EVOLUTION & DEVELOPMENT 4:5, 344–353 (2002)

Expression of the *Artemia trachealess* gene in the salt gland and epipod

Brian Mitchell[1] and Stephen T. Crews*

Department of Biochemistry and Biophysics, Program in Molecular Biology and Biotechnology, Curriculum in Neurobiology, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280, USA

*Author for correspondence (email: steve_crews@unc.edu)

[1]Present address: Section of Molecular Cell and Developmental Biology, University of Texas at Austin, Austin, TX 78712, USA.

SUMMARY The *Drosophila trachealess* gene encodes a basic-helix-loop-helix-PAS transcription factor that controls the formation of the trachea and salivary duct. An ortholog of *trachealess* was identified in the brine shrimp, *Artemia franciscana*, and was shown to be highly conserved by sequence identity. Expression of *Artemia trachealess* was observed at two sites during development: the naupliar salt gland and the juvenile thoracic epipod. These two organs function at their respective times of development in osmoregulation, an important aspect of brine shrimp physiology. This extends the range of putative functions of *trachealess* to include formation of osmoregulatory, respiratory, and ductile organs.

INTRODUCTION

Osmoregulation is a fundamental aspect of organismal physiology. It is particularly important for branchiopod brine shrimp, such as *Artemia franciscana*. *Artemia* survive in the hypersaline environments of salt ponds and brine pits. A requirement for survival of halophilic animals is possession of efficient mechanisms for both osmotic and ionic regulation. Water uptake occurs in the alimentary tract by swallowing salt water, and the excess NaCl is actively excreted (Croghan 1958b; Hootman and Conte 1974). *Artemia* deal with the problem of salt excretion using different organs at different stages of their life cycle. Nauplii, which are the early stages of larval development, rely on a transient structure, the salt gland, for salt excretion (Conte 1984), whereas juveniles and adults rely on the thoracic epipods (Croghan 1958a). The antennal glands and maxillary glands may also contribute to osmoregulation (Martin 1992). *Artemia* cysts possess a fully functional salt gland when they hatch into nauplii (Croghan 1958a). Beginning at the juvenile stage, the salt gland is resorbed concomitant with the appearance of the thoracic epipods (Conte 1984). The cells of the salt gland and the epipods share morphological features consistent with their roles in electrolyte transport, including extensive membrane amplification and a rich supply of mitochondria (Conte 1984). Relatively little is known about the molecular processes that underlie the formation, differentiation, and function of the *Artemia* osmoregulatory organs. However, the *Artemia APH-1* gene is expressed in the salt gland (Chavez et al. 1999). *APH-1* encodes a POU-III subclass homeobox-containing transcription factor highly related to the *Drosophila* Drifter (Dfr) protein (Anderson et al. 1995). One prominent role of *Drosophila* Dfr is in controlling tracheal development and transcription by acting as a coactivator of the Trachealess (D-Trh) transcription factor (Zelzer and Shilo 2000). Here we identify an *Artemia trachealess* (*Af-trh*) ortholog.

The *D-trh* gene is expressed in the salivary tissue, trachea, posterior spiracle, and central nervous system (CNS) (Isaac and Andrew 1996; Wilk et al. 1996). *D-trh* is expressed in the salivary primordium, which includes the precursors to both salivary duct and glands, but later expression is restricted to only the salivary duct. Genetic analysis of *D-trh* mutant embryos revealed that *D-trh* is required for the formation of the salivary duct but not the salivary gland (Isaac and Andrew 1996). During tracheal development, *D-trh* is expressed in the tracheal placodes and continues to be expressed in all tracheal cells. Mutations in *D-trh* result in an absence of tracheal cells and a reduction in expression of numerous genes that are normally expressed in the trachea (Younossi-Hartenstein and Hartenstein 1993; Isaac and Andrew 1996; Wilk et al. 1996). The role of *D-trh* as a master regulator of tracheal development is reinforced by the observation that misexpression of *trh* can induce ectopic formation of tracheal cells (Wilk et al. 1996). Analysis of *trh* in another insect, the silkworm *Bombyx mori*, shows that it is also expressed in the trachea, supracolonic trachea (analogous to *Drosophila* posterior spiracle), and the anterior region of the developing silk gland (Matsunami et al. 1999). The silk gland is derived from the labial segment, as is the Dipteran salivary primordium. Although *Bombyx trh* (*Bm-trh*) is initially expressed in the entire elongating silk gland, expression eventually becomes restricted to just the anterior silk

gland, which functions as a duct for silk protein transport to the spinneret. This is analogous to the restriction of expression and function of *Drosophila trh* in salivary duct formation (Isaac and Andrew 1996).

The *trh* gene encodes a protein of the basic-helix-loop-helix-PAS (bHLH-PAS) family of transcription factors. This class of proteins is highly conserved throughout evolution and is involved in a variety of developmental and physiological processes in organisms as diverse as *Caenorhabditis elegans*, *Drosophila*, and human (Crews 1998; Gu et al. 2000). bHLH-PAS proteins are characterized by a DNA binding basic region, HLH domain for dimerization to bHLH-PAS protein partners, and the PAS-1 and PAS-2 domains that are multifunctional interaction domains. The D-Trh protein functions as a heterodimer in vivo with the Tango (Tgo) bHLH-PAS protein (Ohshiro and Saigo 1997; Sonnenfeld et al. 1997). Together, they bind to DNA sequence elements on target genes and activate transcription. The sequence elements contain an ACGTG core sequence and are referred to as a CNS Midline Element (Wharton et al. 1994), because it is also a target sequence of Single-minded (Sim):Tgo heterodimers, which control CNS midline cell development and transcription. Activation of tracheal gene expression by D-Trh:Tgo requires a direct interaction between D-Trh and Dfr via the D-Trh PAS domain (Zelzer and Shilo 2000).

Given the prominent roles that *D-trh* plays in development of multiple *Drosophila* tissues and the existence of a D-Trh coactivator in *Artemia* (APH-1), we have asked whether there is an *Artemia trh* gene. The results demonstrate the presence of an *Artemia* gene (*Af-trh*) highly related to insect *trh* that is expressed in the salt gland and thoracic epipods. Although morphologically and developmentally distinct, the salt gland and epipods both function in osmoregulation, suggesting that *Af-trh* regulates genes involved in that process. This expands the potential developmental roles of *trh* to include osmoregulatory organs, in addition to controlling insect tracheal, salivary duct, and silk gland development. Because APH-1 is also expressed in the salt gland, this suggests that Af-Trh and APH-1 may interact to control salt gland transcription, similar to their interaction that controls tracheal transcription in *Drosophila*.

MATERIALS AND METHODS

Artemia culture

Artemia franciscana were purchased from Carolina Biological. Cysts (Burlington, NC, USA) were hydrated and cultured in reconstituted seawater at 20°C.

Construction of *Artemia* genomic library

Artemia trh genomic clones were isolated from a library of bacteriophage λ clones containing *Artemia* genomic DNA. *Artemia* were grown for 48 h and collected using a Nitex filter (Tetko, Lancaster, NY, USA). DNA isolation was performed using the Easy-DNA kit (Invitrogen, Carlsbad, CA, USA) and subjected to partial digestion with *Sau*3A. Sucrose gradient fractionation of the resulting fragments was carried out, and fractions containing DNA 20–25 kb in size were collected and pooled. These DNA fragments were ligated into the vector λDashII/*Bam*HI. The recombinant clones were packaged, plated, and amplified.

Isolation and analysis of *Af-trh* genomic clones

Af-trh genomic clones were identified using a ^{32}P-labeled probe containing the bHLH region of the *D-trh* gene (Isaac and Andrew 1996; Wilk et al. 1996). The probe was generated by polymerase chain reaction (PCR) amplification of a *D-trh* cDNA clone, provided by B.-Z. Shilo (Weizmann Institute of Science, Rehovot, Israel), using the primers, 5′-CGCAAGGAGAAGTCGAGG-3′ and 5′-CAGCTTCAGGTAQGCTGAT-3′, corresponding to amino acids 78–129. Hybridization to filters containing 1.5×10^5 recombinant phage was carried out in 50% formamide, 6× SSPE, 0.1% NaPPi, 0.1% SDS, 5× Denhardt's, 100 μg/ml salmon sperm DNA at 42°C for 18 h. After hybridization, filters were washed three times for 15 min each in 2× SSC, 0.1% SDS at room temperature and two times for 30 min each in 2× SSC, 0.1% SDS at 50°C. DNA from positive clones was isolated and subjected to restriction enzyme cleavage, followed by Southern blot analysis and hybridization to the *D-trh* bHLH probe. *Af-trh* fragments were subcloned and sequenced.

Generation and analysis of an *Af-trh* cDNA clone

Reverse transcriptase (RT)-PCR was used to generate an *Af-trh* cDNA clone using RNA from animals collected 1 to 14 days after hydration. Poly(A)+ RNA was purified using a Poly(A) Pure kit (Ambion, Austin, TX, USA). cDNA synthesis was performed using a primer containing oligo(dT) and additional 5′ sequences for subsequent PCR (5′-CCAGTGAGCAGAGTGACGAGGACTCGAGC TCAAGC[T18]-3′) with 5 ng of Poly(A)+ RNA. PCR was carried out on cDNA in two steps as a nested reaction. Primers for the first PCR were (1) 5′-TATTTTTTCAGTATTTTGGAAATG-3′ and (2) 5′-CCAGTGAGCAGAGTGACG-3′. Primers used in the second PCR were (1) 5′-GATCAAGGCGTGGAAAAG-3′ and (2) 5′-GAGGACTCGAGCTCAAGC-3′. The PCR amplification products were subcloned into pCRII-Topo-TA (Invitrogen) and sequenced.

Sequence analysis

The conceptual Af-Trh protein sequence was aligned to existing bHLH-PAS proteins using CLUSTAL W (Thompson et al. 1994). The limits of the PAS-1 and PAS-2 domains correspond to regions defined by Taylor and Zhulin (1999). Phylogenetic trees were generated from CLUSTAL W alignments of bHLH-PAS proteins using the neighbor-joining algorithm (Saitou and Nei 1987).

Whole mount in situ hybridization

The in situ hybridization protocol was modified from Nulsen and Nagy (1999). Animals were collected from 1 to 14 days after hydration and fixed in 4% paraformaldehyde, 1× phosphate-buffered saline for 2 h. They were then processed through an ascending methanol series (25%, 50%, 75%, 100%) and stored at 4°C after washing four times in 100% methanol. Preparation for hybridization involved rehydrating animals through a decreasing series of methanol

into PBT (1× phosphate-buffered saline + 0.1% Tween). After washing four times in PBT, animals were sonicated (Branson probe tip sonicator setting at 1.5, Danbury, CT, USA) until approximately 50% were visibly destroyed (1–2 min). The sonication step enhances probe accessibility. *Artemia* were then washed once in PBT and three times in 0.1 M triethanolamine. Even in the best preparations, only 50% of the animals showed positive staining.

Digoxygenin (DIG)-labeled RNA antisense and sense probes were synthesized corresponding to a 1.5-kb *Af-trh* cDNA fragment and 1.2-kb *Artemia engrailed* (*Af-en*) cDNA fragment. These probes were synthesized using either T7 or T3 RNA polymerase (Promega, Madison, WI, USA) from a linearized pBluescript II template containing either an *Af-trh* or *Af-en* cDNA clone fragment. Animals were incubated at room temperature for 20 min in triethanolamine with acetic anhydride (1:400 v/v), followed by five washes in PBT, 1 wash in 0.5× PBT, 0.5× hybridization buffer (1× hybridization buffer is 50% formamide, 5× SSC, 5× Denhardt's, 0.1% Tween, 100 μg/ml Salmon sperm DNA), and then 1× hybridization buffer. Incubation continued at 75°C for 30 min, followed by prehybridization overnight at 60°C. DIG-labeled probes at a concentration of 50 ng/ml were added to *Artemia* in fresh 1× hybridization buffer, and hybridization was carried out overnight at 60°C. Hybridization was followed by (1) three washes in hybridization buffer at room temperature; (2) five washes 45 min each at 60°C in hybridization buffer; (3) two washes in 2× SSC, 0.1% SDS for 10 min each at 60°C; (4) two washes in 2× SSC, 0.1% SDS for 15 min each at 60°C; and (5) three washes in PBT for 10 min at RT. The animals were incubated overnight in anti-DIG antibody (1:2000 in PBT, 2% bovine serum albumin), rinsed three times in PBT, washed six times in PBT for 10 min each, five times in PBT for 30 min each, and then twice for 10 min each in alkaline phosphatase development buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris, pH 9.5, 0.1% Tween). Color substrate (5-bromo-4-chloro-3-indolylphosphate [BCIP] and nitro blue tetrazolium [NBT]) was added and animals incubated until staining was complete (30 min to several hours). Animals were mounted in 70% glycerol and examined and photographed using a Zeiss (Oberkochen, Germany) Axiophot microscope.

RESULTS

Identification of an *Artemia trh* gene

The strategy for isolating and acquiring sequence information for a putative *Artemia trh* (*Af-trh*) gene involved first isolating genomic clones followed by cDNA clone isolation. This approach was taken because before isolation, the developmental distribution of putative *Af-trh* RNA was unknown. Most bHLH-PAS genes have vertebrate and invertebrate orthologs. Between orthologs, the most conserved region by sequence is the bHLH domain, and they can be over 90% identical in amino acid sequence between *Drosophila* and mammals. Therefore the bHLH domain of *D-trh* was used as a probe to screen an *Artemia* genomic library.

Two bHLH-positive genomic clones were isolated, and sequence analysis indicated the presence of a gene highly related to *D-trh* and *Bm-trh*. The sequence from the *Artemia* gene was used to design PCR primers for RT-PCR. Preliminary experiments indicated that *Af-trh* transcripts were present at all stages of larval development and in the adult (data not shown). PCR of larval Poly(A)+ RNA resulted in amplification of a cDNA clone of 1.5 kb that included sequences encoding the bHLH, PAS-1, and PAS-2 domains. Comparison of the *Artemia* protein sequence with the sequences of *Drosophila* and *Bombyx* Trh proteins and with the human Neuronal PAS1 (NPAS1) (Zhou et al. 1997) and Neuronal PAS3 (NPAS3) (Brunskill et al. 1999) Trh-like proteins revealed high sequence identity (Fig. 1). The *NPAS1* and *NPAS3* bHLH-PAS genes are prominently expressed in the CNS. The strong sequence conservation among these five proteins includes the bHLH, PAS-1, and PAS-2 domains, as well as the spacers between bHLH-PAS1 and PAS-1–PAS-2. The sequence C-terminal to PAS-2 is unconserved, a feature commonly found among bHLH-PAS proteins.

Comparison of the *Artemia* protein with all other bHLH-PAS proteins demonstrates that it is an *Artemia* Trh (Af-Trh) ortholog. Within the bHLH domain, Af-Trh shares 91% sequence identity to D-Trh and Bm-Trh but only 70% identity to *Drosophila* Sim (Table 1). Of the *Drosophila* bHLH-PAS proteins that dimerize with Tgo (Sonnenfeld et al. 1997), including Sim, Similar, and Spineless, all show significantly less sequence identity to Af-Trh than does D-Trh, and comparison of Af-Trh with other bHLH-PAS proteins, such as human ARNT and Clock, show much lower sequence identity (Table 1). Similar relationships are also found when comparing PAS-1 and PAS-2 domains among bHLH-PAS proteins (Table 1). Examination of a phylogenetic tree that compares the full array of bHLH-PAS full-length protein sequences (Fig. 2) demonstrates that Af-Trh clusters with other Trh proteins in a distinct subfamily of bHLH-PAS proteins that include D-Trh, Bm-Trh, and the mammalian NPAS1 and NPAS3 proteins. As expected, the insect D-Trh and Bm-Trh proteins are more closely related to each other than to Af-Trh. Similarly, D-Trh and Bm-Trh are closer in sequence identity to Af-Trh than to mammalian NPAS1 and NPAS3.

Comparison of the bHLH, PAS-1, and PAS-2 domains between Af-Trh and D-Trh show sequence identity of 91%, 58%, and 56%, respectively (Table 1). The lower conservation between PAS domains compared with the bHLH domain is a relationship also found when comparing other bHLH-PAS proteins. The PAS-1 and PAS-2 domain comparisons among the Trh subfamily (Fig. 1) differ with respect to blocks of sequence identity. PAS-2 (102–103 amino acids in length in the proteins compared) has relatively uniform distribution of sequence identity scattered throughout the domain, with the longest stretch of sequence identity three residues. In contrast, PAS-1 (129–159 amino acids in length) begins at an uninterrupted cluster of sequence identity (9 amino acids long), followed closely by another stretch of identity (18 amino acids), and has another block of identity (10 amino ac-

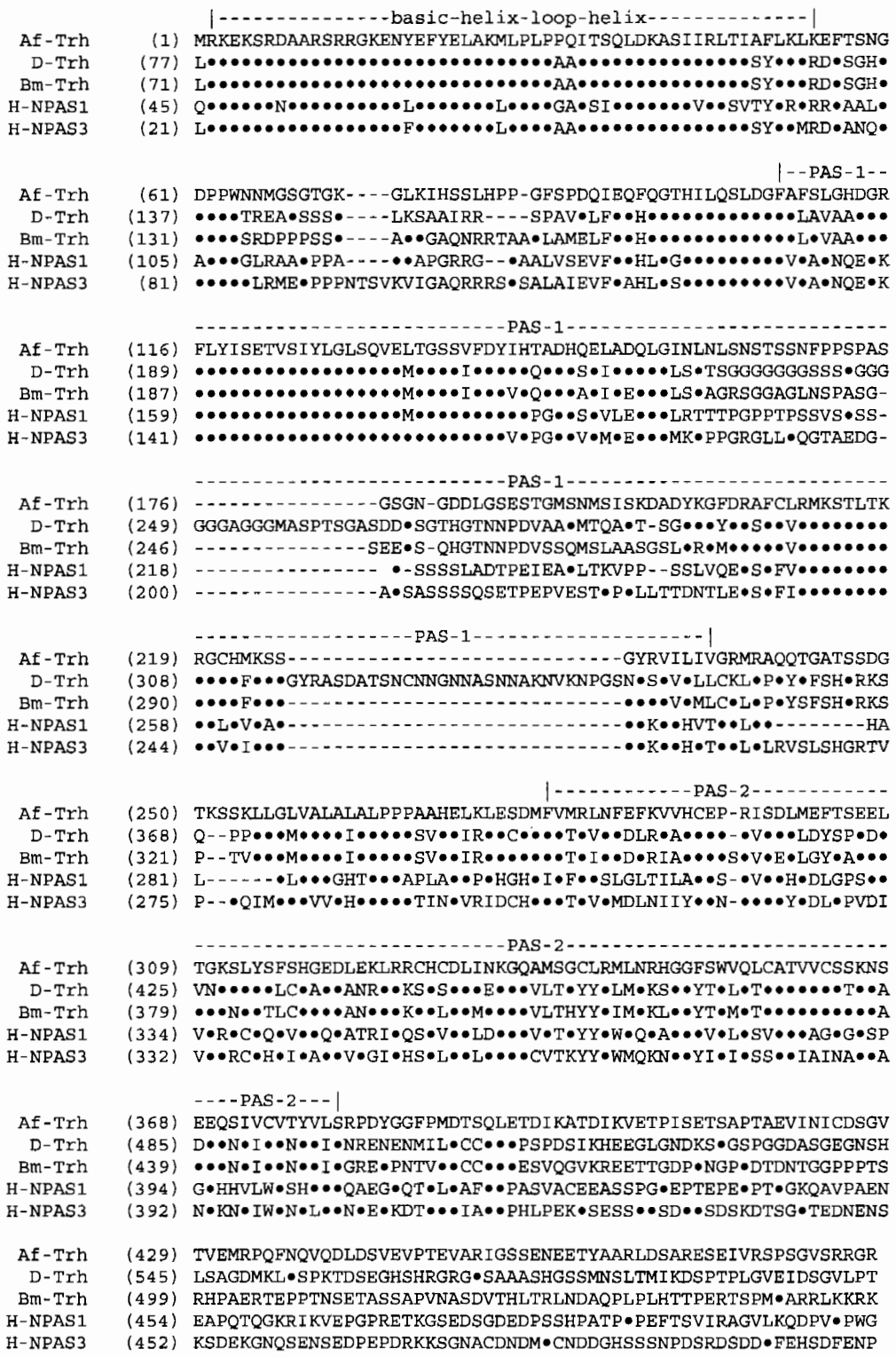

Fig. 1. Protein sequence of the *Artemia* Trh protein is highly related to other Trh and Trh-like proteins. The Af-Trh sequence is shown on the top line and spans 487 amino acids. It likely has additional residues both N-terminal and C-terminal. The Af-Trh sequence is aligned to the Trh proteins of *Drosophila* (D-Trh; NP_523872) and *Bombyx* (Bm-Trh; BAA22946) and the Trh-like human Neuronal PAS1 (H-NPAS1; AAB47248) and Neuronal PAS3 (H-NPAS3; NP_071406) proteins. The alignment was determined using CLUSTAL W. The sequences after Af-Trh residue 395 are shown without gaps because identity is low. •, Sequence identity to Af-Trh; -, Gaps. Each sequence is numbered on the left. At the top are shown the extents of the basic-helix-loop-helix, PAS-1, and PAS-2 domains.

Table 1. The percent sequence identity between Af-Trh and selected insect and mammalian bHLH-PAS proteins is shown quantitatively for the bHLH, PAS-1, and PAS-2 domains

	% Identity to Af-Trh		
Gene	bHLH	PAS-1	PAS-2
Bombyx Trh	91	55	60
Drosophila Trh	91	58	56
Human NPAS3	85	50	42
Human NPAS1	72	48	44
Drosophila Sim	70	29	37
Human HIF-1α	66	24	43
Drosophila Sima	62	25	30
Drosophila Ss	37	22	22
Human Ahr	35	23	19
Human ARNT	26	18	23
Human Clock	24	18	29

Sequences were aligned using ClustalW. The highest % identity was observed in all three domains between Af-Trh and the Trh proteins from the insects, *Bombyx* and *Drosophila*, indicating that the *Artemia* gene encodes a Trh homolog. Sim, Single-minded; HIF-1α, Hypoxia inducible factor-1α; Sima, Similar; Ss, Spineless; Ahr, Aryl hydrocarbon receptor; ARNT, Aryl hydrocarbon nuclear receptor translocator.

ids) near the end. Also contained in PAS-1 are two large blocks of low sequence identity and two insertions in D-Trh of 15 and 29 amino acids not found in the other sequences. These differences may reflect PAS-1 and PAS-2 species-specific biochemical interactions and functions and different functions between PAS-1 and PAS-2.

Af-trh is expressed in the larval salt gland

Expression of the *Af-trh* gene was examined in developing larvae by in situ hybridization (Fig. 3). The early naupliar stages showed high levels of *trh* expression specifically in the salt gland (Fig. 3, A and C). The osmoregulatory salt gland is one of the most prominent morphological features of the nauplius, because of its prominent position on the dorsal surface of the head region. Salt gland expression was observed in emerging stage I naupliar larvae (Fig. 3A), indicating that *Af-trh* expression is present in the pre-nauplius when the salt gland is forming. Expression continued in the salt gland during further naupliar development (Fig. 3C). The early expression pattern of *Af-trh* mRNA is consistent with it playing a role in the development of the salt gland.

The specificity and validity of *Af-trh* in situ hybridization was confirmed by hybridization with sense and antisense probes generated from *Af-trh* and *Artemia engrailed* (*Af-en*) cDNA clones. Expression of *Af-en* in segmentally repeated stripes of *Artemia* larvae has been previously reported using in situ hybridization (Manzanares et al. 1993) and immunostaining (Manzanares et al. 1996) with the 4F11 En monoclonal antibody (Patel et al. 1989). We repeated these results, showing that the *Af-en* antisense probe detects expression in stripes (Fig. 3E) similar to immunostaining with Mab 4F11 (Fig. 3F). More importantly, neither the *Af-en* antisense (Fig. 3E), *Af-trh* sense (Fig. 3, B and D), nor *Af-en* sense (not shown) probes showed expression in the larval salt gland. Furthermore, the sense probes had low background and no discernible staining pattern. These control experiments provide support that *Af-trh* is expressed in the salt gland.

Af-trh is expressed in the thoracic epipods

As the salt gland disappears during larval development, its function in osmoregulation is replaced by the thoracic epipods. Correspondingly, *Af-trh* expression begins to decline in the salt gland of the late nauplius and appears in the epipods of the thoracic appendages (Fig. 4, A and C). Negative control hybridizations with an *Af-trh* sense riboprobe do not show epipodal staining (Fig. 4, B and D). *Artemia* thoracic appendages develop in a graded manner with the anterior segments developing before the posterior ones. Figure 4A shows a gradient of *Af-trh* expression, decreasing from anterior to posterior. The first two thoracic segments strongly express *Af-trh*, the third segment has weak expression, and the remaining posterior segments lack *Af-trh* expression. Older larvae show *Af-trh* expression in the epipods of all segments. The individual lobes of dissected limbs are shown in Figure 4C. Localization of *Af-trh* RNA in these dissected limbs is weakly detected in the pre-epipod and robustly detected in the epipod. Later in development the *Af-trh* message is expressed exclusively in the epipods.

DISCUSSION

Using a *D-trh* bHLH cDNA probe, we isolated an orthologous *trh* gene from *Artemia*. The conceptual Af-Trh protein sequence is 91% identical to the D-Trh and Bm-Trh bHLH domains, a degree of identity considerably higher than to any other bHLH-PAS protein. Similarly, the PAS domains of Af-Trh are significantly more related to insect Trh than to any other bHLH-PAS proteins. Analysis of genomic and cDNA clones provided evidence for only a single *trh*-like gene in *Artemia*, although the possibility of multiple *Af-trh* genes has not been rigorously eliminated. The high degree of sequence conservation between Af-Trh and insect Trh is not surprising, given the high sequence conservation found between highly related bHLH-PAS proteins of *C. elegans*, *Drosophila*, and vertebrates. *Caenorhabditis elegans* has several bHLH-PAS proteins highly related to insect and vertebrate bHLH-PAS genes (Consortium 1998; Powell-Coffman et al. 1998; Epstein et al. 2001; Jiang et al. 2001), but there is no clear Trh ortholog. However, the C-Pas-A protein is in the same phylogenetic branch as Trh and Sim. Its function and expression pattern have not been reported. Are there

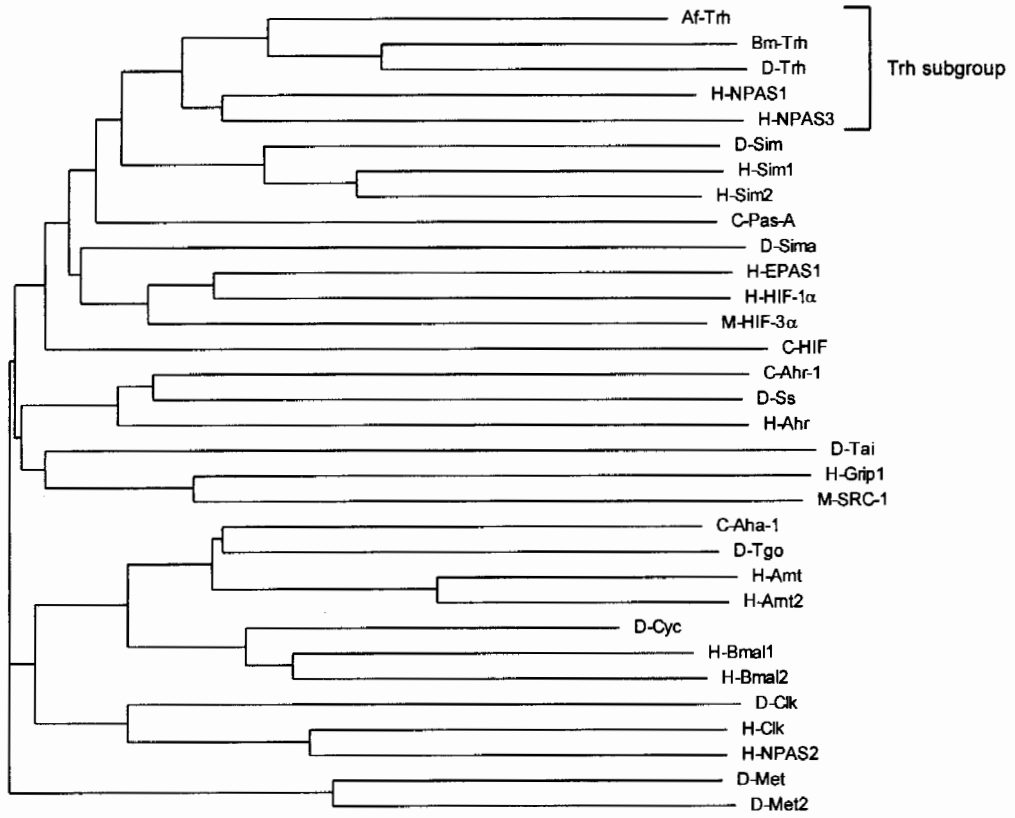

Fig. 2. Phylogenetic tree between Af-Trh and other bHLH-PAS proteins. Full-length sequences of representatives of all published bHLH-PAS proteins were compared with each other using CLUSTAL W and placed into a phylogenetic tree based on the neighbor-joining pair (NJP) method. The results graphically demonstrate that Af-Trh belongs in a Trh subgroup with Bm-Trh, D-Trh, H-NPAS1, and H-NPAS3. Af, *Artemia*; Bm, *Bombyx*; C, *C. elegans*; D, *Drosophila*; H, human; M, murine. Names and references for each abbreviation can be found in Taylor and Zhulin (1999).

vertebrate orthologs of Trh, and do they participate in tubular, osmoregulatory, or respiratory functions? The mammalian NPAS1 and NPAS3 proteins are both more related to arthropod Trh proteins than to any other protein. In the adult, localization of both proteins is highly restricted to the adult CNS and was not detected in lungs or kidney (Zhou et al. 1997; Brunskill et al. 1999). During embryonic development, no expression was observed in the developing respiratory system, although NPAS3 is weakly present in cells associated with the submandibular salivary gland and kidney tubules. Although not orthologous to *trh*, the Hypoxia Inducible Factor (HIF) subfamily of bHLH-PAS proteins plays prominent and evolutionarily conserved physiological and developmental roles in oxygen homeostasis (Nambu et al. 1996; Semenza 1998; Crews and Fan 1999; Epstein et al. 2001; Jiang et al. 2001).

The *Af-trh* gene is expressed initially in the salt gland and later in the epipods. Although evidence is lacking regarding the functional roles of *Af-trh* in *Artemia*, its expression during epipod development and the dramatic roles of *Drosophila trh* in salivary duct and tracheal development suggest that *Af-trh* may play a role in the development and/or function of the salt gland and epipods. Both *Artemia* structures function in osmoregulation and express genes encoding proteins involved in salt transport, such as the Na^+K^+ ATPase (Conte 1984; Holliday et al. 1990; Escalante et al. 1995). Cells of both tissues possess extensive membrane folds that serve to create a large surface area for transport. These cells also have a large number of mitochondria. There are some differences: (1) the salt gland consists of a single cell type, whereas the epipods consist of two distinct cell types (Martin 1992), and (2) the salt gland possesses yolk platelets and glycogen granules, which are absent in epipods (Conte 1984). Thus, although the gross morphology and some properties of the salt gland and epipods are distinct, they share a sufficient number of features to suggest that some of the same genes may be regulated by *Af-trh* in both tissues. In *Drosophila*, salt regulation in the larva occurs in the dorsal region of the large intestine (Murakami and Shiotsuki 2001; Lengyel and Iwaki 2002). The large intestine was not reported to express *D-trh*

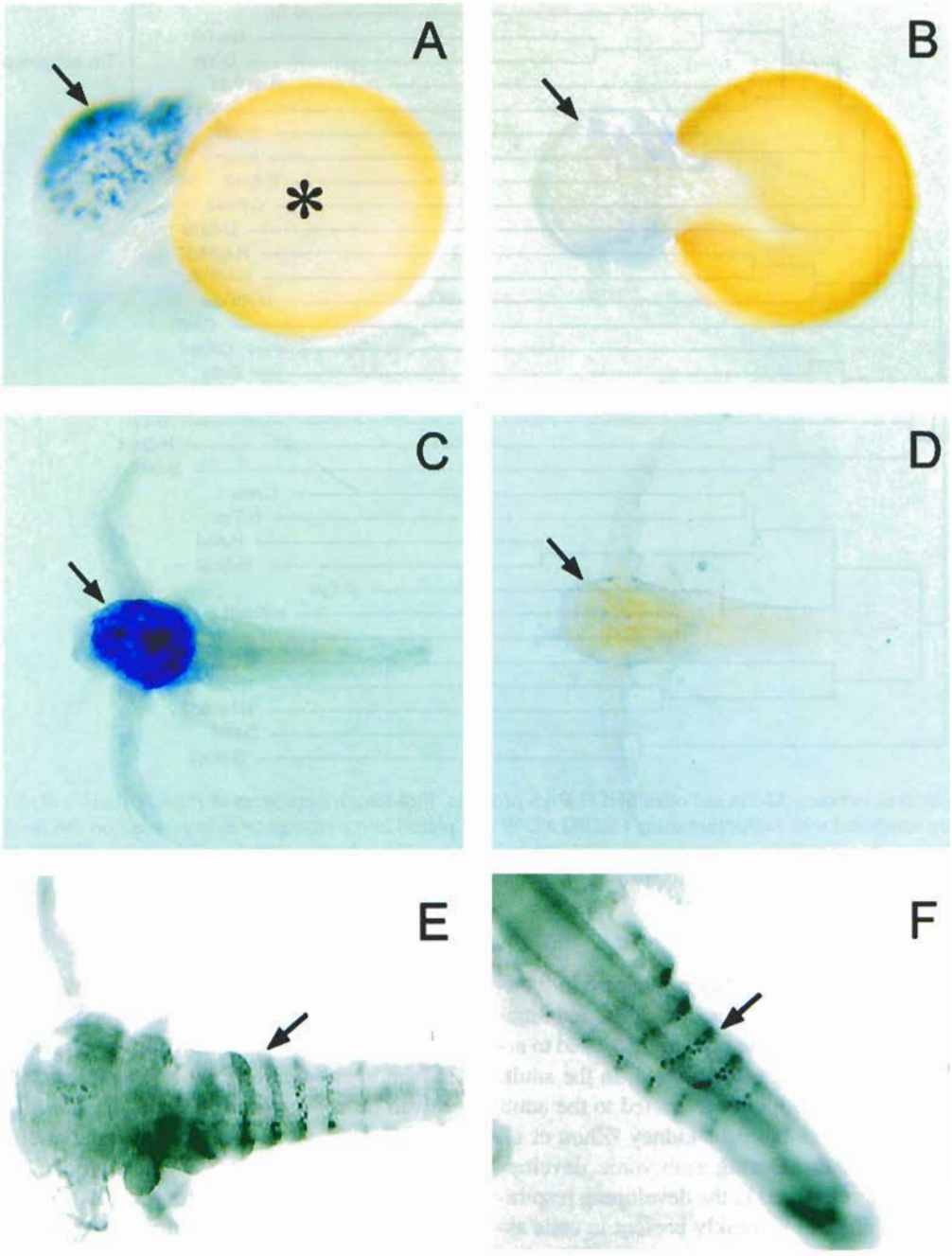

Fig. 3. The *Af-trh* gene is expressed in the larval salt gland. (A) Stage I nauplius was hybridized to an *Af-trh* antisense riboprobe. The larva has just emerged from the cyst (*). Expression is observed in the salt gland (arrow). (B) Stage I nauplius hybridized to a negative control *Af-trh* sense riboprobe showing an absence of hybridization in the salt gland (arrow) or other cells. (C) Dorsal view of a stage II nauplius hybridized to an *Af-trh* antisense probe. Anterior is to the left. Expression of *Af-trh* is observed only in the salt gland (arrow). (D) Stage II nauplius hybridized to an *Af-trh* sense probe. No hybridization to the salt gland (arrow) or other cell types was detected. (E) Control experiment showing in situ hybridization of stage III larva with an *Af-en* antisense riboprobe. The expression pattern of *Af-en* transcripts in segmentally repeated stripes (arrow) corresponds to the localization of Af-En protein shown by 4F11 immunostaining. Staining is detected in the cytoplasm. This experiment validates the in situ hybridization technique. (F) Immunostaining of a stage III larva with monoclonal antibody 4F11 that recognizes Af-En protein. Af-En is localized to nuclei in segmentally repeated stripes at the posterior border of each segment (arrow). (A–E) *Artemia* larvae were subjected to in situ hybridization with DIG-labeled riboprobes followed by reaction with alkaline phosphatase-labeled anti-DIG antibodies, NBT, and X-phosphate. (F) Larva was stained with En 4F11 antibody and treated with horseradish peroxidase-conjugated anti-mouse antibodies followed by diaminobenzidine/H_2O_2 histochemistry.

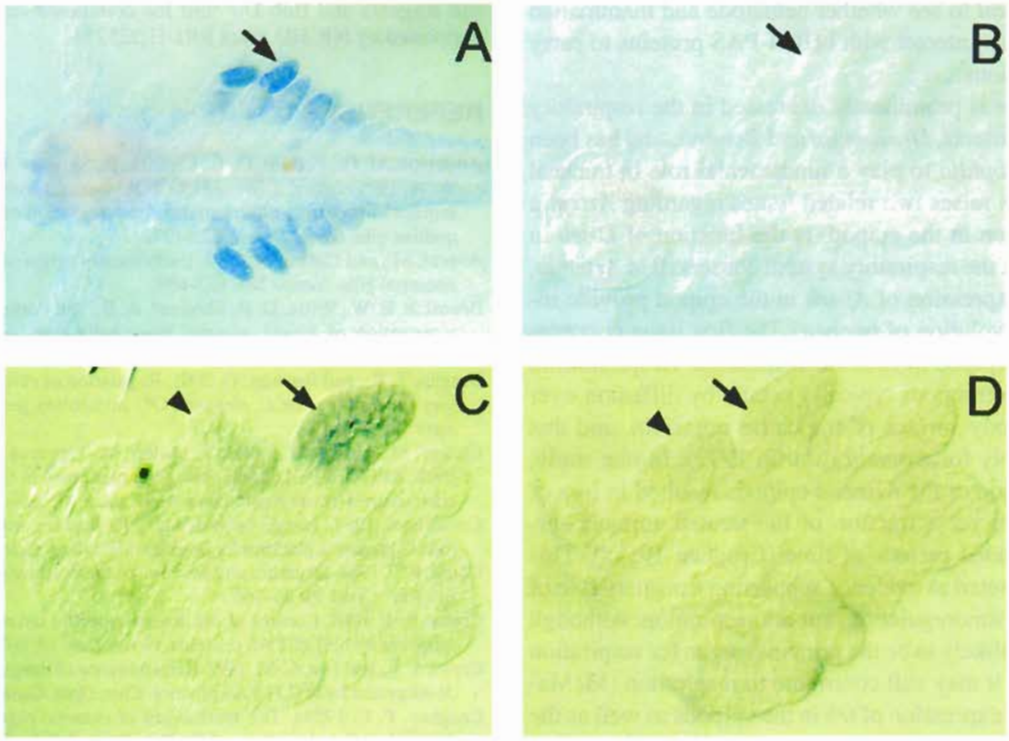

Fig. 4. The *Af-trh* gene is expressed in epipods. (A) Horizontal view of a stage VII larva hybridized in situ to an *Af-trh* antisense riboprobe. Hybridization is detected in the epipods (arrow) of the thoracic limbs. (B) Hybridization to a stage VII larva with a negative control *Af-trh* sense riboprobe showed no detectable expression. (C) High magnification view of a dissected thoracic appendage hybridized to an *Af-trh* antisense probe. High levels of expression are detected in the epipod (arrow) and lower levels are observed in the preepipod (arrowhead). (D) Hybridization using a negative control *Af-trh* sense riboprobe shows no detectable hybridization in epipods (arrow) or preepipods (arrowhead).

(Isaac and Andrew 1996), suggesting that the putative role of *Af-trh* in controlling formation of osmoregulatory organs in *Artemia* is not conserved in *Drosophila.* Because *D-trh* is expressed in the salivary duct and trachea, it was proposed that *trh* might function in tubule formation (Isaac and Andrew 1996). Although one aspect of *D-trh* is to control tubule morphogenesis of those tissues, it is not required for the formation of all tubular structures, because other tubules, such as Malpighian tubules, do not express *D-trh.* The *D-trh* gene is also expressed in the embryonic CNS, a nontubular structure (Isaac and Andrew 1996). Similarly, the sites of *Af-trh* expression, the salt gland and the epipod, are not tubular.

The differential control of gene expression by Trh in *Drosophila* and silkworm may be due, in part, to the use of different Trh-interacting coregulatory proteins in different tissue types. The control of *Drosophila* tracheal gene expression by Trh requires the presence of Dfr, but Dfr is not expressed in the salivary duct, suggesting that a different coregulatory protein(s) is required to interact with Trh to control salivary duct gene expression and formation (Anderson et al. 1995). The *Bombyx* ortholog of Dfr, SGF-3/POU-M1, overlaps with Bm-Trh expression in the trachea and also overlaps with Bm-Trh in the anterior silk gland (Matsunami et al. 1998), suggesting that Trh:Dfr interactions may contribute to tracheal and silk gland expression and development. The *Artemia dfr* homolog, *APH-1*, is expressed in the salt gland, suggesting that it interacts with Af-Trh to control salt gland gene expression. Thus, Trh:Dfr interactions may be conserved in diverse arthropods, including insects and crustaceans, although the targets of their action may be different, reflecting the different tissue types in which they are expressed. Expression of *APH-1* in the epipods was not reported, so that it is unknown whether Af-Trh and APH-1 could potentially interact to control epipod gene expression. Further evidence that POU-III subclass proteins regulate osmoregulatory processes is found in *C. elegans*, where the CEH-6 Dfr ortholog controls formation of an excretory cell involved in osmoregulation (Burglin and Ruvkun 2001). Because vertebrate POU-III proteins have also been implicated in control of osmoregulation, it has been proposed that this is a conserved feature of these proteins (Chavez et al. 1999; Burglin and Ruvkun 2001), although the single *Drosophila* POU-III protein, Dfr, is not expressed in the large intestine (Anderson et al. 1995), the site of larval osmoregulation. It

will be of interest to see whether nematode and mammalian POU-III proteins interact with bHLH-PAS proteins to carry out these functions.

The *trh* gene is prominently expressed in the respiratory organs of two insects, *Drosophila* and *Bombyx*, and has been shown in *Drosophila* to play a fundamental role in tracheal formation. This raises two related issues regarding *Artemia Af-trh* expression in the epipod. Is the function of *D-trh* in development of the respiratory system conserved in *Artemia*, and does the expression of *Af-trh* in the epipod provide insight into the evolution of trachea? The first issue concerns the role of the epipod in *Artemia* respiration. Respiration in small aquatic arthropods typically occurs by diffusion over much of the body surface of the entire organism, and this also seems likely for *Artemia* (Martin 1992). In one study, chemical ablation of the *Artemia* epipods resulted in loss of osmoregulation, yet a fraction of the treated animals survived for extended periods of time (Croghan 1958a). This has been interpreted as evidence supporting a primary role of the epipods in osmoregulation, but not respiration. Although the epipod is unlikely to be the principal organ for respiration (Martin 1992), it may still contribute to respiration (McMahon 2001), and expression of *trh* in the epipods as well as the trachea offers an intriguing link between these two structures. In addition, some crustacea, such as amphipods (Schmitz 1992), do possess epipodite gills, indicating a relationship between epipods and respiration. There are two interpretations for the occurrence of *trh* in the insect trachea and branchiopod epipod. First, the occurrence of *D-trh* in the trachea and *Af-trh* in the epipod is not evolutionarily related but represents co-option of the gene regulatory cassette for use in two distinct tissues. Second, the *trh* expression in the two tissues is evolutionarily related, and a *trh*-expressing epipod-like ancestral structure gave rise to both the insect trachea and *Artemia* epipod. Because of morphological association in the fossil record, it has been proposed that the tracheal spiracle and the insect wing have co-evolved (Kukalova-Peck 1978). Molecular and morphological data have supported a theory that wings evolved from the epipod of an ancestral multibranched appendage that functioned as gills (Wigglesworth 1973; Kukalova-Peck 1983; Averof and Cohen 1997). This structural association of the wings and the spiracles together with the proposed epipodal origin of wings supports a model that the trachea and the wings are derived from an ancestral epipod. Expression of *trh* in the epipods and the trachea is consistent with an evolutionary link between these two tissues. Identification of *trh* in other arthropods and analysis of its expression and function will provide additional insight into this issue.

Acknowledgments

We thank Bob Goldstein, Wayne Johnson, Judith Lengyel, Lisa Nagy, Nipam Patel, Benny Shilo, and Greg Wray for useful advice and reagents and Bob Duronio for comments on the manuscript. Supported by NICHD grant R01-HD25251.

REFERENCES

Anderson, M. G., Perkins, G. L., Chittick, P., Shrigley, R. J., and Johnson, W. A. 1995. *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev.* 9: 123–137.

Averof, M., and Cohen, M. 1997. Evolutionary origin of insect wings from ancestral gills. *Nature* 385: 627–630.

Brunskill, E. W., Witte, D. P., Shreiner, A. B., and Potter, S. S. 1999. Characterization of *Npas3*, a novel basic helix-loop-helix PAS gene expressed in the developing mouse nervous system. *Mech. Dev.* 88: 237–241.

Burglin, T. R., and Ruvkun, G. 2001. Regulation of ectodermal and excretory function by the *C. elegans* POU homeobox gene *ceh-6*. *Development* 128: 779–790.

Chavez, M., Landry, C., Loret, S., Muller, M., Figueroa, J., Peers, B., et al. 1999. APH-1, a POU homeobox gene expressed in the salt gland of the crustacean *Artemia franciscana*. *Mech. Dev.* 87: 207–212.

Consortium, The *C. elegans* Sequencing. 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science* 282: 2012–2018.

Conte, F. P. 1984. Structure and function of the crustacean larval salt gland. *Int. Rev. Cytol.* 91: 45–106.

Crews, S. T. 1998. Control of cell lineage-specific development and transcription by bHLH-PAS proteins. *Genes Dev.* 12: 607–620.

Crews, S. T., and Fan, C.-M. 1999. Remembrance of things PAS: regulation of development by bHLH-PAS proteins. *Curr. Opin. Genet. Dev.* 9: 580–587.

Croghan, P. C. 1958a. The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the branchiae. *J. Exp. Biol.* 35: 234–242.

Croghan, P. C. 1958b. The mechanism of osmotic regulation in *Artemia salina* (L.): the physiology of the gut. *J. Exp. Biol.* 35: 243–249.

Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., et al. 2001. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107: 43–54.

Escalante, R., Garcia-Saez, A., and Sastre, L. 1995. In situ hybridization analyses of Na, K-ATPase alpha-subunit expression during early larval development of *Artemia franciscana*. *J. Histochem. Cytochem.* 43: 391–399.

Gu, Y. Z., Hogenesch, J. B., and Bradfield, C. A. 2000. The PAS superfamily: sensors of environmental and developmental signals. *Annu. Rev. Pharmacol. Toxicol.* 40: 519–561.

Holliday, C. W., Roye, D. B., and Roer, R. D. 1990. Salinity-induced changes in branchial Na/K ATPase activity and transepithelial potential difference in the brine shrimp *Artemia salina*. *J. Exp. Biol.* 151: 279–296.

Hootman, S. R., and Conte, F. P. 1974. Fine structure and function of the alimentary epithelium in *Artemia salina* nauplii. *Cell. Tissue Res.* 155: 423–436.

Isaac, D. D., and Andrew, D. J. 1996. Tubulogenesis in *Drosophila*: a requirement for the *trachealess* gene product. *Genes Dev.* 10: 103–117.

Jiang, H., Guo, R., and Powell-Coffman, J. A. 2001. The *Caenorhabditis elegans hif-1* gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. *Proc. Natl. Acad. Sci. USA* 98: 7916–7921.

Kukalova-Peck, J. 1978. Origin and evolution of insect wings and their relation to metamorphosis, as documented by the fossil record. *J. Morph.* 156: 53–126.

Kukalova-Peck, J. 1983. Origin of the insect wing and wing articulation from the arthropodan leg. *Can. J. Zool.* 61: 1618–1669.

Lengyel, J. A., and Iwaki, D. D. 2002. It takes guts: the *Drosophila* hindgut as a model system for organogenesis. *Dev. Biol.* 243: 1–19.

Manzanares, M., Marco, R., and Garesse, R. 1993. Genomic organization and developmental pattern of expression of the *engrailed* gene from the brine shrimp *Artemia*. *Development* 118: 1209–1219.

Manzanares, M., Williams, T. A., Marco, R., and Garesse, R. 1996. Segmentation in the crustacean *Artemia*: engrailed staining studied with an antibody raised against the Artemia protein. *Rouxs Arch. Dev. Biol.* 205: 424–431.

Martin, J. W. 1992. Branchiopoda. In F. W. Harrison and A. G. Humes (eds.). *Microscopic Anatomy of the Invertebrates, Volume 9: Crustacea*. Wiley-Liss, New York, pp. 25–224.

Matsunami, K., Kokubo, H., Ohno, K., and Suzuki, Y. 1998. Expression pattern analysis of SGF-3/POU-M1 in relation to sericin-1 gene expression in the silk gland. *Dev. Growth Differ.* 40: 591–597.

Matsunami, K., Kokubo, H., Ohno, K., Xu, P., Ueno, K., and Suzuki, Y. 1999. Embryonic silk gland development in *Bombyx*: molecular cloning and expression of the *Bombyx trachealess* gene. *Dev. Genes Evol.* 209: 507–514.

McMahon, B. R. 2001. Control of the cardiovascular function and its evolution in crustacea. *J. Exp. Biol.* 204: 923–932.

Murakami, R., and Shiotsuki, Y. 2001. Ultrastructure of the hindgut of *Drosophila* larva, with special reference to the domains identified by specific gene expression patterns. *J. Morphol.* 248: 144–150.

Nambu, J. R., Chen, W., Hu, S., and Crews, S. T. 1996. The *Drosophila melanogaster similar bHLH-PAS* gene encodes a protein related to human Hypoxia-inducible factor 1a and *Drosophila* Single-minded. *Gene* 172: 249–254.

Nulsen, C., and Nagy, L. M. 1999. The role of wingless in the development of multibranched crustacean limbs. *Dev. Genes Evol.* 209: 340–348.

Ohshiro, T., and Saigo, K. 1997. Transcriptional regulation of *breathless* FGF receptor gene by binding of TRACHEALESS/dARNT heterodimers to three central midline elements in *Drosophila* developing trachea. *Development* 124: 3975–3986.

Patel, N. H., Marin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., et al. 1989. Expression of *engrailed* proteins in arthropods, annelids, and chordates. *Cell* 58: 955–968.

Powell-Coffman, J. A., Bradfield, C. A., and Wood, W. B. 1998. *Caenorhabditis elegans* orthologs of the aryl hydrocarbon receptor and its heterodimerization partner the aryl hydrocarbon receptor nuclear translocator. *Proc. Natl. Acad. Sci. USA* 95: 2844–2849.

Saitou, N., and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406–425.

Schmitz, E. H. 1992. Amphipoda. In F. W. Harrison and A. G. Humes (eds.). *Microscopic Anatomy of the Invertebrates, Volume 9: Crustacea.* Wiley-Liss, New York, pp. 443–528.

Semenza, G. L. 1998. Hypoxia-inducible factor 1: master regulator of O_2 homeostasis. *Curr. Opin. Genet. Dev.* 8: 588–594.

Sonnenfeld, M., Ward, M., Nystrom, G., Mosher, J., Stahl, S., and Crews, S. 1997. The *Drosophila tango* gene encodes a bHLH-PAS protein that is orthologous to mammalian Arnt and controls CNS midline and tracheal development. *Development* 124: 4583–4594.

Taylor, B. L., and Zhulin, I. B. 1999. PAS domains: internal sensors of oxygen, redox potential, and light. *Microbiol. Mol. Biol. Rev.* 63: 479–506.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

Wharton, J., K. A., Franks, R. G., Kasai, Y., and Crews, S. T. 1994. Control of CNS midline transcription by asymmetric E-box elements: similarity to xenobiotic responsive regulation. *Development* 120: 3563–3569.

Wigglesworth, V. B. 1973. Evolution of insect wings and flight. *Nature* 246: 127–129.

Wilk, R., Weizman, I., Glazer, L., and Shilo, B.-Z. 1996. *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* 10: 93–102.

Younossi-Hartenstein, A., and Hartenstein, V. 1993. The role of the tracheae and musculature during pathfinding of *Drosophila* embryonic sensory axons. *Dev. Biol.* 158: 430–437.

Zelzer, E., and Shilo, B. 2000. Interaction between the bHLH-PAS protein Trachealess and the POU-domain protein Drifter, specifies tracheal cell fates. *Mech. Dev.* 19: 163–173.

Zhou, Y.-D., Barnard, M., Tian, H., Ring, H., Francke, U., Shelton, J., et al. 1997. Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. *Proc. Natl. Acad. Sci. USA* 94: 713–718.