*Xho*I sites of the pRSETA vector (Invitrogen), respectively, to generate His-tagged protein fusions. AfCad and AfEve proteins were produced by transforming these constructs into BL21(pLys) cells, inducing with IPTG, and purifying the His-tagged proteins on a Ni-NTA column (QIAGEN), as described in the manufacturer's manual. Antibodies were raised against these bacterially expressed and purified proteins by repeated immunisations in rabbits; 750 μg of each protein were used to carry out eight immunisations over a period of 8 months (carried out by Davids Biotechnologie).

The anti-AfCad serum obtained was affinity purified on an Affigel-10 column (Biorad) carrying bacterially expressed and purified AfCad (Harlow and Lane, 1988); the affinity purified serum was used for immunochemical stainings at 1:100 dilution. The anti-AfEve serum was pre-absorbed overnight on acetone powder prepared from *Artemia* larvae (Harlow and Lane, 1988) and used at 1:1000 dilution.

Antibodies and immunochemical stainings

Production of antibodies against AbdB, AfCad and AfEve are described above. Other antibodies used: rabbit anti-Dll (Panganiban et al., 1995), mouse monoclonal FP6.87 (Ubx/AbdA) (Kelsh et al., 1994), mouse monoclonal 4F11 (En) (Patel et al., 1989).

Whole-mount immunochemical staining was carried out following standard protocols (Patel, 1994), using sonication to break the exoskeleton of the larvae and long (4×30 minute) washes to reduce non-specific signals. All reported stainings had nuclear localisation and were observed reproducibly in a significant number of larvae.

Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out by adapting existing protocols (Felgenhauer, 1987). Specimens were fixed and

kept in 4% neutralised paraformaldehyde. The samples were then dehydrated in a graded series of alcohol and in amyl acetate before critical-point drying. Specimens were mounted on copper stubs with silver paint, coated with 300 A of gold in a Polaron sputtering apparatus, and examined on a JEOL JSM 6100 scanning electron microscope at 15 kV.

Drosophila experiments

The UAS-AfCad construct was prepared by cloning the full-length *AfCad* cDNA into *Xho*I/*Eco*RI sites of the pUAST vector (Brand and Perrimon, 1993). The construct was transformed into flies and several independent transgenic lines were obtained. Crossing these lines to MS-248 GAL4 drives expression of *AfCad* broadly in eye-antennal and wing discs, causing malformations in the head and thorax of the adults. Strongest phenotypes, including the appearance of ectopic anal plates, were obtained when the progeny of these crosses were raised at 30°C, using at least three independent UAS-AfCad lines. The effects of *AfCad* mis-expression were also analysed in flies carrying Dll-*lacZ* (Moreno and Morata, 1999) or Byn-*lacZ* (Murakami et al., 1995) reporters, using the MS-248 or the apterous-GAL4 drivers.

To test whether mis-expression of *AfCad* activates the endogenous *Cad* gene, immunochemical stainings were carried out using an antibody against *Drosophila* Cad (kindly provided by Gary Struhl). No *Drosophila* Cad could be detected in imaginal discs expressing *AfCad*, although significant expression could be seen in discs mis-expressing *Drosophila Cad*. We should note, however, that the sensitivity of these stainings was low (the normal expression of *Cad* in the genital disc was barely detectable).

PCR screen for posteriorly expressed Hox genes

The posterior part of the body, including the developing genital and post-genital regions, was dissected from *Artemia* larvae at stage L9-L10 and snap-frozen in dry ice. First-strand cDNA was prepared from this material, as described earlier. The 'universal' Hox primers HoxF1(ELEKEF) [GGAATTCGA(A/G)CTIGA(A/G)AA(A/G)GA-(A/G)TT] and HoxR1(WFQNRR) [GCTCTAGACGICG(A/G)-TTTTG(A/G)AACCA] (Averof and Akam, 1993) were used for PCR on the first-strand cDNA prepared from posterior regions, with an early annealing temperature of 40°C. The ~130 bp products of the reaction were cloned into the pGEMT-easy vector (Promega). Sixty independent clones were analysed by PCR using specific *Artemia AbdB* and *AfCad* primers and/or by sequencing.

Results

Artemia AbdB is not expressed in the post-genital segments

We have previously shown by in situ hybridisation that *AbdB* is expressed specifically in the genital segments of *Artemia* (Averof and Akam, 1995). Technical difficulties, however, had not allowed us to study its expression during late larval stages, leaving open the possibility that late *AbdB* expression may extend into the post-genital region. We have now raised an antibody that recognises AbdB proteins in diverse arthropod species, including *Artemia*. Using this antibody, we are able to study the expression of *AbdB* throughout larval development, by immunochemical stainings.

AbdB protein is first detected during mid-late larval stages (stage L8) and is restricted to the newly formed genital segments (Fig. 2A); this is consistent with the previously reported patterns of *AbdB* mRNA distribution (Averof and Akam, 1995). Expression becomes stronger in the genital segments as larval development proceeds (Fig. 2B), and expands anteriorly to become expressed in some cells of posterior thoracic/trunk segments (Fig. 2C). *AbdB* expression is never observed to extend posteriorly, into the segments of the post-genital region. These results argue against a direct role of *AbdB* in specifying the identity of the post-genital segments.

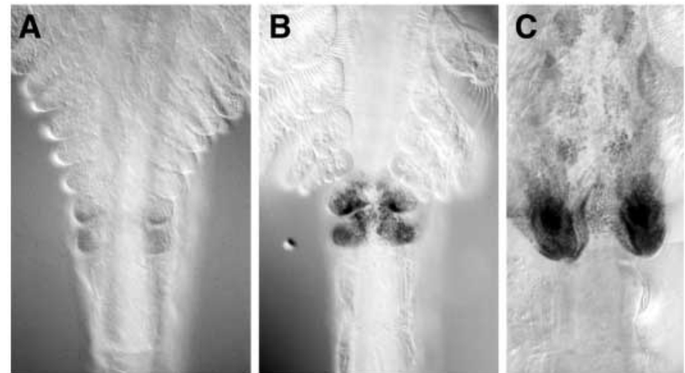

Fig. 2. Expression of AbdB in *Artemia*. (A) Early AbdB expression in the two genital segments, shortly after these segments have formed (stage L9). (B) Later, stronger AbdB expression restricted to the two genital segments (stage L11). (C) Late expression of AbdB (after stage L13) persists in the differentiated genital segments and also extends anteriorly to cells of posterior thoracic/trunk segments. AbdB expression is never seen posterior to the genital segments. Anterior is upwards.

Cloning and expression of *Artemia Cad*, *Eve* and *Sal* homologues

To examine the possible role of *Cad/Cdx*, *Eve/Evx* and *Sal* genes in defining the identity of the post-genital segments, we used PCR with degenerate primers, followed by RACE, inverse PCR and/or screening of cDNA libraries, to isolate homologues of these genes from *Artemia*. We then studied the expression of these genes and found that they are all expressed in the posterior growth zone, from which the thoracic/trunk, genital and post-genital segments are generated. This is consistent with a role of these genes in the process of segment formation. However, we could not detect any segment-specific expression patterns in the post-genital segments, indicating that these genes are unlikely to play a direct role in defining the identity of these segments.

Caudal (*AfCad*)

We isolated three full-length cDNAs and a number of smaller fragments, which correspond to a single *Artemia Cad* gene (sequence Accession Number AJ567452). Sequence comparisons with other members of the Cad/Cdx family suggest that this gene is orthologous to *Drosophila Cad* and to vertebrate *Cdx* genes (Fig. 3A).

We used whole-mount in situ hybridisation to study the expression of *AfCad* during early larval development. Owing to technical difficulties we were not able to use in situ hybridisation in later stages, but we generated an antibody that recognises the AfCad protein, which enabled us to study its distribution throughout larval development using immunochemical staining. From the earliest stages of larval development, *AfCad* is expressed in a domain that lies posterior to the differentiated segments of the body and anterior to the telson, and corresponds to the growth zone (Fig. 4A). *AfCad* expression persists in the growth zone throughout the early-

A. Cad/Cdx

```
                              hexapeptide region         5'     homeodomain                                                    3'
Drosophila      insect        RTSPSKPPYFDWMKKPAYPAQPQP   GKTRT  KDKYRVVYTDFQRLELEKEYCTSRYITIRRKSELAQTLSLSERQVKIWFQNRRAKERTSN  KKGSD
Bombyx          insect        PPGPPRSPY-EWMKKPNYQTQPNP   GKTRT  KDKYRVVYSDHQRLELEKEFHYSRYITIRRKAELAVSLGLSERQVKIWFQNRRAKERKQV  KKREE
Tribolium       insect        RPPPARSPY-EWIKKTSYQSQPNP   GKTRT  KDKYRVVYTDHQRVELEKEFYYSRYITIRRKAELANSLGLSERQVKIWFQNRRAKERKQV  KKREE
Schistocerca    insect        RPLPVRSPY-EWMKKPSYQSQPNP   GKTRT  KDKYRVVYSDHQRLELEKEFHYSRYITIRRKAELAANLGLSERQV
Artemia         crustacean    RPLSIRSPY-EWMKKQSFHCNMPP   SKTRT  KDKYRVVYTDHQRLELEKEFHYNKYITIRRKSELATVLGLSERQVKIWFQNRRAKDRKQM  KKREE
Procambarus     crustacean              KKPSYQAQPNT    GKTRT  KDKYRVVYSDHQRLELEKEFHYSRYITIRRKSELASMLGLSERQVKIW
Achaearanea     spider        RPQPARSPY-EWIKRTSYQSQPNP   GRTRT  KDKYRIVYTDHQRLELEKEFHYSRYITIRRKVELAASLNLSERQIKIWFQNRRAKERRQA  KKREE
C.elegans/pal1  nematode      ------------------------   VRVRT  ADKYRMVYSDYQRLELEKEFHTSPFITSDRKSQLSTMLSLTERQIKIWFQNRRAKDRRDK  QKIRL
Patella         mollusc       -----RAPY-EWMKPA--------   GKTRT  KDKYRVAYTDHQRLELEKEFHYSRYITIRRKAEIAAQLALSERQVKIWFQNRRAKERKQN  KKGKD
Halocynthia     ascidian      -----RIPY-DWINMN--------   GKTRT  KDKYRVVYSDHQRLELEKEFHFSRYITIRRKSELAMQLSLSERQIKIWFQNRRAKERKIS  KKQTG
Mouse/Cdx1      vertebrate    -----RTPY-EWMRRS--------   GKTRT  KDKYRVVYTDHQRLELEKEFHYSRYITIRRKSELAANLGLTERQVKIWFQNRRAKERKVN  KKKQQ
Mouse/Cdx2      vertebrate    -----RNLC-EWMRKP--------   VKTRT  KDKYRVVYTDHQRLELEKEFHFSRYITIRRKSELAATLGLSERQVKIWFQNRRAKERKIK  KKQQQ
Mouse/Cdx4      vertebrate    -----HSPY-AWMRKT--------   GKTRT  KEKYRVVYTDHQRLELEKEFHCNRYITIRRKSELAVNLGLSERQVKIWFQNRRAKERKMI  KKKIS
```

B. Eve/Evx

```
                                         homeodomain (part)                      3'          repression domain        C-term.
Drosophila      insect        LGRLEKEFYKENYVSRPRRCELAAQLNLPESTIKVWFQNRRMKDKRQR  -IAVAWPY  DPAFAASILQAAANSVGMPYPP  PKLFKPYKTE
Bombyx          insect        LARLEKEFMKENYVSRPRRCELAAQLQLPESTIKVWFQNRRMKDKRQR  -IAVAWPY  DPAFAASILQAAASSVGLPYGY  ----------
Tribolium       insect        LARLEKEFFKENYVSRPRRCELAAQLNLPESTIKVWFQNRRMKDKRQR  -MAIAWPY  DPAFAASIFHAAATSLPLHYPP  PKLFKPYKSE
Schistocerca    insect        LARLEKEFYKENYVSRPRRCELASQLNLPESTIKVWFQNRRMKDKRQR  -MAMAWPY  DPTLAASLLQAAMYVPCPPYYP  PKLFQPYKSD
Artemia         crustacean    LGRLEKEFLQENYVSRPRRCELAAQLGLQESTIKVWFQNRRMKDKRQR  -MAMAWPM  DPMFAAYLLQVAGCAY--PYPP  PKLFQPYKLG
Lithobius       centipede            YRENYVSRPRRCELAASLNLPESTIKVWFQNRRMKDKRQR  -MALAWPY  DPHFAAYVLQAAAAXGAYPYHL  GSPLQPTPQL
Cupiennius      spider        LARLEKEFMRENYVSRPRRCELATALNLPESTIKVWFQNRRMKDKRQR  -MSLPWPY  DPHLAAYVINAAYSGYPLPPPF  RTLFQPYKTD
C.elegans/vab7  nematode      IGRLEREFAKENYVSRKTRGELAAELNLPEGTIKVWFQNRRMKDKRQR  VGGLAWPF  ----------------------  SSSPSPYSTD
Helobdella      annelid       LAKLEKEFLKENYVSRPRRCELASQLDLPECTIKVWFQNRRMKDKRQR  ISMAAWSY  ----------------------  ----------
Ilyanassa       mollusc       ISRLEKEFAKENYISRPKRCELAATMGLPESTIKVWFQNRRMKDKRQR  -MSLTWLY  ----------------------  ----------
Herdmania       ascidian      VACLEKEFHRENYVSRPRRCELAQELGLPETTIKVWFQNRRMKDKRQR  -MTTAWPL  SPSPFFPMSSYYGQIDARFGLPM  ----------
Mouse/Evx1      vertebrate    IARLEKEFYRENYVSRPRRCELAAALNLPETTIKVWFQNRRMKDKRQR  -LAMTWPH  DPAFYTYMMSHAAAAGGLPYPF  ----------
Mouse/Evx2      vertebrate    IARLEKEFYRENYVSRPRRCELAAALNLPETTIKVWFQNRRMKDKRQR  -LAMSWPH  DPSFYTYMMTHAAATGSLPYPF  ----------
```

C. Sal

```
                                          3'                                                              zinc finger 2 region
Drosophila/sal   insect        LLPRPHSNDNSWENFIEVSNTCETMKLKELMKN--  KKISDPNQCVVCDRVLSCKSALQMHYRTHTGERPFKCRICGRAFTTKGNLKTHMAVHKIRPPMRNFHQCPVCHKKYSNALVLQQHIRLHTGEPTD
Drosophila/salr  insect        LAQMERIIDKSWEDLIEIDKTSETSKLQQLVDNIE  NKLTDPNQCIFCQKVMSCRSSLQMHIRTHTGERPFRCKICGRAFATKGNLKAHMSIHKIKPPMRSQFKCPVCHQKFSNGIILQQHIRIHTMDDGS
Artemia          crustacean    LLPRPGSNDNAWETLIEVTKASDTTKLQQLVDSIE  NKMSDPNQCVICHRVLSCKSALQMHYRTHTGERPFKCKICGRAFTTKGNLKTHMGVHRAKPPSRLLHICPVCHRKFTNGIVLQQHIRLHTGEPTD
C.elegans/sem4   nematode      -----HDNDDWEALMEISTSDEAEKIRALVGDKA  VPTTDPNQCILCRRVLSCKSALQMHYRTHTGERPFKCKICQRAFTTKGNLKTHMGVHRSKHSFR-------------------------------
Mouse/Sal1       vertebrate    ----------------------ETSKLQQLVENID  KKATDPNECIICHRVLSCQSALKMHYRTHTGERPFKCKICGRAFTTKGNLKTHYSVHRAMPPLRVQHSCPICQKKFTNAVVLQQHIRMHMGGQIP
Mouse/Sal2       vertebrate    ----------------------ETSKLQQLVEKID  SSASGPNQCVICLRVLSCPRALRLHYGQHGGERPFKCKVCGRAFSTRGNLRAHFVGHKTSPAARAQNSCPICQKKFTNAVTLQQHVRMHLGGQIP
Mouse/Sal3       vertebrate    ----------------------ETSKLQQLVENID  KKMTDPNQCVICHRVLSCQSALKMHYRTHTGERPFKCKICGRAFTTKGNLKTHFGVHRAKPPLRVQHSCPICQKKFTNAVVLQQHIRMHMGGQIP
```

Fig. 3. Sequence alignments among *Cad/Cdx*, *Eve/Evx* and *Sal* homologues from *Artemia* and diverse species. Amino acid sequence alignments confirm that we have cloned orthologues of the *Cad/Cdx*, *Eve/Evx* and *Sal* genes, respectively. Only significantly conserved regions are shown, with conserved amino acids highlighted in grey. Sequence Accession Numbers: AJ567452 (*AfCad*), AJ567453 (*AfEve*), AJ567454 (*AfSal*).

mid stages of larval development (stages L1-L9), while the thoracic/trunk, genital and post-genital segments are being formed. Double immunochemical stainings show that the Hox genes *Ubx* and *AbdA*, which are expressed in thoracic/trunk segments, are transiently co-expressed with *AfCad* in the anterior part of the growth zone (Fig. 4G). This confirms that *AfCad* is expressed in the progenitors of thoracic/trunk segments.

The domain of *AfCad* expression in the growth zone has sharp anterior and posterior borders. Expression within these borders is relatively uniform in most stages, although occasionally two different levels of expression can be discerned: high levels in most of the growth zone and lower levels in a band of cells that lie at the anterior edge of the growth zone (Fig. 4E). This is likely to represent the transient pattern, seen when cells exit the growth zone and switch off *AfCad* expression. The early patterns observed by in situ hybridisation and immunochemical stainings are indistinguishable (Fig. 4C,D), suggesting that no significant post-transcriptional regulation of *AfCad* takes place during the early stages of trunk segmentation.

The growth zone expression domain becomes less sharp and eventually fades away at around the time when all the segments have been generated (stage L10). During later stages, *AfCad* is also expressed in a small number of mesodermal and ectodermal cells (data not shown) and in developing anal structures of the adult (see later).

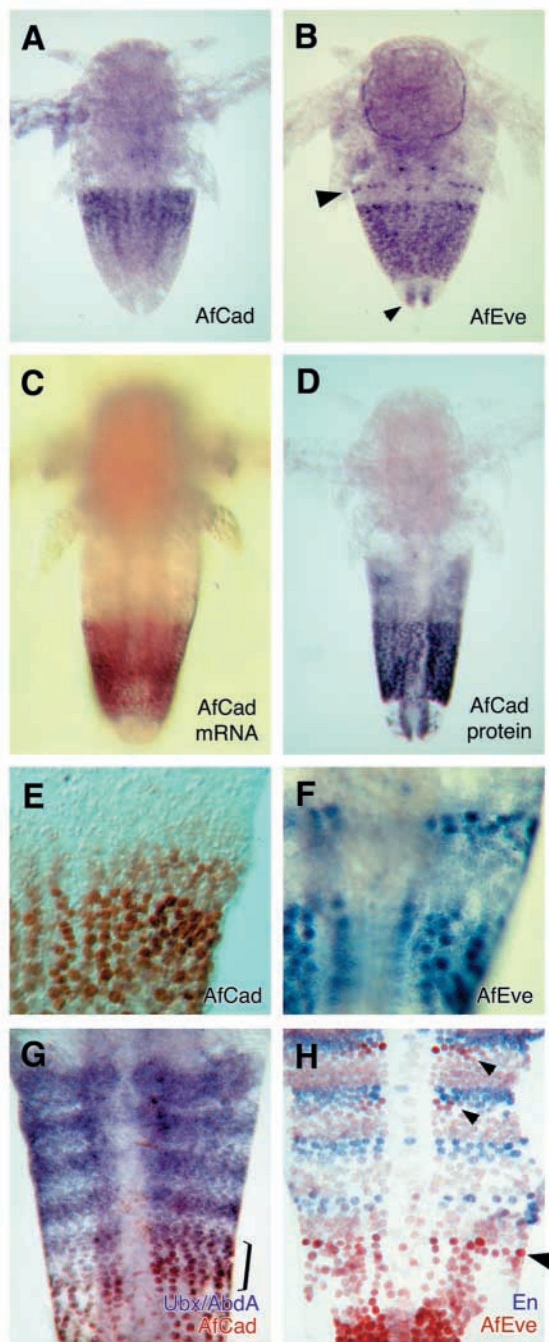

Fig. 4. Expression patterns of Cad and Eve homologues in *Artemia*. (A,B) Immunochemical stainings with antibodies against AfCad and AfEve in newly hatched nauplii, showing expression of both genes in the posterior growth zone that will generate most body segments. AfEve shows additional expression in a single-cell wide segmental stripe (large arrowhead) and in the hindgut (small arrowhead). (C) Distribution of *AfCad* mRNA in an early larva (stage L2), visualised by in situ hybridisation. (D) Distribution of AfCad protein during the same stage, visualised by immunochemical staining. *AfCad* mRNA and protein distributions appear the same. (E) Magnification of the anterior boundary of expression of AfCad in the growth zone, showing two zones expressing different levels of AfCad protein. The zone with lower levels of AfCad is seen only in some individuals and is presumed to be a transient feature. (F) Magnification of the anterior boundary of expression of AfEve, including a single segmental stripe that has separated from the growth zone. This stripe is only seen in some individuals, and is thought to appear very transiently. (G) Double immunochemical staining with antibodies against Ubx and AbdA (in purple), and AfCad (in dark red), showing overlap of their expression domains over one or two segments (bracket). (H) Double immunochemical staining with antibodies against Engrailed (in blue) and AfEve (in red). The stripe of AfEve expression disappears before the onset of Engrailed expression. Expression of AfEve can be seen in the growth zone, in a transient segmental stripe (large arrowhead) and in specific cells in the CNS (small arrowheads). Ventral views, anterior towards the top.

Even-skipped (*AfEve*)

We cloned partial cDNA fragments of *Artemia Eve*, corresponding to part of the homeobox and the 3′ end of the coding sequence (sequence Accession Number AJ567453). Sequence comparisons indicate that this gene is orthologous to the *Drosophila Eve* and to the vertebrate *Evx* genes (Fig. 3B).

We used whole-mount in situ hybridisation to study the expression of *AfEve* during early larval development (data not shown), and generated an antibody that recognises the AfEve protein to examine its expression pattern in detail, throughout larval development. *AfEve* is expressed in the growth zone of *Artemia* during early-mid larval stages, in a pattern that largely overlaps with the expression of *AfCad* (Fig. 4B). Additionally, *AfEve* expression is occasionally detected in narrow (single- or few-cell wide) stripes that appear to 'split' from the anterior edge of the growth zone domain as *AfEve* is switched off in the intervening ('inter-stripe') cells (Fig. 4B,F). These stripes appear to be very transient and are visible only one at a time, as new segments are being generated from the growth zone.

The *AfEve* stripes appear before any morphological signs of segmentation become apparent. To examine the relationship between these stripes and the process of segmentation, we

compared the expression of *AfEve* with the expression of the segmentation gene *engrailed* (*en*), using double immunochemical stainings. The *AfEve* stripes appear earlier than *engrailed* (i.e. several cell diameters posterior to the youngest *engrailed* stripe) and have disappeared by the time *engrailed* is turned on in any particular segment (Fig. 4H). We are therefore not able to determine the precise position and segmental register of these stripes.

AfEve is also expressed in cells of the developing hindgut (Fig. 4B), in mesodermal cells that give rise to the dorsal vessel/heart (not shown), and in a small set of segmentally repeated cells in the central nervous system (Fig. 4H). Similar patterns in the central nervous system and heart have been documented in other arthropods (Frasch et al., 1987; Patel et al., 1992; Patel et al., 1994; Duman-Scheel and Patel, 1999; Hughes and Kaufman, 2002a), suggesting that these aspects of *Eve* expression are evolutionarily conserved.

Spalt (*AfSal*)

We isolated several genomic fragments containing the second zinc finger of *Sal* from *Artemia* (sequence Accession Number AJ567454). Sequence comparisons suggest that this is orthologous to *Drosophila Sal* and *Salr*, and to the vertebrate *Sal* genes (Fig. 3C).

We used in situ hybridisation, to examine the expression of *AfSal* in early larvae (stage L1). Early *AfSal* expression is restricted to the posterior growth zone and is very similar to the expression of *AfCad* (data not shown). Technical difficulties with in situ hybridisation did not allow us to examine the expression of *AfSal* during later stages.

Late *AfCad* expression marks the differentiation of the adult anal appendages

During mid-late stages of larval development, *AfCad* gradually ceases to be expressed in the growth zone (which presumably disappears after the formation of all body segments) and starts to be expressed in the posterior part of the telson, in the region surrounding the anus (Fig. 5C). This expression begins at stage L6 and coincides with the beginning of *Distal-less* (*Dll*) expression in the same part of the telson (Fig. 5A); it is associated with the development of a pair of appendage-like structures, the caudal furca, that surround the anus in the adults (Fig. 5D-F). Unlike the dynamic expression of *AfCad* in the growth zone, expression in the posterior telson is relatively stable throughout mid-late larval stages. This is reminiscent of the homeotic function of *Drosophila Cad* in the primordia of the adult anal structures, where *Cad* expression is also associated with the expression of *Dll* (Gorfinkiel et al., 1999; Moreno and Morata, 1999). *Dll* expression is not observed in the post-genital segments of *Artemia*.

Homeotic transformations induced by expression of *AfCad* in *Drosophila*

In order to test the relatedness of the *Artemia* and *Drosophila* Cad proteins at the functional level, we decided to compare the effects of mis-expressing these proteins in *Drosophila*. Mis-expressing *Drosophila Cad* in the eye-antennal and wing imaginal discs during larval development, using the UAS/GAL4 system, is known to produce striking homeotic transformations of head cuticle and notum towards anal plates, and to induce the ectopic expression of its target genes *Distal-*

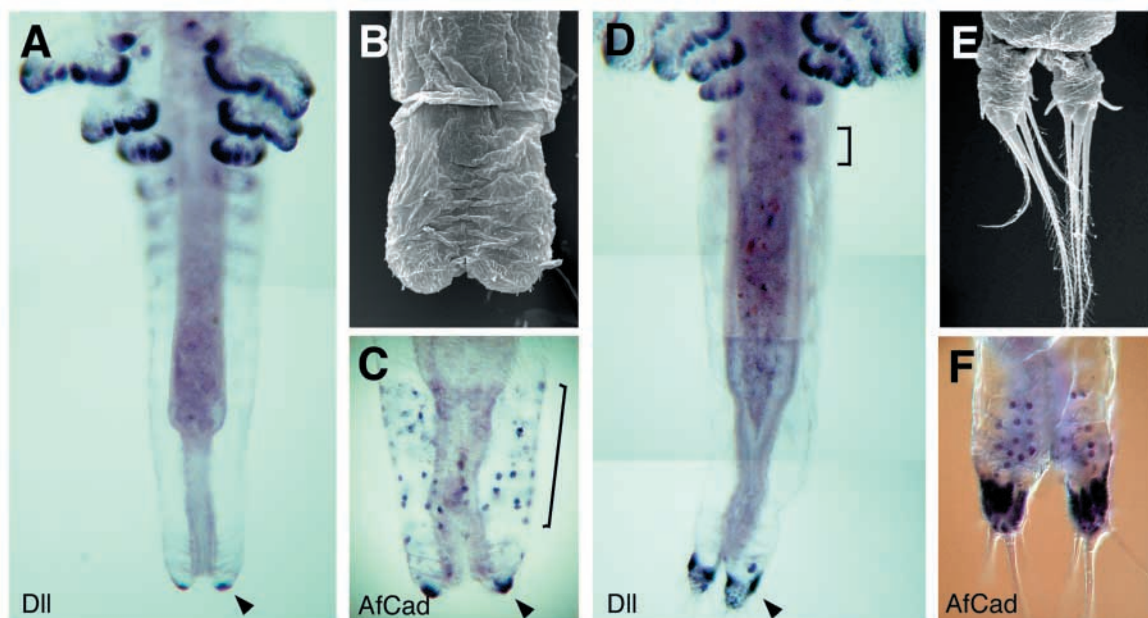

Fig. 5. Expression of AfCad and development of the anal appendages in *Artemia*. (A) Immunochemical staining with an antibody against Dll, showing the early expression of Dll in the anal structures (arrowhead), in stage L6. Dll expression is also seen in the first few thoracic appendages that have formed at this stage. (B) Scanning electron micrograph of the anal region in stage L6-7. (C) Immunochemical staining showing the earliest expression of AfCad in the anal structures (arrowhead) at stage L6. Expression is still seen in the growth zone (bracket). (D) Immunochemical staining for Dll in a fully-segmented larva (around stage L11). Dll expression is seen in the caudal furca (arrowhead), in the thoracic appendages and in the two genital segments (bracket). No Dll expression is seen in the post-genital segments. (E) Scanning electron micrograph of the anal region in stage L11. (F) Immunochemical staining showing the late expression of AfCad in the caudal furca (around stage L11).

less (*Dll*) and *Brachyenteron* (*Byn*) (Moreno and Morata, 1999).

We prepared a UAS-AfCad construct and carried out the same experiments, expressing *Artemia Cad* in the eye-antennal and wing imaginal discs using the same GAL4 drivers. The results we obtained are strikingly similar to those observed by mis-expressing of *Drosophila Cad*: appearance of ectopic anal plates in the head and notum (Fig. 6A) and ectopic expression of *Dll* and *Byn* (Fig. 6B,C). The penetrance of these phenotypes was higher than that observed using *Drosophila Cad* (although this may depend on the particular transgenic lines that were used) and no significant activation of *Drosophila Cad* was detected in imaginal discs mis-expressing *AfCad*, suggesting that these effects are mediated directly by *AfCad* expression. We conclude that the *Drosophila* and *Artemia* Cad proteins behave very similarly in this in vivo assay, indicating that these proteins have inherited similar biochemical properties from their common ancestor, in their ability to bind and regulate the relevant downstream targets.

PCR screen for the isolation of posteriorly-expressed Hox genes

Our study of expression patterns suggests that the candidate genes *AbdB*, *Cad*, *Eve* and *Sal* are unlikely to play a direct role in defining the identity of post-genital segments. We therefore wondered whether previously unidentified Hox genes might be responsible for this function, and set out to search for new Hox genes specifically expressed in this region.

Previous screens to isolate Hox genes from *Artemia* relied on genomic DNA as a template for PCR using degenerate primers (Averof and Akam, 1993). We reasoned that such screens could be strongly enriched for posteriorly-expressed genes if, instead of genomic DNA, the starting material was mRNA derived specifically from posterior parts of the body. We therefore obtained larvae at around the time when the post-genital segments are being formed (stage L9-L10), dissected the posterior part of their body (including part of the genital region, the post-genital region and telson), and prepared first-strand cDNA from this material. We then carried out a PCR screen on this cDNA, using degenerate primers that are expected to recover homeobox fragments from all classes of Hox genes and Cad (including some divergent Hox genes) (see de Rosa et al., 1999; Averof and Akam, 1993), aiming to isolate any posteriorly expressed Hox genes that were missed by our previous screens. Out of the 60 independent clones that we analysed, 59 turned out to be *AfCad* and one was *AbdB*. This result confirms that the PCR screen is highly selective for posteriorly expressed genes, and shows that our general Hox primers are not able to detect any new Hox genes expressed in this region.

Similarly, PCR screens using primers that target specifically AbdB/posterior class Hox genes did not yield any new genes (T.C. and M.A., unpublished). The failure to identify new posteriorly expressed Hox genes may be due to a number of reasons: such genes may not exist in *Artemia*, or they may be too divergent to be amplified by our Hox primers.

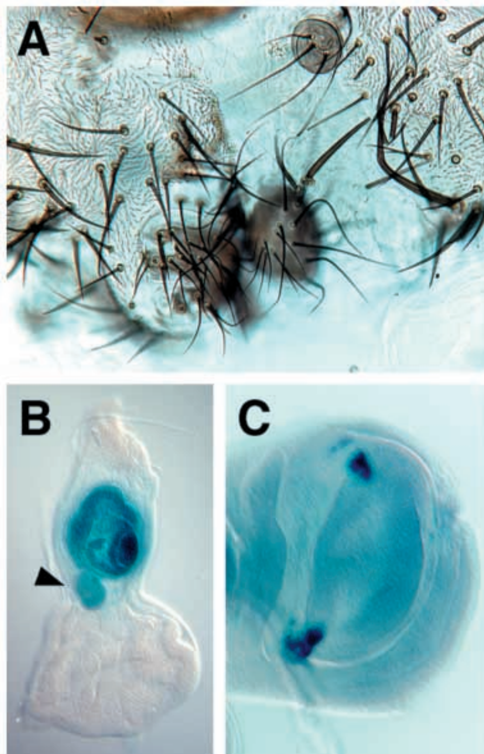

Fig. 6. Mis-expression of AfCad in *Drosophila*. (A) Ectopic anal plates in the dorsal head cuticle of a fly caused by mis-expression of AfCad using the MS-248 GAL4 driver. The anal plates are characterised by being darkly pigmented, having no trichomes and carrying long wavy bristles. (B) Ectopic activation of Dll-*lacZ* following mis-expression of AfCad using the MS248 driver. Dll is activated in an ectopic patch of cells (arrowhead) close to the normal domain of Dll expression in the antennal primordium. (C) Ectopic activation of Byn-*lacZ* in the wing disc, following mis-expression of AfCad using the apterous-GAL4 driver. Byn is induced in two patches of cells at the lateral edges of the wing pouch.

Discussion

AbdB expression in the genital and posterior thoracic/trunk segments

Our observations on *AbdB* expression support the idea that this gene is involved in defining the identity of the two genital segments in *Artemia*, where it is specifically expressed from early stages of their formation until their final differentiation (Fig. 2A-C). This is consistent with previous observations using in situ hybridisation (Averof and Akam, 1995). Apart from its stable expression in the genital segments, during late stages AbdB is also observed in cells of the most posterior thoracic/trunk segments (Fig. 2C). This anterior expansion of AbdB is reminiscent of the distribution of the AbdB-m (otherwise known as AbdB-I) isoform in *Drosophila*, which extends into several posterior abdominal segments (Celniker et al., 1990; Delorenzi and Bienz, 1990). This expansion has not been observed in other insects, such as the locust *Schistocerca gragaria* or the firebrat *Thermobia domestica* (Kelsh et al., 1993; Peterson et al., 1999), suggesting that it may be a convergent similarity between *Artemia* and *Drosophila AbdB* expression. In *Artemia*, *AbdB* expression does not extend into the post-genital region at any developmental stage, indicating that *AbdB* has no direct role in the specification of the post-genital segmental identity.

Possible roles of *Cad* and *Eve* in the sequential generation of body segments

The expression pattern of *AfCad* and *AfEve* in the growth zone does not suggest a specific, homeotic-like role of these genes in defining the identity of the post-genital segments. Unlike homeotic genes, the expression of these genes is not restricted to regions with a particular segmental identity, it appears before segmentation, and it is not maintained during the subsequent development and differentiation of segments. Furthermore, there is no indication that these genes are expressed any differently in the growth zone when the post-genital region is being formed (compared with when other regions are forming), so there is no evidence that they could play a specific role in distinguishing this region from other parts of the body. These expression patterns, however, suggest that these genes could play a role in segmentation that is comparable with the early functions of *Cad* and *Eve* in *Drosophila* and in other arthropods.

The expression of *AfCad* in the growth zone of *Artemia* is very similar to expression patterns of *Cad* observed in other arthropods that generate their trunk segments sequentially from a posterior growth zone ('short-germ' arthropods), most notably in the beetle *Tribolium castaneum* and in the locust *Schistocerca gregaria* (Schulz et al., 1998; Dearden and Akam, 2001). *Cad* expression is also localised to the growth zone, in these species, and the most obvious difference from *Artemia* is that this expression is not excluded from the posterior-most tip of the body during the early stages of segmentation. Expression of *AfCad* in the growth zone is also similar to the expression of vertebrate Cdx genes in posterior parts of the primitive streak, prior to the formation of somites (Meyer and Gruss, 1993; Marom et al., 1997). These similarities may reflect an ancestral role of *Cad/*Cdx genes during the progressive generation of body parts (segments) from a posterior growth zone.

Somewhat different patterns of expression are observed in *Drosophila*, where segments are not generated sequentially from a growth zone (Macdonald and Struhl, 1986). Maternal *Cad* mRNA is uniformly distributed in early *Drosophila* embryos, but translational repression mediated by the anterior morphogen Bicoid transforms this uniform distribution into a gradient, with highest levels of Cad protein at the posterior end of the embryo (Macdonald and Struhl, 1986; Rivera-Pomar et al., 1996). The zygotic expression of *Cad* is transcriptionally regulated and is also restricted to posterior parts of the embryo. The combined zygotic and maternal patterns of *Cad* are known to regulate a number of early segmentation and gap genes (Macdonald and Struhl, 1986; Rivera-Pomar et al., 1995). *Cad/*Cdx genes are also known to directly regulate Hox genes in *C. elegans* and in vertebrates (Hunter et al., 1999; Subramanian et al., 1995; Epstein et al., 1997; van den Akker et al., 2002), and may also have a similar function in some arthropods.

In *Artemia* we have found no evidence for translational repression of *AfCad* mRNA or for a graded distribution of the AfCad protein (Fig. 4A,C,D), indicating that the mechanisms regulating *Cad* expression during segmentation are substantially different in *Drosophila* and *Artemia*. Nevertheless, *Cad* could have equivalent roles in regulating the expression of segmentation genes and Hox genes in these species. In *Drosophila* this regulation occurs simultaneously throughout the embryo, with different concentrations of Cad eliciting different responses on different targets along the anteroposterior axis of the embryo. In 'short-germ' arthropods, like *Artemia*, the regulation of these targets would have to occur in a temporal sequence, as individual segments exit from the growth zone, but it is conceivable that a 'temporal gradient' of Cad activity in these organisms could function in an analogous manner to the spatial concentration gradient of Cad in *Drosophila*: the progenitor cells that give rise to posterior parts of the body spend more time in the growth zone, and thus experience *Cad* expression for longer periods than the anterior progenitor cells. If target enhancers are capable of integrating Cad activity over time, the effect of this 'temporal gradient' could be similar to that of a Cad concentration gradient. Thus, in arthropods like *Artemia*, expression of *Cad* in the growth zone may help to define the spatial limits for the activation of segmentation genes, in an analogous manner to the spatial gradient of Cad protein operating in early *Drosophila* embryos. In addition, it may help to set temporal limits required for the sequential activation of segmentation genes and Hox genes, as segments exit from the growth zone.

AfEve expression is also observed in the growth zone of *Artemia*, with transient stripes of expression emerging from this posterior domain. This is highly reminiscent of *Eve/Evx* expression in short germ arthropods, like the beetle *Tribolium castaneum* and the centipede *Lithobius atkinsoni* (Patel et al., 1994; Hughes and Kaufman, 2002a). The main difference between these species appears to be in the stability of these stripes: the *Eve* stripes of *Artemia* appear to be very transient, while the stripes in *Lithobius* and *Tribolium* persist for longer, and consequently a number of stripes can be detected at any one time. Another issue concerns the segmental periodicity of *Eve* stripes. In *Drosophila*, *Eve* is expressed in alternate segments and is well known for its role as a pair-rule segmentation gene (Frasch et al., 1987), but expression with double-segment periodicity has not been observed beyond higher insects. In *Tribolium*, the stripes that have just emerged from the growth zone are broad and have a double-segment periodicity, but subsequently each of these stripes splits into two narrower stripes with single-segment periodicity (Patel et al., 1994). In the centipede, the *Eve* stripes have single-segment periodicity (Hughes and Kaufman, 2002a). In *Artemia*, the transient appearance of *AfEve* stripes, prior to the appearance of any morphological signs of segmentation or *engrailed* expression (Fig. 4H), does not allow us to determine whether these stripes have single- or double-segment periodicity. In spite of differences in the segmental periodicity of *Eve* stripes, the observation that *Eve* is expressed in stripes associated with segmentation in diverse arthropods (including insects, crustaceans, myriapods and chelicerates) (Patel et al., 1994; Damen et al., 2000; Hughes and Kaufman, 2002a) provides strong evidence for a conserved role of this gene in the process of segment formation.

Beyond arthropods, *Eve/*Evx genes appear not to be involved in segmentation, but instead play a role in the development of posterior structures (Ahringer, 1996; Ferrier et al., 2001; Ruiz i Altaba and Melton, 1989; Bastian and Gruss, 1990; Beck and Slack, 1999). A role in posterior development may also exist in arthropods, where posterior expression is a prominent feature of *Eve* expression. Although no function has been assigned to *Eve* expression in the anal plates and hindgut of

Drosophila, conservation of this posterior expression in *Artemia* and in insects (Frasch et al., 1987; Moreno and Morata, 1999; Patel et al., 1994) suggests that it is likely to play a conserved role in the development of posterior structures. Other aspects of *Eve* function, in the heart and in the CNS, are also likely to be conserved (Frasch et al., 1987; Patel et al., 1992; Patel et al., 1994; Duman-Scheel and Patel, 1999; Hughes and Kaufman, 2002a).

Finally, the early expression of *AfSal* is very similar to the early expression of *AfCad*, encompassing the growth zone at least during the time when thoracic/trunk segments are being generated. This suggests that, like *AfCad* and *AfEve*, *AfSal* may have a role in the process of segment formation, but is unlikely to have a direct role in defining the identity of the post-genital segments.

Conserved role of *Cad* in the specification of anal structures

Besides the dynamic expression of *AfCad* in the growth zone, during later stages this gene is also expressed in the posterior part of the telson. This expression pattern appears shortly before the anal appendages of the adult begin to differentiate in this region, and remains relatively stable during the development of these structures (Fig. 5). This aspect of *AfCad* expression also coincides with the onset of *Dll* expression in precisely the same part of the telson, in striking parallel to *Drosophila*, where *Cad* is associated with the expression of *Dll* in the anal plates (Gorfinkiel et al., 1999; Moreno and Morata, 1999). *Drosophila Cad* is known to have an important homeotic-like function in defining the identity of anal structures (Moreno and Morata, 1999). These similarities, therefore, suggest that *AfCad* could play a similar role in defining the identity of anal structures in *Artemia*. Similar expression patterns have also been observed in other arthropods (insects, crustaceans and chelicerates) (Xu et al., 1994; Schroder et al., 2000; Dearden and Akam, 2001; Abzhanov and Kaufman, 2000b; Akiyama-Oda and Oda, 2003), suggesting that this role is likely to be ancient and phylogenetically conserved among arthropods. The effects of mis-expressing *AfCad* in *Drosophila* (Fig. 6) also suggest that the functional properties of Cad proteins are likely to be widely conserved.

Identification of a unique body region that expresses no known Hox genes

In this work, we set out to investigate the nature of the post-genital segments of *Artemia*, asking whether any of the known Hox genes or related candidate genes could play a role in defining the identity of these segments. The expression patterns of *AbdB*, *Cad*, *Eve* and *Sal* homologues suggest that none of these genes are likely to have such function. The expression patterns of *AbdB* and *AfCad*, however, provide useful landmarks that allow us to place the post-genital segments in the context of other regions within the body plan of *Artemia*.

AbdB, the most posterior acting of all Hox genes that have been identified in arthropods, is expressed specifically in the two genital segments of *Artemia*, supporting the notion that these segments may be related to the *AbdB*-expressing genital segments of insects (Fig. 7). Consistently, all the other Hox genes are expressed anterior to this region and never extend beyond the posterior boundary of *AbdB* expression (Hughes and Kaufman, 2002b). *AbdB* is not expressed – even transiently – in the post-genital segments, suggesting that these segments bear no direct relationship to the genital segments.

The expression of *AfCad* and *AfEve* in the growth zone points to a role of these genes in the generation of body segments, but it does not suggest a direct, homeotic-like role of these genes in specifying the identity of post-genital segments. More relevant is the expression pattern of *AfCad* in the anal region, which is comparable with the expression of *Cad* in the anal structures of other arthropods (Macdonald and

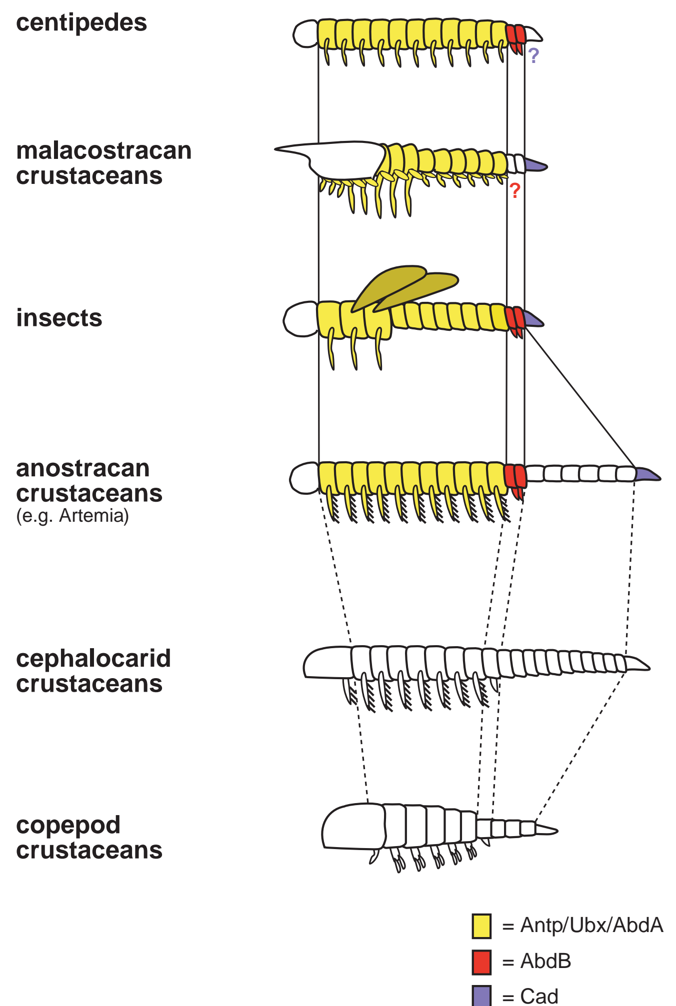

Fig. 7. Regional homologies among diverse arthropod body plans. The proposed relationships between insects, myriapods, anostracans and malacostracan crustaceans are based on patterns of Hox gene expression, indicated by colours: Antp, Ubx and AbdA in yellow; AbdB in red; Cad in purple (Averof and Akam, 1995; Abzhanov and Kaufman, 2000a; Abzhanov and Kaufman, 2000b; Hughes and Kaufman, 2002c). The post-genital region appears to be a unique region arising between the AbdB- and Cad-expressing domains in anostracan crustaceans, with no obvious counterpart in insects, myriapods and malacostracan crustaceans. The proposed relationships with body regions of cephalocarids and copepods are hypothetical (broken lines), based on similarities in the overall patterns of tagmosis and segmental specialisation among these groups (Averof and Akam, 1995; Walossek and Muller, 1997).

Struhl, 1986; Moreno and Morata, 1999; Xu et al., 1994; Schroder et al., 2000; Dearden and Akam, 2001; Abzhanov and Kaufman, 2000b; Akiyama-Oda and Oda, 2003), suggesting a direct relationship of these structures among diverse arthropod groups (Fig. 7). Again, the absence of *AfCad* staining from the post-genital segments suggests that these segments are probably not directly related to the anal region.

In all arthropods where the expression of the relevant genes is known, *AbdB* and *Cad* are expressed in abutting domains that define the most posterior parts of the body (Fig. 7). Uniquely, in *Artemia*, a series of six post-genital segments have become intercalated between these *AbdB* and *Cad* domains. These segments do not express either of these genes or any other known Hox-related gene. Thus, we consider that the post-genital segments of *Artemia* constitute a unique body region that bears no relationship with any of the regions that have been previously characterised by Hox gene expression in other arthropods (Fig. 7). Certainly, we can find no counterpart for this region in insects, where the role of Hox genes has been studied in most detail.

Origin and affinities of the post-genital region

The identification of the post-genital segments as a unique body region that is not specified by any of the known Hox genes, raises a number of questions. First, we know nothing about how the identity of these segments is specified. Are there new, yet unidentified, Hox genes that have adopted this role, or are these segments able to develop without any input from Hox genes? A precedent for the latter is the specification of the antennal segment of insects, where no Hox genes appear to have a direct role (Struhl, 1982; Stuart et al., 1991).

Another question relates to the origin of this region. Is it a novelty that appeared in *Artemia* and its closest relatives, or could it be an ancient feature that is shared with other arthropod groups? Anostracan crustaceans, to which *Artemia* belongs, all share the same general body plan consisting of the head, a series of limb-bearing thoracic/trunk segments, the genital segments, the limb-less post-genital segments and the telson/anus. Yet the homologues of the post-genital segments are more difficult to trace in other groups of branchiopod crustaceans (Brusca and Brusca, 1990), like the Conchostraca and Notostraca, which have a series of thoracic-like limb-bearing segments posterior to their genital segments, followed by a smaller number of limb-less segments; or the Cladocera, which have such a modified and reduced segmental pattern that it is difficult to identify any counterparts of the post-genital segments.

In spite of the differences observed among branchiopods, however, there is evidence to suggest that the post-genital region could have an ancient origin, and may not be strictly restricted to anostracan crustaceans. One line of evidence comes from fossils: the body plan of *Lepidocaris* (Scourfield, 1926) shows striking similarities to the body plan of present-day anostracans, suggesting that the origin of the post-genital segments can be traced at least back to the early Devonian (~390 million years before present). Similarly, a number of other groups of crustaceans, including copepods and cephalocarids (thought to be among the earliest-branching groups of crustaceans) (Brusca and Brusca, 1990), as well as some controversial fossils whose phylogenetic/taxonomic status is not well resolved (e.g. McNamara and Trewin, 1993; Hou and Bergstrom, 1997; Walossek and Muller, 1998), have a segmental organisation that is comparable with that of anostracans, showing differences mainly in the number of segments found in their thoracic/trunk, genital and post-genital regions (Fig. 7). These similarities raise the possibility that the limb-less post-genital region may be an ancient feature shared by a number of divergent arthropod groups.

Our study is the first to propose the existence of a distinct segmental identity, that is not dependent on any of the known Hox genes, between the domains of *AbdB* and *Cad* expression in *Artemia*. Similar studies of Hox gene expression in other arthropods have not identified a comparable body region in major groups like the insects, myriapods, chelicerates and malacostracan crustaceans (Damen et al., 1998; Telford and Thomas, 1998; Abzhanov and Kaufman, 2000a; Hughes and Kaufman, 2002c; Hughes and Kaufman, 2002b), but a number of phylogenetically interesting groups, like copepods and cephalocarids, have not yet been examined. Extending studies of developmental gene expression to diverse groups may be a key to understanding the origin of evolutionary innovations, like the post-genital segments, which contribute to the enormous morphological diversity of arthropod body plans.

We are grateful to Michael Akam in whose laboratory this work was initiated, and to Eduardo Moreno for his active involvement in an earlier phase of this project. We also thank Guillaume Balavoine, Michael Akam and Nipam Patel for communicating unpublished information; Ed Lewis in whose laboratory the AbdB antibody was made; Barry Condron and Nipam Patel for help in testing that antibody; Habib Boulekbache and David Montero for help with SEM observations; Jean Deutsch and Maryline Blin for hospitality and advice on in situ hybridisations; Tassos Pavlopoulos for help with immunisations; Yiannis Livadaras for injection of *Drosophila* embryos; Leandro Sastre and Clive Trotman for cDNA libraries; Nipam Patel, Rob White, Grace Panganiban and Gary Struhl for antibodies; Eduardo Moreno, Gines Morata and Ryutaro Murakami for fly stocks; and Stephen Ekker for the AbdB homeodomain polypeptide. Fernando Casares, Michael Akam and Maura Strigini provided valuable advice and comments on the manuscript. This work was supported by an EMBO Young Investigators' Award to M.A., by Université Paris 7 and by IMBB intramural funds.

References

Abzhanov, A. and Kaufman, T. C. (1999). Novel regulation of the homeotic gene *Scr* associated with a crustacean leg-to-maxilliped appendage transformation. *Development* **126**, 1121-1128.

Abzhanov, A. and Kaufman, T. C. (2000a). Crustacean (malacostracan) Hox genes and the evolution of the arthropod trunk. *Development* **127**, 2239-2249.

Abzhanov, A. and Kaufman, T. C. (2000b). Embryonic expression patterns of the Hox genes of the crayfish *Procambarus clarkii* (Crustacea, Decapoda). *Evol. Dev.* **2**, 271-283.

Ahringer, J. (1996). Posterior patterning by the *Caenorhabditis elegans even-skipped* homolog *vab-7*. *Genes Dev.* **10**, 1120-1130.

Akiyama-Oda, Y. and Oda, H. (2003). Early patterning of the spider embryo: a cluster of mesenchymal cells at the cumulus produces Dpp signals received by germ disc epithelial cells. *Development* **130**, 1735-1747.

Averof, M. and Akam, M. (1993). HOM/Hox genes of *Artemia*: implications for the origin of insect and crustacean body plans. *Curr. Biol.* **3**, 73-78.

Averof, M. and Akam, M. (1995). Hox genes and the diversification of insect and crustacean body plans. *Nature* **376**, 420-423.

Averof, M. and Patel, N. H. (1997). Crustacean appendage evolution associated with changes in Hox gene expression. *Nature* **388**, 682-686.

Bastian, H. and Gruss, P. (1990). A murine *even-skipped* homologue, *Evx 1*, is expressed during early embryogenesis and neurogenesis in a biphasic manner. *EMBO J.* **9**, 1839-1852.

Beck, C. W. and Slack, J. M. (1999). A developmental pathway controlling outgrowth of the *Xenopus* tail bud. *Development* **126**, 1611-1620.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brooke, N. M., Garcia-Fernandez, J. and Holland, P. W. (1998). The ParaHox gene cluster is an evolutionary sister of the Hox gene cluster. *Nature* **392**, 920-922.

Brusca, R. C. and Brusca, G. J. (1990). *Invertebrates*. Sunderland: Sinauer Associates.

Celniker, S. E., Sharma, S., Keelan, D. J. and Lewis, E. B. (1990). The molecular genetics of the bithorax complex of *Drosophila*: *cis*-regulation in the *Abdominal-B* domain. *EMBO J.* **9**, 4277-4286.

Chawengsaksophak, K., James, R., Hammond, V. E., Kontgen, F. and Beck, F. (1997). Homeosis and intestinal tumours in *Cdx2* mutant mice. *Nature* **386**, 84-87.

Criel, G. R. H. (1991). Morphology of *Artemia*. In *Artemia Biology* (ed. P. Browne, P. Sorgeloos and C. N. A. Trotman), pp. 155-185. Boca Raton, FL: CRC Press.

Damen, W. and Tautz, D. (1999). *Abdominal-B* expression in a spider suggests a general role for *Abdominal-B* in specifying the genital structure. *Journal of Exp. Zool.* **285**, 85-91.

Damen, W. G., Hausdorf, M., Seyfarth, E. A. and Tautz, D. (1998). A conserved mode of head segmentation in arthropods revealed by the expression pattern of Hox genes in a spider. *Proc. Natl. Acad. Sci. USA* **95**, 10665-10670.

Damen, W. G., Weller, M. and Tautz, D. (2000). Expression patterns of *hairy*, *even-skipped*, and *runt* in the spider *Cupiennius salei* imply that these genes were segmentation genes in a basal arthropod. *Proc. Natl. Acad. Sci. USA* **97**, 4515-4519.

de Rosa, R., Grenier, J. K., Andreeva, T., Cook, C. E., Adoutte, A., Akam, M., Carroll, S. B. and Balavoine, G. (1999). Hox genes in brachiopods and priapulids and protostome evolution. *Nature* **399**, 772-776.

Dearden, P. K. and Akam, M. (2001). Early embryo patterning in the grasshopper, *Schistocerca gregaria*: *wingless*, *decapentaplegic* and *caudal* expression. *Development* **128**, 3435-3444.

Delorenzi, M. and Bienz, M. (1990). Expression of Abdominal-B homeoproteins in *Drosophila* embryos. *Development* **108**, 323-329.

Duman-Scheel, M. and Patel, N. H. (1999). Analysis of molecular marker expression reveals neuronal homology in distantly related arthropods. *Development* **126**, 2327-2334.

Edgar, L. G., Carr, S., Wang, H. and Wood, W. B. (2001). Zygotic expression of the *caudal* homolog *pal-1* is required for posterior patterning in *Caenorhabditis elegans* embryogenesis. *Dev. Biol.* **229**, 71-88.

Ekker, S. C., Jackson, D. G., von Kessler, D. P., Sun, B. I., Young, K. E. and Beachy, P. A. (1994). The degree of variation in DNA sequence recognition among four *Drosophila* homeotic proteins. *EMBO J.* **13**, 3551-3560.

Epstein, M., Pillemer, G., Yelin, R., Yisraeli, J. K. and Fainsod, A. (1997). Patterning of the embryo along the anterior-posterior axis: the role of the *caudal* genes. *Development* **124**, 3805-3814.

Escalante, R. and Sastre, L. (1993). Similar alternative splicing events generate two sarcoplasmic or endoplasmic reticulum Ca-ATPase isoforms in the crustacean *Artemia franciscana* and in vertebrates. *J. Biol. Chem.* **268**, 14090-14095.

Felgenhauer, B. E. (1987). Techniques for preparing crustaceans for scanning electron microscopy. *J. Crust. Biol.* **7**, 71-76.

Ferrier, D. E., Minguillon, C., Cebrian, C. and Garcia-Fernandez, J. (2001). Amphioxus *Evx* genes: implications for the evolution of the midbrain-hindbrain boundary and the chordate tailbud. *Dev. Biol.* **237**, 270-281.

Frasch, M., Hoey, T., Rushlow, C., Doyle, H. and Levine, M. (1987). Characterization and localization of the even-skipped protein of *Drosophila*. *EMBO J.* **6**, 749-759.

Gibert, J. M., Mouchel-Vielh, E., Queinnec, E. and Deutsch, J. S. (2000). Barnacle duplicate *engrailed* genes: divergent expression patterns and evidence for a vestigial abdomen. *Evol. Dev.* **2**, 194-202.

Gorfinkiel, N., Sanchez, L. and Guerrero, I. (1999). *Drosophila* terminalia as an appendage-like structure. *Mech. Dev.* **86**, 113-123.

Grenier, J. K., Garber, T. L., Warren, R., Whitington, P. M. and Carroll, S. (1997). Evolution of the entire arthropod Hox gene set predated the origin and radiation of the onychophoran/arthropod clade. *Curr. Biol.* **7**, 547-553.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hou, X. and Bergstrom, J. (1997). Arthropods of the Lower Cambrian Chengjiang fauna, southwest China. *Fossils Strata* **45**, 1-116.

Hughes, C. and Kaufman, T. (2002a). Exploring myriapod segmentation: the expression patterns of *even-skipped*, *engrailed*, and *wingless* in a centipede. *Dev. Biol.* **247**, 47-61.

Hughes, C. and Kaufman, T. (2002b). Hox genes and the evolution of the arthropod body plan. *Evol. Dev.* **4**, 459-499.

Hughes, C. L. and Kaufman, T. C. (2002c). Exploring the myriapod body plan: expression patterns of the ten Hox genes in a centipede. *Development* **129**, 1225-1238.

Hunter, C. P. and Kenyon, C. (1996). Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage in *C. elegans* embryos. *Cell* **87**, 217-226.

Hunter, C. P., Harris, J. M., Maloof, J. N. and Kenyon, C. (1999). Hox gene expression in a single *Caenorhabditis elegans* cell is regulated by a *caudal* homolog and intercellular signals that inhibit wnt signaling. *Development* **126**, 805-814.

Jurgens, G. (1988). Head and tail development of the *Drosophila* embryo involves *spalt*, a novel homeotic gene. *EMBO J.* **7**, 189-196.

Kelsh, R., Dawson, I. and Akam, M. (1993). An analysis of *abdominal-B* expression in the locust *Schistocerca gregaria*. *Development* **117**, 293-305.

Kelsh, R., Weinzierl, R. O., White, R. A. and Akam, M. (1994). Homeotic gene expression in the locust *Schistocerca*: an antibody that detects conserved epitopes in *Ultrabithorax* and *abdominal-A* proteins. *Dev. Genet.* **15**, 19-31.

Kuhnlein, R. P., Frommer, G., Friedrich, M., Gonzalez-Gaitan, M., Weber, A., Wagner-Bernholz, J. F., Gehring, W. J., Jackle, H. and Schuh, R. (1994). *spalt* encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. *EMBO J.* **13**, 168-179.

Macdonald, P. M. and Struhl, G. (1986). A molecular gradient in early *Drosophila* embryos and its role in specifying the body pattern. *Nature* **324**, 537-545.

Marom, K., Shapira, E. and Fainsod, A. (1997). The chicken *caudal* genes establish an anterior-posterior gradient by partially overlapping temporal and spatial patterns of expression. *Mech. Dev.* **64**, 41-52.

McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

McNamara, K. J. and Trewin, N. H. (1993). A euthycarcinoid arthropod from the Silurian of western Australia. *Palaeontology* **36**, 319-335.

Meyer, B. I. and Gruss, P. (1993). Mouse *Cdx-1* expression during gastrulation. *Development* **117**, 191-203.

Mitchell, B. and Crews, S. T. (2002). Expression of the *Artemia trachealess* gene in the salt gland and epipod. *Evol. Dev.* **4**, 344-353.

Moreno, E. and Morata, G. (1999). *Caudal* is the Hox gene that specifies the most posterior *Drosophila* segment. *Nature* **400**, 873-877.

Murakami, R., Shigenaga, A., Kawakita, M., Takimoto, K., Yamaoka, I., Akasaka, K. and Shimada, H. (1995). *aproctous*, a locus that is necessary for the development of the proctodeum in *Drosophila* embryos, encodes a homolog of the vertebrate *Brachyury* gene. *Roux Arch. Dev. Biol.* **205**, 89-96.

Panganiban, G., Sebring, A., Nagy, L. and Carroll, S. (1995). The development of crustacean limbs and the evolution of arthropods. *Science* **270**, 1363-1366.

Patel, N., Ball, E. and Goodman, C. (1992). Changing role of *even-skipped* during the evolution of insect pattern formation. *Nature* **357**, 339-342.

Patel, N. H. (1994). Imaging neuronal subsets and other cell types in whole mount *Drosophila* embryos and larvae using antibody probes. In *Methods in Cell Biology*, Vol. 44 (ed. L. S. B. Goldstein and E. A. Fyberg), pp. 445-487. San Diego, CA: Academic Press.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* **58**, 955-968.

Patel, N. H., Condron, B. G. and Zinn, K. (1994). Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ beetles. *Nature* **367**, 429-434.

Peterson, M. D., Rogers, B. T., Popadic, A. and Kaufman, T. C. (1999). The embryonic expression pattern of *labial*, posterior homeotic complex genes and the *teashirt* homologue in an apterygote insect. *Dev. Genes Evol.* **209**, 77-90.

Rivera-Pomar, R., Lu, X., Perrimon, N., Taubert, H. and Jackle, H. (1995). Activation of posterior gap gene expression in the *Drosophila* blastoderm. *Nature* **376**, 253-256.

Rivera-Pomar, R., Niessing, D., Schmidt-Ott, U., Gehring, W. J. and Jackle, H. (1996). RNA binding and translational suppression by bicoid. *Nature* **379**, 746-749.

Ruiz i Altaba, A. and Melton, D. A. (1989). Bimodal and graded expression of the *Xenopus* homeobox gene *Xhox3* during embryonic development. *Development* **106**, 173-183.

Schrehardt, A. (1987). A scanning electron-microscope study of the post-embryonic development of *Artemia*. In *Artemia Research and its Applications*, Vol. 1 (ed. P. Sorgeloos, D. A. Bengtson, W. Decleir and E. Jaspers), pp. 5-32. Wetteren, Belgium: Universa Press.

Schroder, R., Eckert, C., Wolff, C. and Tautz, D. (2000). Conserved and divergent aspects of terminal patterning in the beetle *Tribolium castaneum*. *Proc. Natl. Acad. Sci. USA* **97**, 6591-6596.

Schulz, C., Schroder, R., Hausdorf, B., Wolff, C. and Tautz, D. (1998). A *caudal* homologue in the short germ band beetle *Tribolium* shows similarities to both, the *Drosophila* and the vertebrate *caudal* expression patterns. *Dev. Genes Evol.* **208**, 283-289.

Scourfield, D. J. (1926). On a new type of crustacean from the Old Red Sandstone (Rhynie Chert Bed, Aberdeenshire) – *Lepidocaris rhyniensis* gen. et sp. nov. *Philos. Trans. R. Soc. Lond. B* **214**, 153-187.

Struhl, G. (1982). Genes controlling segmental specification in the *Drosophila* thorax. *Proc. Natl. Acad. Sci. USA* **79**, 7380-7384.

Stuart, J. J., Brown, S. J., Beeman, R. W. and Denell, R. E. (1991). A deficiency of the homeotic complex of the beetle *Tribolium*. *Nature* **350**, 72-74.

Subramanian, V., Meyer, B. I. and Gruss, P. (1995). Disruption of the murine homeobox gene *Cdx1* affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell* **83**, 641-653.

Telford, M. J. and Thomas, R. H. (1998). Expression of homeobox genes shows chelicerate arthropods retain their deutocerebral segment. *Proc. Natl. Acad. Sci. USA* **95**, 10671-10675.

van den Akker, E., Forlani, S., Chawengsaksophak, K., de Graaff, W., Beck, F., Meyer, B. I. and Deschamps, J. (2002). *Cdx1* and *Cdx2* have overlapping functions in anteroposterior patterning and posterior axis elongation. *Development* **129**, 2181-2193.

Walossek, D. and Muller, K. J. (1997). Cambrian 'Orsten'-type arthropods and the phylogeny of Crustacea. In *Arthropod Relationships* (ed. R. A. Fortey and R. H. Thomas), pp. 139-153. London: Chapman & Hall.

Walossek, D. and Muller, K. J. (1998). Early arthropod phylogeny in light of the Cambrian 'Orsten' fossils. In *Arthropod Fossils and Phylogeny* (ed. G. D. Edgecombe), pp. 185-231. New York: Columbia University Press.

Xu, X., Xu, P. X. and Suzuki, Y. (1994). A maternal homeobox gene, *Bombyx caudal*, forms both mRNA and protein concentration gradients spanning anteroposterior axis during gastrulation. *Development* **120**, 277-285.