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SHORT COMMUNICATION

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Expression of *hunchback* during trunk segmentation in the branchiopod crustacean *Artemia franciscana*

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Abstract Comparative studies have shown that some aspects of segmentation are widely conserved among arthropods. Yet, it is still unclear whether the molecular prepatterns that are required for segmentation in *Drosophila* are likely to be similarly conserved in other arthropod groups. Homologues of the *Drosophila* gap genes, like *hunchback*, show regionally restricted expression patterns during the early phases of segmentation in diverse insects, but their expression patterns in other arthropod groups are not yet known. Here, we report the cloning of a *hunchback* orthologue from the crustacean *Artemia franciscana* and its expression during the formation of trunk segments. *Artemia hunchback* is expressed in a series of segmental stripes that correspond to individual thoracic/trunk, genital, and postgenital segments. However, this expression is not associated with the segmenting ectoderm but is restricted to mesodermal cells that associate with the ectoderm in a regular metameric pattern. All cells in the early segmental mesoderm appear to express *hunchback*. Later, mesodermal expression fades, and a complex expression pattern appears in the central nervous system (CNS), which is comparable to *hunchback* expression in the CNS of insects. No regionally restricted expression, reminiscent of gap gene expression, is observed during trunk segmentation. These patterns suggest that the expression patterns of *hunchback* in the mesoderm and in the CNS are likely to be ancient and conserved among crustaceans and insects. In contrast, we find no evidence for a conserved role of *hunchback* in axial patterning in the trunk ectoderm.

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Introduction

Comparative studies have shown that crucial aspects of segmentation are likely to be homologous and widely conserved among diverse arthropod groups. For example, this is clearly reflected in the striking conservation of *engrailed* expression among insects, crustaceans, and spiders (Patel et al. 1989b; Damen 2002). In spite of this conservation, however, it has been argued that earlier events and gene expression patterns that precede segmentation are likely to be less widely conserved. The conservation of gap gene functions, in particular, has been questioned: these functions depend on the formation of broad gradients by diffusion through a syncytial environment in *Drosophila* (Pankratz and Jackle 1993), but this environment appears to be absent during the relevant stages of segmentation in most other groups of insects or arthropods (Ho et al. 1997; Davis and Patel 2002). Nevertheless, homologues of some gap genes have been shown to have regionally restricted expression patterns during segmentation in several insects (Sommer and Tautz 1993; Kraft and Jackle 1994; Wolff et al. 1995; Patel et al. 2001; Bucher and Klingler 2004; Liu and Kaufman 2004a,b; Mito et al. 2005). These patterns are consistent with a possible role of these genes in segmental patterning and/or segmental specialization, albeit in a cellularized environment that precludes the formation of gradients by simple diffusion.

Here, we focus our attention on *hunchback*. In *Drosophila*, *hunchback* is expressed in an anterior domain that encompasses the prospective head, gnathal, and anterior thoracic segments and plays an important role in the formation of these segments (Lehmann and Nusslein-Volhard 1987; Tautz et al. 1987). In addition, a posterior domain of *hunchback* expression is associated with patterning of posterior abdominal segments (Lehmann and Nusslein-Volhard 1987; Tautz et al. 1987), and important roles for *hunchback* have been described in the central nervous

system (CNS; Isshiki et al. 2001). *hunchback* homologues have also been cloned and studied in several other insect species, including *Manduca sexta* (Lepidoptera), *Tribolium castaneum* (Coleoptera), *Oncopeltus fasciatus* (Hemiptera), *Schistocerca americana*, and *Gryllus bimaculatus* (Orthoptera), where they are expressed in similar segmental domains and in the CNS (Kraft and Jackle 1994; Wolff et al. 1995; Patel et al. 2001; Schroder 2003; Liu and Kaufman 2004a; Mito et al. 2005). Although important differences have been highlighted in the roles of *hunchback* in segmentation (Liu and Kaufman 2004a; Mito et al. 2005), *hunchback* appears to play a role in segmental specialization in all insects studied to date. In contrast, data from *Caenorhabditis elegans* and annelids suggest that *hunchback* is unlikely to play any role in segmental/axial patterning in these organisms (Fay et al. 1999; Savage and Shankland 1996; Iwasa et al. 2000; Werbrock et al. 2001; Shimizu and Savage 2002).

Data from intermediate lineages—most importantly from other arthropod classes—would provide important clues about the evolution of gap gene functions. But, so far, there are no reports on the expression of gap gene homologues in any arthropod species besides insects. To address this problem, we have cloned and studied the expression of *hunchback* in a crustacean, a group that probably represents the sister group of insects (Regier and Schultz 1997; Boore et al. 1998; Mallatt et al. 2004).

Materials and methods

Cloning of *Artemia hunchback* fragment

Degenerate primers hbF1 (5′-cgggatccAARCAYCAYYTI GARTAYCA-3′), hbR1 (5′-ggaattcCYRTGRTAICCCATR TG-3′), and hbR2 (5′-ggaattcTGRCARTAYTTIGTIGCR TA-3′) were designed to target sequences encoding conserved regions of the Hunchback protein (see Fig. 1); these primers contained inosine at sites of fourfold degeneracy. A small fragment of *Artemia hunchback (Af-hb)* was amplified by a primary polymerase chain reaction (PCR) using primers hbF1 and hbR1 on 100 ng of *Artemia* genomic DNA followed by a nested PCR using primers hbF1 and hbR2 on 1 μl of the primary PCR. This fragment was cloned in pGEM-T-Easy (Promega) and sequenced, and a specific forward primer, AfHbF1 (5′-cggatccAACAAG TGCAGTTACTC-3′), was designed on the basis of its sequence. Primer AfHbF1 was used with the degenerate primer hbR1 (described above) on 100 ng of *Artemia* genomic DNA to amplify a larger (1.1 kb) fragment of *Artemia hunchback*, which was also cloned in pGEM-T-Easy and sequenced. Finally, a specific reverse primer, AfhbR3 (5′-ggaattcATTGTATACATGATGGCAT-3′), was designed and used in combination with AfHbF1 (described above) to amplify the 1.1-kb fragment of *Artemia hunchback*. This was cloned in pBluescript II KS+ (Stratagene) and pET-23a (Novagen), using the *Eco*RI and *Bam*HI sites present at the 5′-ends of the primers (indicated with small letters). Some of the sequenced clones contained a 4-bp deletion (leading to frameshift and truncation of the inferred AfHb protein) and may correspond to a related pseudogene. The *Artemia hunchback* sequence has been deposited in the European Molecular Biology Laboratory/GenBank databases (sequence accession no. AM055593).

Antibodies and *Artemia hunchback* expression

As described above, the 1.1-kb fragment of *Artemia hunchback* was cloned into the *Eco*RI and *Bam*HI sites of the pET-23a expression vector (Novagen), bringing the *Artemia hunchback* coding sequence in frame with the 6× His sequence of the pET-23a vector. The resulting construct (pET-23a–AfHb) was introduced into *Escherichia coli* BL21pLys cells, and expression was induced with 0.5 mM isopropylthiogalactoside. The His-tagged AfHb protein fragment was purified on a Nickel–nitrilotriacetic acid column (QIAGEN). Three mice and one rabbit were repeatedly immunized with approximately 50- and 150-μg doses (respectively) of the purified protein in Freund's adjuvant. Polyclonal sera were collected after 3–5 immunizations and used for immunochemical stainings at 1:500 or 1:1,000 dilution. Whole mount immunochemical stainings on *Artemia* larvae were carried out as described previously (Copf et al. 2003). Consistent patterns were obtained using the sera from all immunized animals (three mice and one rabbit). Double immunostainings were carried out with the 4F11 monoclonal antibody for Engrailed (Patel et al. 1989a) or a rabbit polyclonal antibody for Caudal (Copf et al. 2003). Costaining with fluorescent markers for actin or DNA was carried out by adding phalloidin–fluorescein isothiocyanate (Sigma) or TO-PRO-3 (Molecular Probes) together with the secondary antibody. Brightfield/differential interference contrast images were obtained on a Leitz Diaplan microscope and fluorescence images were obtained on a Zeiss Axioskop with a Bio-Rad Radiance 2100 confocal system.

Results and discussion

Cloning of an *Artemia hunchback* homologue

hunchback encodes a transcription factor with several conserved zinc finger domains. We used PCR with degenerate primers to clone a 1.1-kb fragment of *hunchback* from the branchiopod crustacean *Artemia franciscana* (Anostraca), spanning the region between the MF2 and CF1 zinc fingers of *hunchback* (as defined by Patel et al. 2001). Sequence comparisons with previously cloned *hunchback* sequences from insects, *C. elegans* and leech (Fig. 1), show that the *Artemia* Hunchback protein contains characteristic conserved sequences corresponding to part of the MF2 and the MF3, MF4 and CF1 zinc finger domains, a block of conserved sequence immediately downstream of MF4 (known as the "C box") and a conserved stretch that is rich in basic residues (the "Basic box"). In addition, the *Artemia* Hunchback sequence contains an extra putative zinc finger

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                    ----MF2----        ---------MF3---------        ----------MF4----------
                          H   H        C  C            H   H        C  C              H     H
Dm (Diptera)        KHHLEYHIRKHKNQKPFQCDKCSYTCVNKSMLNSHRKSHSSVYQYRCADCDYATKYCHSFKLHLRKYGHK
Ms (Lepidoptera)          HMRNHLGSKPFQCSQCSYSCVNKSMLNSHLKSHSNVYQYRCADCNYATKYCHSLKLHLRKYQHN
Tc (Coleoptera)     KHHLEYHLRNHAGSKPFQCNKCDYTCVNKSMLNSHMKSHSNVYRYSCRDCSYATKYCHSLKIHLRRYGHT
Of (Hemiptera)      KHHLEYHLRNHFGSKPFKCDKCSYSCVNKSMLNSHLKSHSNVYQFRCADCTYATKYCHSLKLHLRKYGHN
Sa (Orthoptera)     KHHLEYHLRNHSGWKPFHCDKCDYQCVNKSMLNSHLKSHSNIYQYRCADCTYATKYCHSLKLHLRKYHHT
Af (crustacean)            MRNHFGSKPFKCNKCSYSCVNKSMLNSHMKSHSNIYQYRCADCTYATKYCHSLELHLRKYNHK
Ce (nematode)       KHHLEYHYRNHIGSKPFQCKKCAYNCVNKSMLNSHMKSHTNHYQFRCMDCTYATKYCHSLKLHLKKYNHR
Ht (leech)          KHHLEYHIRVHIGSKPFKCPKCNYSCVNKSMLNSHMKSHTNVYQFRCRDCNYATKYSHSLKLHLIKKKHL
                    -hbF1->                                                <-hbR2-

                      --------------C-Box-------------
Dm (Diptera)        --PGMVLDEDGTPNPS-----LVIDVYGTRRGPKSKNG
Ms (Lepidoptera)    --PAMVLNLDGTPNPL-----PIIDVYGTRRGPKQKPF
Tc (Coleoptera)     --PNVVLDEEGNPCPD-----IIIDVHGTRRGPKIKTQ
Of (Hemiptera)      --PAMVLNPDGSPNPL-----PIVDVYGTRRGPKQKPK          ---------ExF---------
Sa (Orthoptera)     --PDVVLNPDGSPNPL-----PIIDVYGTRRGPKQRPR            C  C             H   Hh
Af (crustacean)     --PAMVLTNEGNPAPS------MIDLYSPRRGPRPMKK   50    LKCQLCGFSTFASNQFAEHILSHS
Ce (nematode)       RVPEGIEMSGGDSSPPFTSDATITFSPLMKQEIKTETV   47    LKCSACDFVASSADEKMRHSMSHI
Ht (leech)          --ADVVLNSDGSLPADGTGHFDSISRRGPPRGPRTMKK   94    FDCSFCIEKFTTPIELKCHVEETH

                            --Basic--            ---------CF1---------
                               Box               C  C             H   H
Dm (Diptera)        200  ASRRKGRVLKLDT   112    CKYCDIFFKDAVLYTIHMGYHS
Ms (Lepidoptera)    152  KNRRKGRAFKLQP    38    CQFCDITFGDLTMHTIHMGFHG
Tc (Coleoptera)      71  KSRRKGPAFKVDP    45    CQYCNIAFGDAVLYTIHMGYHG
Of (Hemiptera)       96  KNRRKGKAFKLDR    58    CTYCDISFKDIVMYTMHMGYHG
Sa (Orthoptera)     211  KHRRKGKAYKLER    65    CEHCENTFRDVIMYSLHKGYHG
Af (crustacean)     131  KNRRKGKAVKLER    35    CQFCDIAFKDAIMYTM
Ce (nematode)                            173    CDHCKIPFDTQQVLDSHMRFHT
Ht (leech)           85  KRARKGRAFKIQT    61    CRHCEMAFADQMTHRLHMGYHG
                                                                <-hbR1
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Fig. 1 Comparison of *Artemia* Hunchback with homologues from diverse insects, a nematode, and a leech. Amino acid sequence alignments are shown for regions with significant sequence conservation among these diverse groups (sequence identity highlighted by *grey shading*). The *Artemia* sequence is highlighted in *bold*. Previously identified motifs, including zinc fingers MF2-4 and CF1, an extra zinc finger (ExF), and a conserved C box and Basic box sequences are marked *above* the alignment. The number of amino acid residues separating noncontiguous domains is indicated; the positions targeted by degenerate primers are marked *below* the alignment. Sequence accession numbers: Dm (*Drosophila melanogaster*) NM_169233, Ms (*M. sexta*) Z30281, Tc (*T. castaneum*) X91618, Of (*O. fasciatus*) AY460341, Sa (*S. americana*) AY040606, Af (*A. franciscana*) AM055593, Ce (*C. elegans*) AF097737, Ht (*Helobdella triserialis*) X91395

domain (ExF), located between the C box and the Basic box, which is not present in the insect Hunchback sequences. This domain shows some sequence similarity with a putative zinc finger domain that is located in the same region in the *C. elegans* and leech Hunchback proteins, suggesting that the presence of this domain is an ancestral feature that was secondarily lost in the insect lineage.

Expression of *Artemia hunchback* during trunk segment formation

To study the expression of *hunchback* during *Artemia* development, we raised polyclonal antisera against bacterially expressed *Artemia* Hunchback protein. Using these sera, we carried out immunochemical stainings in *Artemia* larvae during the stages when the thoracic, genital, and postgenital segments are forming. In *Artemia*, as in other short-germ arthropods, these segments are generated sequentially from a posteriorly located growth zone (Copf et al. 2003).

At hatching, the *Artemia* nauplius larva emerges with three morphologically distinct anterior head segments (antennal, antennular, and mandibular), and, during the first larval stage, the maxillary and first two thoracic segments are generated and begin to express the segmental marker *engrailed* (Manzanares et al. 1993). At this stage, expression of Hunchback is detected in a characteristic pattern in the head (possibly neuronal) and in a loosely defined band of cells that corresponds to the first thoracic segment (Fig. 2a). By the end of this stage, a second band corresponding to the second thoracic segment is visible. During subsequent stages, successive stripes of Hunchback expression appear as new segments emerge from the posterior growth zone (Fig. 2b,c)—one stripe corresponding to each thoracic, genital, and postgenital segment. Double stainings with Engrailed suggest that each stripe of Hunchback expression appears at approximately the same time as Engrailed and prior to overt morphological segmentation (Fig. 2h,i). Unlike Engrailed, however, the Hunchback stripes do not appear in the segmented ectoderm but are restricted to mesodermal cells that lie immediately beneath the ectoderm of the newly forming segments (Fig. 2g–j). It appears that all cells in the segmented somatic mesoderm express Hunchback during the early phases of segment formation, and we can see no unstained mesodermal cells in the interstripe intervals (Fig. 2i,j). Thus, the striped appearance of this pattern directly reflects the distribution of mesodermal cells underlying each forming segment. As segments mature, the mesodermal expression of Hunchback fades, but a new and increasingly complex expression pattern appears within the developing CNS (Fig. 2c–e).

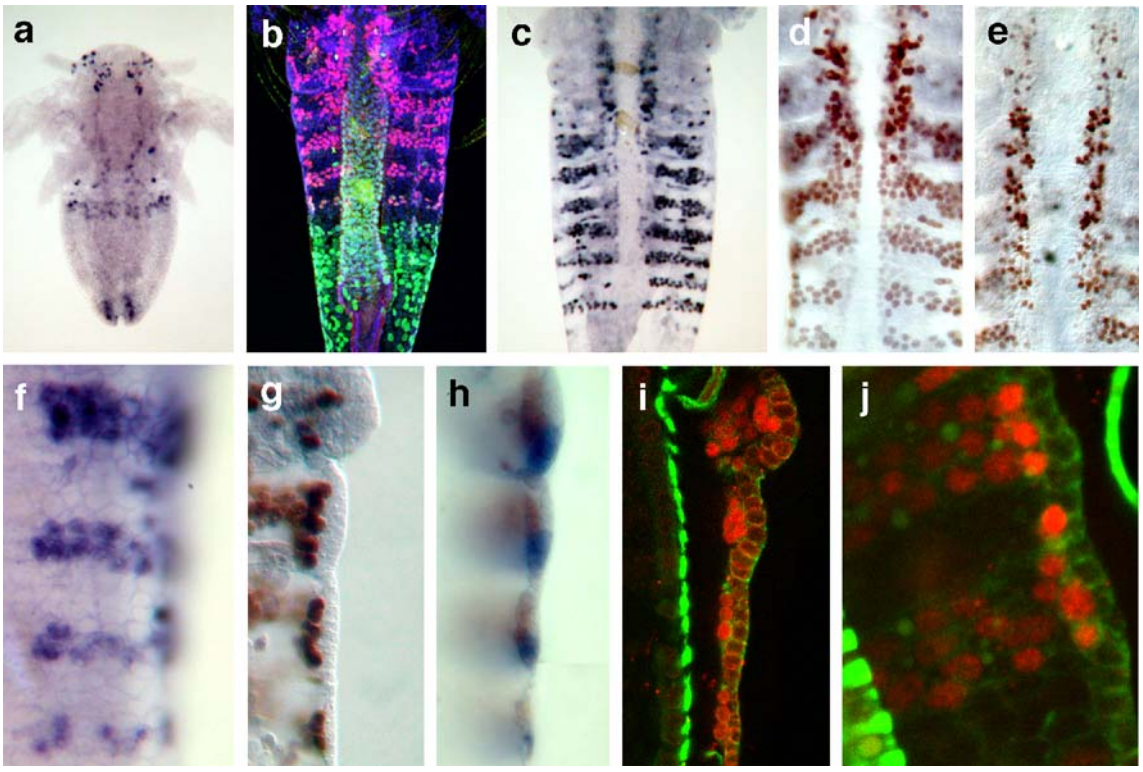

Fig. 2 Hunchback expression during *Artemia* larval development. **a** Hunchback staining in newly hatched nauplius larva, showing expression in the head, a mesodermal stripe corresponding to the emerging first thoracic segment, and posterior staining probably associated with the hindgut (the hindgut staining is transient). **b** Somewhat later larva stained for Hunchback (*red*), Caudal (*green*), and nuclei/DNA using TO-PRO-3 (*blue*); stripes of Hunchback expression correspond to newly formed thoracic segments emerging from the growth zone (the last stripe corresponds to the fifth thoracic segment). The growth zone is visualized by Caudal expression. **c** Hunchback expression in midlarval stage, showing a striped pattern in young (posterior thoracic and genital) segments and CNS expression in more mature (midthoracic) segments. **d** Hunchback staining in maturing anterior thoracic segments, showing the fading of Hunchback expression in the mesodermal stripes and successive phases of expression in the CNS. **e** Similar view during later larval stage. **f–i** Hunchback staining in four successive thoracic hemisegments of decreasing maturity (*top to bottom*). **f** Surface view. **g** Optical section through body wall, showing mesodermal localization of staining; the ectoderm is unstained. **h** Similar optical section of double staining for Hunchback (*brown*) and Engrailed (*blue*). **i** Confocal optical section through body wall, stained with an antiserum for Hunchback (*red*) and phalloidin (*green*); this particular antiserum gives specific nuclear staining for Hunchback (in mesodermal cells) and nonspecific cytoplasmic staining (which outlines the ectodermal cells). **j** Confocal image of band of mesodermal cells, stained with a specific antiserum for Hunchback (*red*) and phalloidin (*green*). Anterior is up in all panels

In the patterns described, staining was localized to cell nuclei (as expected for a transcription factor), and consistent patterns were obtained using antisera from three mice and one rabbit. We are therefore confident that these represent genuine patterns of Hunchback expression in *Artemia*. During the course of larval development, no expression of Hunchback was detected in regionally restricted segmental domains or in the growth zone (Fig. 2b).

Conserved and divergent aspects of hunchback expression

The expression patterns described above provide no evidence for a role of *hunchback* in region-specific segmental patterning in *Artemia*. Unlike the expression patterns observed in insects, we see no evidence for specific anterior or posteriorly retricted domains of expression during larval stages when body segments are forming. In particular, we find no evidence of specific expression in the ectoderm of maxillary and anterior thoracic segments or in posterior thoracic and genital segments (which correspond to the posterior abdominal segments of insects; Averof and Akam 1995; Copf et al. 2003), which are known to express *hunchback* in insects (Tautz et al. 1987; Wolff et al. 1995; Patel et al. 2001; Liu and Kaufman 2004a; Mito et al. 2005). In addition, unlike some short-germ insects where *hunchback* is known to have a role in the posterior growth zone during the formation of trunk segments (Liu and Kaufman 2004a; Mito et al. 2005), in *Artemia*, we find no evidence of *hunchback* expression in the growth zone. Thus, we can find no evidence for a role of *hunchback* in segmental patterning or segmental specialization in the ectoderm of *Artemia*, consistent with the possibility that these functions have evolved specifically in insects. In this study, however, we were not able to examine *hunchback* expression during the formation of anterior head segments, which occurs during earlier (embryonic) stages in *Artemia*. Thus, we cannot exclude a role of *hunchback* in the formation of anterior head segments in the embryo.

The expression patterns of *Artemia hunchback* in the segmented somatic mesoderm and in the CNS are rem-

iniscent of similar expression patterns in these tissues in insects, arguing that these patterns are likely to have been inherited from the common ancestors of crustaceans and insects. The role of *hunchback* in the specification of neuroblast identity in the CNS has been studied extensively in *Drosophila* (Isshiki et al. 2001), and corresponding expression patterns have been documented in all insects studied to date (Kraft and Jackle 1994; Wolff et al. 1995; Patel et al. 2001; Liu and Kaufman 2004a; Mito et al. 2005). In contrast, the mesodermal expression of Hunchback has been less well studied in insects: expression in segmented mesodermal blocks has been well documented only in *Schistocerca* (Patel et al. 2001), but similar expression may also occur in other insects (Sommer and Tautz 1991; Wolff et al. 1995; Liu and Kaufman 2004a). In both *Schistocerca* and *Artemia*, the "striped" appearance of Hunchback in the somatic mesoderm reflects the arrangement of mesodermal cells in segmentally reiterated bands during the early stages of segment formation. This striped arrangement is very prominent in *Artemia* and has been observed in at least one other crustacean (Gerberding et al. 2002). Strikingly, this precise segmental arrangement of mesodermal cells appears prior to overt morphological segmentation in the ectoderm (Fig. 2h,i), suggesting that it occurs by an active patterning process and not simply by physical constraints imposed by segmentation in the ectoderm.

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