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Hemps, a novel EGF-like protein, plays a central role in ascidian metamorphosis

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SUMMARY

All chordates share several characteristic features including a dorsal hollow neural tube, a notochord, a pharynx and an endostyle. Unlike other chordate taxa, ascidians have a biphasic life-history with two distinct body plans. During metamorphosis, the larval nerve cord and notochord degenerate and the pharyngeal gill slits and endostyle form. While ascidians, like other marine invertebrates, metamorphose in response to specific environmental cues, it remains unclear how these cues trigger metamorphosis. We have identified a novel gene (*Hemps)* which encodes a protein with a putative secretion signal sequence and four epidermal growth factor (EGF)-like repeats which is a key regulator of metamorphosis in the ascidian *Herdmania curvata.* Expression of *Hemps* increases markedly when the swimming tadpole larva becomes competent to undergo metamorphosis and then during the first 24 hours of metamorphosis. The Hemps protein is localised to the larval papillae and anterior epidermis of the larva in the region known to be required for metamorphosis. When the larva contacts an inductive cue the protein is released, spreading posteriorly and into the tunic as metamorphosis progresses. Metamorphosis is blocked by incubating larvae in anti-Hemps antibodies prior to the addition of the cue. Addition of recombinant Hemps protein to competent larvae induces metamorphosis in a concentration-dependent manner. A subgroup of genes are specifically induced during this process. These results demonstrate that the Hemps protein is a key regulator of ascidian metamorphosis and is distinct from previously described inducers of this process in terrestrial arthropods and aquatic vertebrates.

Key words: Ascidian, *Herdmania curvata*, EGF-like protein, Metamorphosis

INTRODUCTION

The most common life cycle pattern in the animal kingdom is that of indirect development, which includes a larva that undergoes metamorphosis to form a morphologically distinct adult (Davidson et al., 1995; Fell, 1997). In most marine invertebrate taxa, this biphasic life history appears to be primitive (Brusca and Brusca, 1990; Davidson et al., 1995; Fell, 1997; Peterson et al., 1997). Ascidians are marine invertebrate chordates that have a biphasic life history that appears to be secondarily derived. Molecular phylogenetic analyses strongly suggest that the ancestral chordate was free-swimming and that ascidians (and thaliaceans) acquired biphasic development after diverging from the direct-developing appendicularian urochordates (larvaceans) (Wada and Satoh, 1994; Wada, 1998). As such, ascidians undergo two major morphogenetic phases, firstly during embryogenesis and secondly at metamorphosis. Both ontological events produce a distinct set of chordate-specific characters, with axial structures (notochord and dorsal hollow nerve cord) forming during embryogenesis and visceral structures (pharyngeal gill slits and endostyle) forming during metamorphosis. While embryogenesis has been studied extensively in ascidians, largely because of the simple nature of early development (see Satoh, 1994; Satoh and Jeffery, 1995; DiGegorio and Levine, 1998), little attention has been devoted to metamorphosis and postlarval development (Ogasawara et al., 1999; Hinman and Degnan, 1998). For example the expression patterns of conserved regulatory genes in ascidian notochord and nerve cord development appear to be similar to those occurring in vertebrates and often can be attributed to cell lineage-based and/or close range inductive mechanisms occurring during early embryogenesis (e.g. Yasuo and Satoh, 1994; Wada et al., 1996; Corbo et al., 1997; Gionti et al., 1998; Wada et al., 1998). In contrast, the developmental mechanisms underlying metamorphosis are probably regulated by extrinsic signals that interact with larval receptors and intrinsic signaling molecules in the larva and postlarva that coordinate morphogenetic changes during metamorphosis.

In many marine invertebrates, including ascidians, the

planktonic larva develops the ability to sense, discriminate and respond to environmental cues that induce settlement and/or metamorphosis, and initiate a postlarval morphogenetic program (Morse, 1990; Degnan and Morse, 1995; Degnan et al., 1997; Leitz, 1997). Often development of these abilities occurs after the larva is morphologically mature; the larva is considered competent when it can be induced to settle and metamorphose. While the types of cues that induce settlement and metamorphosis, and the mechanisms by which disparate marine invertebrate larvae perceive these cues have been characterised (Miller and Hadfield, 1986; Johnson and Sutton, 1994; Morse et al., 1994; Holm et al., 1998), the extracellular signals within the larva that initiate and co-ordinate metamorphosis have been less well characterized. This gap in the understanding of marine invertebrate metamorphosis contrasts with what is known about the molecular biology of metamorphosis in terrestrial (i.e. *Drosophila*) and aquatic (i.e. *Xenopus*) organisms, where steroid hormone concentrations have been shown to play critical roles in inducing metamorphosis (Baker and Tata, 1992; Truman et al., 1994; Thummel, 1996).

Ascidians undergo metamorphosis into a sessile benthic adult either upon contact with a specific environmental cue or by an unknown autonomous process (i.e. some larvae undergo spontaneous metamorphosis; Cloney and Carey, 1982; Satoh, 1994; Degnan et al., 1997). In the tropical ascidian *Herdmania curvata* (Kott 1985; Degnan and Lavin, 1995), tadpole larvae become competent to settle and metamorphose a few hours after hatching (Degnan et al., 1996, 1997). Contact with either natural (e.g. a released factor from the ectoproct *Margaretta triplex*) or artificial (40 mM KCl-elevated filtered sea water) cues results in rapid induction of metamorphosis (Degnan et al., 1997; unpublished data), providing an ideal model for investigating metamorphosis in marine invertebrates, and the study of chordate body plan evolution.

We have used differential display RT-PCR to identify genes in *H. curvata* (Arnold et al., 1997a) that may be involved in the development of competence and the induction of metamorphosis. One of these genes, **He**rdmania curvata **m**etamorphosis **p**apillae **s**ignalling *(Hemps)*, previously called *HmEGFL-1* encodes a novel protein of 337 amino acids containing four EGF-like repeats, three novel cysteine-rich repeats and a putative secretion signal sequence, and is expressed in the papillae and anterior-most trunk of the larva (Arnold et al., 1997b). The signal that initiates ascidian metamorphosis originates at the anterior end of the larval trunk, where the papillae are located (Degnan et al., 1997). When larvae are severed at various points along the anterior-posterior axis and maintained in a common test only the anterior, papillae-containing fragments can be induced to undergo metamorphosis. Posterior larval fragments only undergo metamorphosis when fused with anterior fragments suggesting that an anterior signaling centre associated with the papillae is critical for induction of metamorphosis. Since the *Hemps* transcript is localised to this region, we investigated whether this gene might be involved in regulating metamorphosis. Northern blot analysis and immunoblotting demonstrates that *Hemps* expression reached a maximum in early postlarvae that initiate metamorphosis and was undetectable in juveniles. Consistent with previous in situ hybridization data for *Hemps* transcripts we show that Hemps protein is localized to the papillae and anterior epidermis of competent tadpole larvae. Anti-Hemps antibodies blocked metamorphosis while recombinant Hemps protein caused competent larvae to metamorphose at rates significantly faster than the spontaneous rate. These results point to a key role for Hemps in the regulation of ascidian metamorphosis.

MATERIALS AND METHODS

Expression of *Hemps* mRNA

Animals were harvested and eggs fertilized as described previously (Degnan et al., 1996). Embryos, larvae and postlarvae were cultured in filtered sea water (FSW) at 25°C and metamorphosis was induced in competent larvae (3 hours post-hatching) by elevating the concentration of KCl to 40 mM above ambient FSW (Degnan et al., 1997). RNA was isolated from various developmental stages and electrophoresed, blotted and hybridized to a *Hemps* probe as described by Arnold et al. (1997b).

Hemps antibody preparation

The *Hemps* open reading frame (except the predicted signal peptide) was PCR amplified from the full-length cDNA using *Hemps*-specific primers, 5′-GATGGATCCGTCGGTGGAGCGATAGCG and 5′-CCCCAGATGAATTCGAGTGAATTCCTG, and cloned into the *Bam*HI and *Eco*RI sites of pGEX-2T (Pharmacia). Expression of Hemps-GST fusion protein was induced with 0.1 mM IPTG and purified by binding to glutathione-Sepharose beads and eluted from the beads with reduced glutathione (Smith and Johnson, 1988; Frangioni and Neel, 1993). The purified Hemps-GST fusion protein was used for the production of polyclonal antibodies in rabbits. The protein was mixed 1:1 with Freund's adjuvant and injected intramuscularly. Sera were tested by ELISA. The antibody was purified by initially pre-absorbing against GST protein coupled to activated CH-Sepharose followed by affinity purification against recombinant Hemps-GST also coupled to activated CH-Sepharose (Harlow and Lane, 1988).

Immunoblotting

Western blot analysis was performed on stage-specific protein extracts made from ascidian embryos, larvae and postlarvae. The embryos, larvae and postlarvae were homogenised in the NETS lysis buffer as described by Sambrook et al. (1989), and DNA precipitated from the organic phase with ethanol. Proteins were then precipitated from this supernatant with isopropanol, washed with 0.1 M guanidine hydrochloride in ethanol, dissolved in 1% SDS and quantitated by spectrophotometry (Chomczynski 1993). 15 μg of stage-specific protein extracts were loaded per lane onto a 10% SDS-PAGE. The separated protein were transferred to a nitrocellulose membrane by electroblotting. The amount of protein in each lane was checked by staining of the membranes with Ponceau S. The membranes were blocked with 5% skim milk powder in PBS containing 0.1% Tween 20 (PBT) and then incubated in 1:200 of the primary antibody for 16 hours. After washing of the membrane, it was incubated in a 1:2000 dilution of the HRP-conjugated anti-rabbit secondary antibody (Silenus). The membrane was then washed again and enzyme chemiluminescent detection carried out (Dupont, Renaissance Kit).

Immunohistochemistry

Larvae and postlarvae were fixed in PBS containing 50 mM EGTA and 9.25% formaldehyle for 1 hour, washed in 0.6% H_2O_2 in 80% methanol for 2 minutes then washed and stored in methanol at −20°C (Swalla and Jeffery, 1996). Larvae and postlarvae were rehydrated into PBS and incubated in a 1:100 dilution of the anti-Hemps antibody for 48 hours. They were then washed in PBT and incubated in a 1:100 dilution of donkey anti-rabbit IgG (Silenus) antibody conjugated to

horseradish peroxidase for 16 hours and washed again in PBT. Immunocytochemical staining was carried out using a metal enhanced diaminobenzene kit (Sigma) until optimal colour development was achieved and then mounted in 80% glycerol in PBS and examined.

Inhibition of metamorphosis using anti-Hemps antibody

Newly hatched larvae were cultured in FSW or in FSW containing anti-Hemps, anti-Hmserp1 or anti-GST (each at 40 μg/ml, 500 μl incubations) and at 3 hours post-hatching (competence) 40 mM KCl-elevated FSW or live ectoproct was added to the appropriate samples to induce metamorphosis. Experiments were carried out at various times over an 18 month period. For each experiment, 30 larvae for each treatment in triplicate were employed. Metamorphosis was scored for different morphological events including tail resorption, retraction of papillae and rounding of the trunk. The reversibility of anti-Hemps inhibition was determined by incubating newly hatched and pre-competent larvae for 5, 10, 20 and 30 minutes in either the anti-Hemps, anti-Hmserp1 or anti-GST antibodies and then washing the larvae twice in FSW. In these experiments 40 mM KCl-elevated FSW was added at competence to induce metamorphosis. Anti-Hemps antibody (40 μg/ml) was also added simultaneously with 40 mM KCl-elevated FSW at competence and also at 3 hour intervals up to 12 hours post-induction. To assess possible toxicity at earlier stages of embryogenesis all 3 antibodies were added to both the 2 cell stage embryo and prior to gastrulation at both 20 μg/ml and 40 μg/ml.

Effect of recombinant Hemps on rate of metamorphosis

Hemps-GST fusion protein was prepared as described under antibody preparation. Fusion protein was eluted from GST beads and run on a 10% SDS-PAGE to assess purity. Newly hatched larvae were allowed to attain competence (3 hours) prior to addition of GST-Hemps, GST-Hmserp1 and GST alone. Other controls included 40 mM KCl-elevated FSW and larvae in FSW alone. In each case metamorphosis, as judged by tail resorption, was monitored for an additional 4 hours. GST-Hemps was also incubated with competent larvae for either 10, 20 or 30 minutes prior to washout with FSW to determine the optimal incubation time with recombinant protein and metamorphosis was determined over the same time period using 30 larvae, in triplicate for each time point.

Reverse transcription and differential display PCR

Differential display of gene expression was performed essentially as described by Liang and Pardee (1992); Mou et al. (1994). Reverse transcription was carried out using 1 μg total RNA using a degenerate anchored primer (T_{12}MT and T_{12}MG) (Operon Technology) using Moloney Murine Leukemia Virus (MMVL)-reverse transcriptase. Duplicate PCR reactions were performed on the first strand cDNA using one of the anchored primers together with both of the following arbitrary 10mers: OPB-06 (5′ TGCTCTGCCC); OPB-07 (5′ GGTGACGCAG). Incorporation of [α^{33}P] dATP during the PCR enabled visualization of the products. Cycling was carried out at 94°C for 30 seconds, 40°C for 60 seconds and 72°C for 30 seconds for 40 cycles, followed by a final cycle of 5 minutes at 72°C. PCR products were resolved on a 6% sequencing gel and exposed to film.

RESULTS

Normal metamorphosis

Herdmania curvata larvae hatch about 10 hours after fertilization (at 24°C) and acquire competence to settle and metamorphose 3-4 hours later. Settlement and metamorphosis are induced rapidly upon contact with either a factor released from the ectoproct *Margaretta triplex* or 40 mM KCl-elevated filtered sea water, with about 75% of the larvae initiating metamorphosis within an hour of contacting these cues (Degnan et al., 1997; unpublished data). The first morphological indications of metamorphic induction are resorption of the larval tail and retraction of the anterior papillae. During the first 30 hours of metamorphosis, the *H. curvata* postlarvae undergo dramatic morphological changes that include a thickening of the caudal epidermis, programmed cell death and autolysis of notochord, nerve cord and tail muscle cells, projection of ampullae from the larval anterior and a rotation of the endodermal rudiment approximately 90° (Degnan et al., 1996; Hinman and Degnan, 1998). This rotation establishes the adult body axes, with the larval anterior becoming the attached ventral side of the adult. An additional 4 days of development are required for the juvenile body to be formed so that external feeding can begin (Degnan et al., 1996).

Expression of Hemps is induced at competence and during early metamorphosis

We have previously shown that the *Hemps* transcript is localised to the papillae and anterior-most trunk of the larva (Arnold et al., 1997b) and also demonstrated that the signal that initiates ascidian metamorphosis originates at the anterior end of the larval trunk, where the papillae are located (Degnan et al., 1997). Northern blot analysis was carried out to characterize the temporal pattern of *Hemps* expression during embryonic larval and post-larval development. This analysis reveals the presence of transcripts initially in the late tailbud embryo, with expression increasing dramatically, first in competent larvae (3 hours post-hatching) and then in early postlarvae that initiated metamorphosis (Fig. 1A). *Hemps* mRNA levels remain high for the first 24 hours of metamorphosis, decreasing markedly between 24 and 48 hours post-induction and are not detectable in 30 day old juveniles. Analysis of a time course of protein expression, by immunoblotting using anti-Hemps polyclonal antiserum prepared against an Hemps-GST construct, essentially parallels that observed for *Hemps* transcripts (Fig. 1B). A single protein of approximately 40 kDa, which corresponds to the 35.6 kDa size predicted from the Hemps amino acid sequence, is detected first in the hatched larva, becomes more abundant in the 3 hours old larva and 12 hours postlarva and decreases in the 30 day old juvenile. The specificity of the antibody was confirmed using anti-Hmserp1 (a serine protease that is expressed during *H. curvata* development (Arnold et al., 1997a) and preimmune serum. The anti-Hmserp1 antibody reacted with a single band of 42.9 kDa only at the neurula stage of development (Fig. 1C) as predicted from mRNA expression studies (Arnold et al., 1997a). Both the anti-GST antibody and the preimmune serum failed to react with protein extract from any stage (Fig. 1D,E) No cross-reaction with GST or a GST-fusion with Hmserp1 was observed and antibodies to these proteins do not cross-react with the GST-Hemps fusion protein (results not shown). The detection of a protein band but not a mRNA band in the juvenile suggests that the protein has considerable stability.

Hemps is released from the larval anterior at induction of metamorphosis

Immunocytochemical analyses of *H. curvata* larvae and postlarvae with anti-Hemps polyclonal antibodies show that Hemps protein is localised to the papillae and anterior epidermis of newly hatched and competent tadpole larvae,

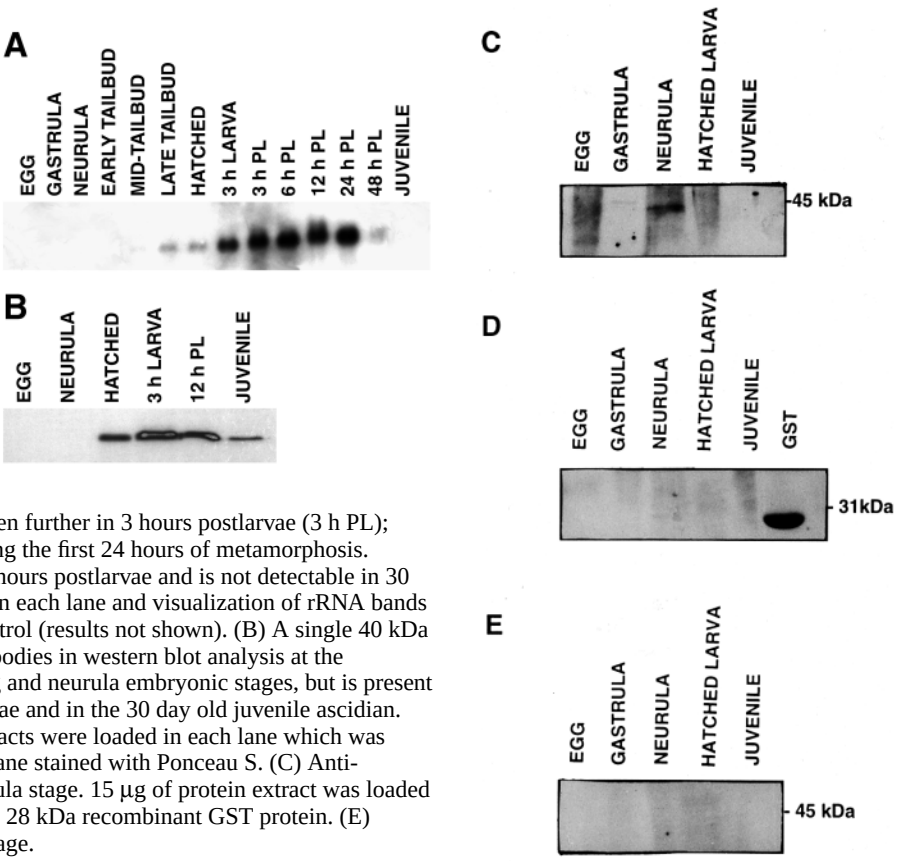

Fig. 1. Hemps is expressed predominantly in competent larvae and during early metamorphosis. (A) *Hemps* transcripts are detected by northern blot hybridization at the indicated stages. *Hemps* mRNA is not observed in egg to early tailbud stages, and relatively low amounts of the 1.2 kb transcript are present in the mid and late tailbud and newly hatched larval stages. Transcript prevalence first increases markedly in competent larvae (3 h LARVA) and then further in 3 hours postlarvae (3 h PL); mRNA levels are maintained at highest levels during the first 24 hours of metamorphosis. *Hemps* mRNA abundance decreases sharply in 48 hours postlarvae and is not detectable in 30 day old juveniles. 10 μg of total RNA was loaded in each lane and visualization of rRNA bands by methylene blue staining served as a loading control (results not shown). (B) A single 40 kDa protein is detected by Anti-Hemps polyclonal antibodies in western blot analysis at the indicated stages. This protein is not detected in egg and neurula embryonic stages, but is present in hatched and competent larvae, 12 hours postlarvae and in the 30 day old juvenile ascidian. Approximately 15 μg of stage-specific protein extracts were loaded in each lane which was confirmed by visualizing the nitrocellulose membrane stained with Ponceau S. (C) Anti-Hmserp1 detects a 45 kDa protein only at the neurula stage. 15 μg of protein extract was loaded in each lane. (D) Anti-GST antibody detects only a 28 kDa recombinant GST protein. (E) Preimmune serum does not detect protein at any stage.

predominantly in a novel papillae-associated tissue (PAT) (Fig. 2A-C). This is consistent with previous results using in situ hybridization of hatched larvae which reveal that Hemps transcripts are present in the papillae and anterior epidermis (Arnold et al., 1997b). Staining of anterior structures is more intense in competent larvae (Fig. 2C) compared to newly hatched larvae (Fig. 2A,B), in accordance with northern and western blot data which demonstrate that there is a large increase in transcript and protein levels at the acquisition of competence (Fig. 1). Within 15 minutes of initiating metamorphosis, the papillae have retracted and the tail has partially resorbed. Prior to these morphological events, but after addition of natural and artificial inductive cues, we detect an increase in Hemps protein levels in PAT, a small cluster of cells that are centered between the three papillae (Fig. 2D). Hemps localisation to these cells was not evident prior to the addition of an inductive cue (Fig. 2B). During the first 30 minutes of metamorphosis these cells are partially extruded anteriorly into the tunic (Fig. 2E,F); the tunic becomes immunoreactive approximately at the same time these cells are released. Ultrastructural analysis (Fig. 2G,H) of this cell cluster reveals that it is composed of several cells that are loosely associated at competence (Fig. 2G) and form a tight ball during tail resorption (Fig. 2H). The developing juvenile tunic forms a pocket in which these cells are located, within the anterior epidermis (Fig. 2F and unpublished).

There is a marked increase in Hemps protein abundance within 15 minutes of induction of metamorphosis (Fig. 2I-O). As metamorphosis proceeds Hemps protein is detected progressively more posteriorly, although staining remains more intense in the most anterior epidermis and tunic (Fig. 2I,J,M,N). Endodermal cells adjacent to the anterior epidermis also stain in the early postlarva (Fig. 2I). These cells are sliding past the epidermis during early metamorphosis (in a counter-clockwise manner when the larval anterior is to the right as shown). 2-3 hours post-induction Hemps is transiently expressed in a small subset of granular tunic cells but not in the predominant vacuolated morula cells (Fig. 2K,L). Immunocytochemical staining is present around the anterior half of the postlarva by 8-10 hours post-induction (Fig. 2M-O), with a line of Hemps expressing cells appearing along the posterior epidermal boundary of expression (Fig. 2O). This posterior boundary corresponds closely with the pre-metamorphosis larval trunk-tail boundary (Fig. 2N). By 16 hours after induction (Fig. 2P), most of the postlarval endoderm and epidermis appears to be immunoreactive to the anti-Hemps antibodies and there is no longer a gradient of staining intensity from anterior to posterior. The staining pattern is similar in 24-30 hours postlarvae (Fig. 2Q). A control anti-serotonin antibody shows specific reactivity to different cells in the *H. curvata* larva (V. F. H. and B. M. D., unpublished) demonstrating the specificity of the anti-Hemps antibody.

Anti-Hemps antibody inhibits metamorphosis

In view of the immunocytochemical evidence presented in Fig. 2 and the observation that Hemps contains a putative signal sequence (Arnold et al., 1997b), and thus is likely to be a secreted protein, we used the anti-Hemps antibody to investigate this proteins role during early metamorphosis. Our expectation was that the antibody would bind to and sequester

the protein and inhibit metamorphosis. Competent larvae subjected to 40 mM KCl-elevated FSW underwent a rapid induction of metamorphosis (as monitored by tail resorption) with approximately 80 and 93% of the larvae initiating metamorphosis within 1 and 3 hours of treatment, respectively (Fig. 3A). Larvae maintained in FSW only, underwent lower rates of spontaneous metamorphosis with approximately 20% initiating metamorphosis 6 hours after hatching (equivalent to 3 hours post-induction). 62% and 74% of the larvae have begun metamorphosing at 1 and 3 hours respectively, after being subjected to live ectoproct *Margaretta triplex*. In contrast, *H. curvata* larvae incubated in anti-Hemps antibodies (20 μg/ml or 40 μg/ml) for 3 hours before the acquisition of competence failed to undergo metamorphosis when subjected to 40 mM KCl-elevated FSW at competence (Fig. 3A) or to live *Margaretta triplex* (results not shown). The same experiment was repeated six times over the course of 18 months to ensure that cohort- or season-specific effects did not occur. While some differences in rates of spontaneous and induced metamorphosis were observed, incubation in anti-Hemps antibodies strongly inhibited metamorphosis in all cases. This inhibition was reversible in larvae exposed to the antibody for 20 minutes or less and retardation in the rate of metamorphosis was observed when antibody was added to post-larvae as late as 12 hours post-hatching (results not shown). Larvae pre-incubated in the same concentration of anti-GST antibodies and then subjected to 40 mM KCl-elevated FSW at competence initiate metamorphosis at rates similar to larvae cultured in FSW, with 74% and 83% initiating metamorphosis within 1 and 3 hours of treatment (Fig. 3A). Larvae treated with the

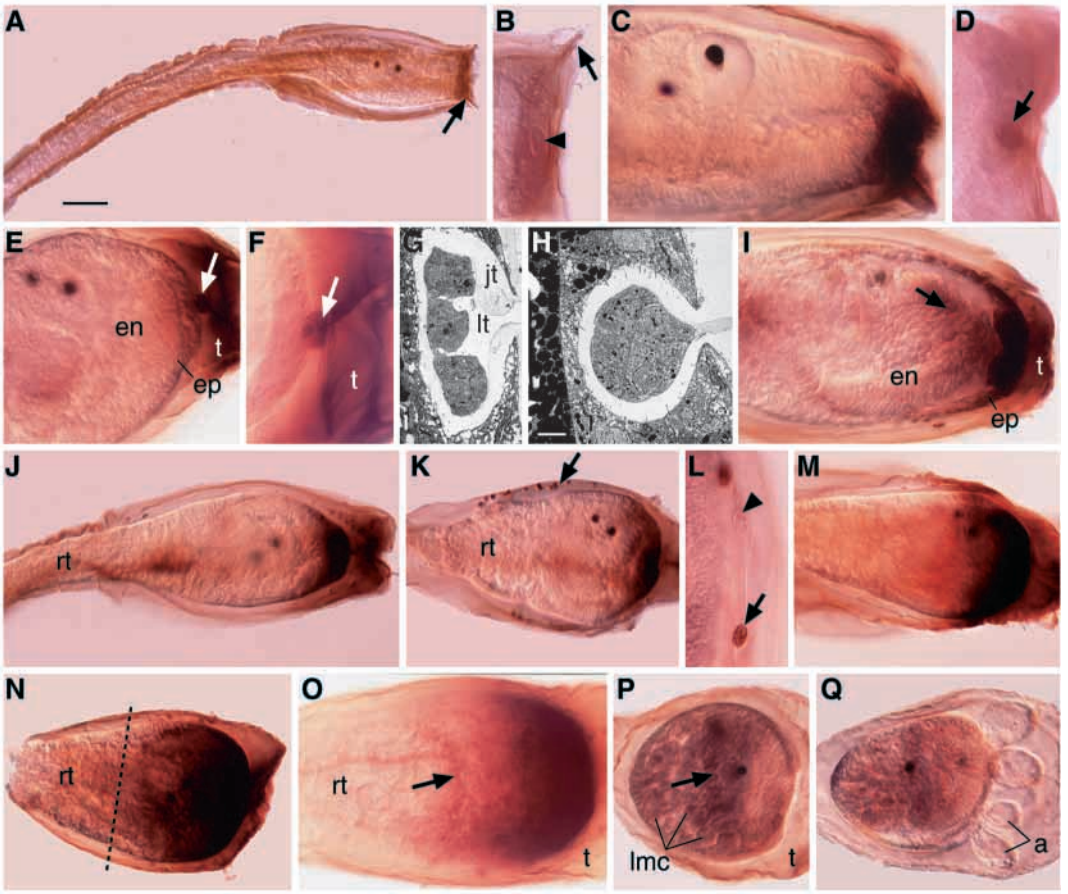

Fig. 2. Immunohistochemical staining with anti-Hemps antibodies monitors the release of Hemps upon induction of metamorphosis. (A,B) At hatching, Hemps is detected in the larval anterior epidermis and papillae (arrows). Immunocytochemical staining is not more intense in the papillae associated tissue (PAT) (arrowhead). (C) At competence, the protein is still localized at the papillae but staining is more intense; hatched and competent larvae were stained under identical conditions. (D) Fifteen minutes later (after the introduction of an environmental inductive cue) Hemps becomes more abundant in the PAT (arrow). Note that the larva in D is stained for a shorter time relative to that in C to visualize the PAT. (E,F) Within 1 hour of the introduction of an inductive cue the PAT (white arrows) is extruded from the anterior epidermis. Immunocytochemical staining is in the epidermis but most intense in the PAT and anterior tunic. (G) As visualized by transmission electron microscopy, cells of the PAT are loosely associated in the competent larva and located outside the juvenile tunic but within the larval tunic. (H) During tail resorption these cells form a discrete structure within the anterior epidermis. (I,J) During tail resorption, Hemps is most abundant in the anterior epidermis. There appears to be a concentration gradient forming along the anteroposterior axis in epidermal cells and the acellular tunic. Endoderm adjacent to the anterior epidermis also stain (arrow). (K,L) During late tail resorption (3-4 hours after initiation of metamorphosis) a subset of test cells express Hemps (arrows); vacuolated morula cells do not express Hemps (arrowhead). (M-O) 8-10 hours post-induction. Immunocytochemical staining occurs more posteriorly in these older postlarvae and is present in both epidermal and endodermal tissues, and tunic; the relative staining of the tunic compared to postlarval tissues is less in these stages. (N) Hemps localization eventually extends approximately half way along the larval anteroposterior axis. Tissues left (posterior) of the dashed line include degenerating larval muscle cells and epidermal cells that covered the larval tail. (O) A line of Hemps-expressing cells can be seen along this posterior trunk-tail boundary (arrow). (P) 16 hours post-induction. Staining is weaker and localized predominantly to a band of differentiating endoderm (arrow) and most of the epidermal cells. In the epidermis, Hemps appears to be least abundant in the most anterior cells; these larval anterior cells are being respecified to be the ventral epidermis in the postlarva. (Q) 24-30 hours post-induction. Immunocytochemical staining remains most intense in the endodermal band in the middle of the animal. a, ampullae; en, endoderm; ep, epidermis; jt, juvenile tunic; lmc, degenerating larval muscle cells; lt, larval tunic; rt, resorbed larval tail; t, tunic. Scale bars: A, 100 μm; H, 4 μm.

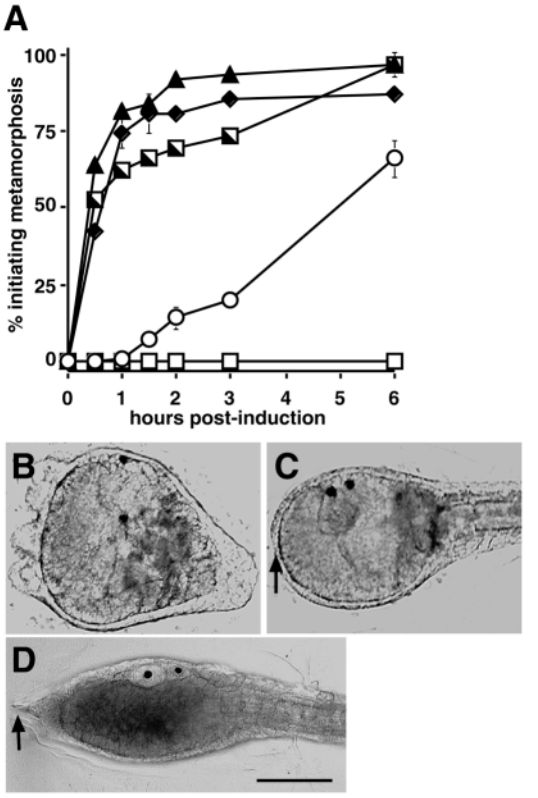

Fig. 3. Anti-Hemps antibodies block the initiation of metamorphosis. (A) Percentage of competent larvae initiating metamorphosis as monitored by tail resorption. Newly hatched larvae are cultured continuously in FSW or FSW with either anti-Hemps or anti-GST antibodies, and 3 hours after hatching are induced to initiate metamorphosis with either 40 mM KCl-elevated FSW or live ectoproct (except untreated controls, which are cultured in FSW); the results are expressed as the mean ± 1 s.d. The results shown represent a typical experiment out of a total of 6 separate experiments over 18 months. In each experiment we employed 30 larvae per treatment in triplicate. Open circles, uninduced controls, which reflect rates of spontaneous metamorphosis; filled triangles, larvae cultured in FSW and induced with KCl-elevated FSW; half filled boxes, larvae cultured in FSW and induced with live ectoproct; filled diamonds, larvae cultured in 40 μg/ml anti-GST antibody and induced with KCl-elevated FSW; open boxes, larvae cultured in 20 μg/ml or 40 μg/ml anti-Hemps antibody and induced. Larvae that are cultured in FSW with either anti-Hmserp1 or anti-GST antibodies respond similarly to live ectoproct and KCl-elevated FSW (results not shown). (B-D) Gross morphologies of living larvae and postlarva; anterior to left. (B) Normal postlarva 8 hours after induction with KCl-elevated FSW, with anterior ampullae, turned endodermal rudiment, retracted tail and thickened epidermis. (C) Anterior half of a similar aged larva that has been cultured in the presence of anti-Hemps antibodies and treated with KCl-elevated FSW. It has retracted papillae (arrow) and rounded trunk, but unchanged larval axial structures and epidermis, and no ampullae. (D) A normal tadpole larva which failed to initiate metamorphosis with descended papillae (arrow). Scale, 100 μm.

same concentration of antibody to another ascidian protein, Hmserp l (Arnold et al., 1997a), followed by KCl induction, underwent metamorphosis at a similar rate to the anti-GST antibody treated larvae. When anti-Hemps, anti-Hmserp1 or anti-GST antibodies (40 μg/ml) were added to early embryos abnormal cleavage occurred. However, when 20 μg/ml antibody was used normal development occurred in each case (results not shown). When either of these concentrations of anti-Hemps antibody was added at hatching there was complete inhibition of metamorphosis whereas the other 2 antibodies had no effect on metamorphosis (Fig. 3 and unpublished results).

Gross morphological analysis of individuals preincubated in anti-GST antibodies and subjected to inductive cues indicates that these antibodies do not affect normal metamorphosis (Fig. 3B). In contrast, individuals preincubated in anti-Hemps antibodies exhibit none of the milestones of early metamorphosis, except retraction of the papillae and rounding of the trunk at 8 hours post-induction (Fig. 3C). A few of these individuals undergo tail resorption about 12 hours after induction, possibly due to degradation of the antibody in the seawater. This contrasts with the small percentage (<1%) of normal larvae that fail to initiate

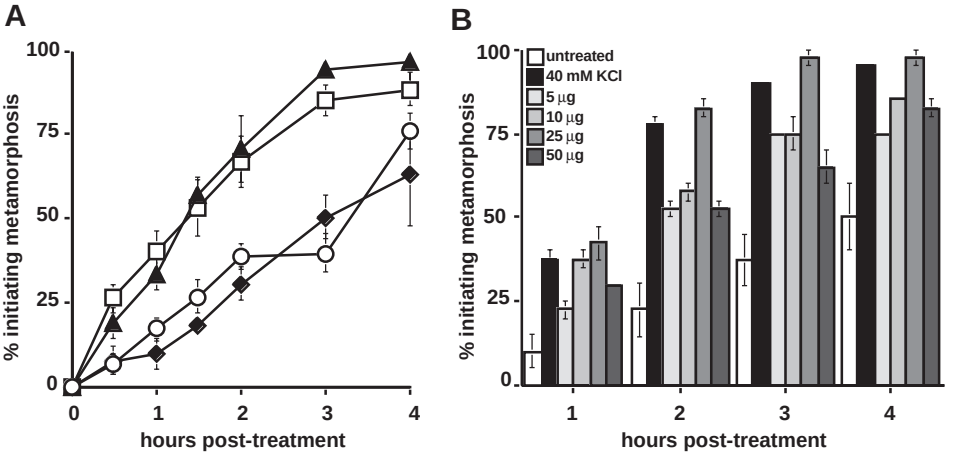

Fig. 4. Recombinant Hemps-GST fusion protein induces competent larvae to metamorphose in a concentration-dependant manner. Competent larvae were subjected to a range of treatments and the percentage of larvae initiating metamorphosis, as monitored by tail resorption, were scored; the results are expressed as the mean ± 1 s.d. (A) Recombinant Hemps-GST, but not GST, induce metamorphosis in a similar manner as 40 mM KCl-elevated FSW. Open circles, untreated controls, showing rates of spontaneous metamorphosis; filled triangles, larvae treated with 40 mM KCl-elevated FSW; filled diamonds, larvae cultured in 25 μg/ml GST in FSW; open boxes, larvae treated with 25 μg/ml Hemps-GST in FSW. GST-Hmserp1 was also used but the data were not plotted since they are approximately the same as GST. (B) Comparison of how the different concentrations of Hemps-GST induce metamorphosis. Competent larvae were subjected to 40 mM KCl or 5-50 μg/ml Hemps-GST.

Table 1. Significance of the effect of recombinant Hemps on rate of metamorphosis compared to other treatments

	Treatment		
Time (h)	Spontaneous (*P* values)	GST-Hmserp1 (*P* values)	GST (*P* values)
0.5	<0.005*	<0.004	<0.006
1	<0.02	<0.006	<0.01
2	<0.03	<0.01	<0.09
3	<0.002	<0.006	<0.001
4	<0.001	<0.02	<0.0001

*Significance has been determined by comparing the results from all 5 experiments carried out with the various treatments. Significance was established using a Student's *t*-test with two samples assuming unequal variances.

metamorphosis, maintaining a papillary structure and having a more elongated trunk (Fig. 3D).

Recombinant Hemps induces metamorphosis

Having shown that the anti-Hemps antibody specifically inhibits the initiation of ascidian metamorphosis, we then determined whether treatment with recombinant Hemps could accelerate the rate of initiation of metamorphosis above the spontaneous rate. Competent larvae can be induced to metamorphose at rates significantly higher than spontaneous rates by subjecting them to FSW with dissolved Hemps-GST fusion protein (Fig. 4A). This effect was observed when recombinant protein was added for 20 minutes or greater. Competent larvae were subjected to recombinant Hemps for 10 min and then washed in FSW and induction of metamorphosis was reduced to approximately 60% of that for larvae incubated with Hemps for 20 minutes or greater (results not shown). The effect of recombinant protein on rate of metamorphosis is significant when compared to either the spontaneous, GST-Hmserp1 or GST treatments (Table 1). This enhancement occurs in a concentration-dependent manner between 5 and 50 μg/ml, with larvae that are cultured in 25 μg/ml exhibiting rates comparable to those produced by incubating larvae in 40 mM KCl-elevated FSW (Fig. 4A,B). Competent larvae subjected to the same concentrations of GST protein (Fig. 4A) or bovine serum albumin (results not shown) metamorphose at rates similar to those for the spontaneous rates. Higher concentrations of Hemps-GST (100 μg/ml) completely inhibit metamorphosis and appear to be toxic to the larvae (results not shown). Newly hatched and pre-competent larvae do not respond to the concentrations of Hemps-GST that induce competent larvae to metamorphose (data not shown).

Expression of a subset of genes triggered by Hemps during metamorprohosis

A complete hierarchy of gene expression has been reported during *Drosophila* metamorphosis induced by the steroid hormone ecdysone (Karin and Thummel, 1992; Von Kalm et al., 1994; Lam et al., 1997). Exogenously added thyroid hormone-induced morphological and physiological changes during metamorphosis in *Xenopus laevis* tadpoles is also accompanied by the activation of a genetic program that involves the upregulation and downregulation of a set of specific genes (Brown et al., 1996; Stolow et al., 1996; Furlow et al., 1997). As a candidate for triggering metamorphosis in ascidians, it would be expected that only a subset of genes would respond to Hemps.

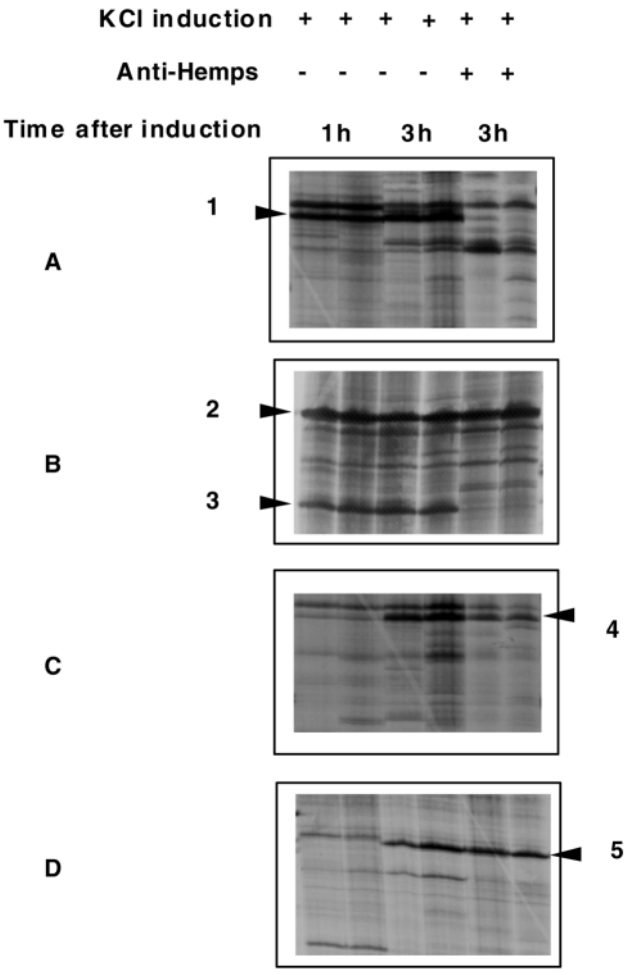

Fig. 5. Autoradiograms of differential display reverse transcriptase-PCR patterns for *H. curvata* induced to undergo metamorphosis in the presence and absence of anti-Hemps antibody. Antibody was added to hatched larvae and KCl added 3 hours later at competence to induce metamorphosis. Induction was for either 1 or 3 hours as indicated with antibody used only in the 3 hour time point. The primer sets for DDRT-PCR for A-D were: A. T_{12}MT and OPB: 06; B. T_{12}MT and OPB: 07; C. T_{12}MG and OPB:07 and D. T_{12}MG and OPB:07. Samples were loaded in duplicate. Labelled fragments were analysed on a 6% sequencing gel.

To address this we employed differential display-reverse transcriptase PCR (DDRT-PCR) which we have previously used to identify genes differentially expressed during ascidian development (Arnold et al., 1997a). *H. curvata* larvae were cultured for 3 hours before the acquisition of competence either in the presence or absence of anti-Hemps antibodies and then treated with 40 mM KCl-elevated sea water to induce metamorphosis. mRNA was isolated from both larvae cultured with and without the antibodies at 1 hour and 3 hours post-induction and subjected to DDRT-PCR, using combinations of anchored and arbitrary primers to generate the different banding patterns. Three distinct patterns of early metamorphic gene expression were detected by differential display (Fig. 5). Cultivation with anti-Hemps antibodies either: (1) decreased the abundance of transcripts that were normally present during early metamorphosis (Fig. 5A, band 1; 5B, band 3); (2) had no effect

on transcript abundance (Fig. 5B, band 2); or (3) down-regulated genes that normally were induced at 3 hours post-induction (Fig. 5C, band 4; Fig. 5D, band 5). Non-specific antibody failed to perturb gene expression induced during metamorphosis (results not shown).

DISCUSSION

In ascidians, the development of chordate-specific structures has become temporally dissociated, with the notochord and nerve cord developing during embryogenesis, and the pharynx and endostyle during metamorphosis. Environmental induction of settlement and metamorphosis in ascidians results in the programmed degradation of redundant larval structures, including the dorsal nerve cord, notochord and tail muscle, and the morphogenesis of adult primordia, including the pharynx and endostyle (reviewed by Cloney, 1982). In this study we have identified a factor, the Hemps signaling peptide, that has all the hallmarks of being an intrinsic regulator of the induction of metamorphosis. Although it has been clearly demonstrated that a wide range of marine invertebrate taxa possess chemosensory systems that allow the larva to effectively use specific exogenous chemical cues as morphogens that induce settlement behaviour and metamorphosis (e.g. Miller and Hadfield, 1986; Johnson and Sutton, 1994; Morse et al., 1994; Holm et al., 1998), the steps following this initial chemosensory stimulation have not been clearly dissected in any marine invertebrate larvae. For example, while two convergent chemosensory pathways have been identified in the gastropod mollusc *Haliotis rufescens* that increase the propensity of the larvae to settle in a potentially favourable environment (Baxter and Morse, 1987; Trapido-Rosenthal and Morse, 1986; Morse, 1993), it is unknown how these pathways affect downstream metamorphic gene expression (Degnan and Morse, 1995). In the cnidarian *Hydractinia echinata*, peptides with a GLWamide terminus have been implicated in the initiation of metamorphosis (Schmich et al., 1998).

Both in situ hybridisation (Arnold et al., 1997b) and immunocytochemical analyses localise Hemps transcripts and protein to the most anterior papillar region of the larva. Previously, we had shown that mechanical separation of *H. curvata* larval trunks and tails from the anterior papillae inhibits metamorphosis (Degnan et al., 1997). Fusion of these posterior larval fragments to papillae-containing anterior fragments allows normal metamorphosis to progress, strongly suggesting that initiation of metamorphosis is controlled by a diffusible factor which is released from an anterior signaling center located in the vicinity of the chemosensory papillae (Degnan et al., 1997).

Since the *Hemps* transcript encodes a protein with EGF-like repeats and a putative secretion signal sequence, we investigated whether this gene might encode the diffusible factor that is released from the anterior signaling center. The localised expression of Hemps and its gene product is in a pattern that would be predicted for a factor controlling metamorphosis. Firstly, the temporal pattern of expression of *Hemps* is consistent with that expected for a gene involved in initiation of metamorphosis. Changes in *Hemps* transcript abundance coincides closely with (i) the acquisition of competence (ii) the induction of metamorphosis and (iii) the transition from larval to juvenile body plan, which occurs over the first 30 hours of metamorphosis (Degnan et al., 1996; Hinman and Degnan, 1998). Secondly, anti-Hemps antibodies localize Hemps to the larval anterior epidermis and papillae at hatching and at competence. Prior to papillae retraction and tail resorption Hemps protein is detected in a defined group of cells associated within the anterior epidermis, centered between the three papillae which we have named papillary associated tissue (PAT). To our knowledge, a similar structure has not been described previously in any ascidian larvae. These cells form a discrete structure within the anterior epidermis during early tail resorption and are subsequently exuded into the tunic at the time of development of a concentration gradient of Hemps along the anterior-posterior axis of the tunic and epidermal cells. Differential localization of Hemps in PAT during the initial stages of settlement and metamorphosis suggests that exogenous morphogens induce these cells to first begin synthesising this factor prior to its appearance in other anterior epidermal cells. Detection of Hemps in the tunic shortly after induction of metamorphosis suggests that PAT is secreting this soluble factor. The diffusion of Hemps posteriorly over the first 24 hours of metamorphosis lends further support for a role of this factor in initiating metamorphosis. We did not detect Hemps protein in the vicinity of the resorbed tail, suggesting other signaling molecules may be required to induce the programmed degradation of larval tail tissues at metamorphosis. One potential candidate for this role is the HrPost-1 protein, which is localised to the tail region of the closely related ascidian *Halocynthia roretzi* and appears to be a secreted protein (Takahashi et al., 1997).

We completely abrogated Hemps ability to induce metamorphosis by adding anti-Hemps antibody into larval cultures prior to the development of competence, except the initial retraction of the papillae and the associated rounding of the anterior trunk. We envisage that the antibody can diffuse through the tunic and epidermis, and thus sequester secreted Hemps before it diffuses posteriorly in the ascidian, thereby neutralizing the biological activity of Hemps. Transient exposure of larvae to the anti-Hemps antibody for 20 minutes or less did not inhibit metamorphosis, suggesting that this amount of time was required for a critical amount of antibody to diffuse through the tunic. Our observations can be compared to other studies where protein activity can be antagonised in cells and explants bathed in the presence of neutralising antibodies. For example, antibodies against human EGF have been used to demonstrate a role for an EGF-like signaling pathway in gastrulation and spiculogenesis in *Lytechinus* sea urchin embryos (Ramachandran et al., 1995). In addition, Swalla and Jeffery (1996) demonstrated that ascidian embryos take up macromolecules from sea water by abrogating the function of the *Manx* gene in embryos cultured in the presence of antisense oligodeoxyribonucleotides. If the anti-Hemps antibody is sequestering secreted Hemps, this suggests that papillae retraction does not require Hemps while all subsequent metamorphic processes do. Given the close proximity of PAT to the papillae, retraction of these structures may play a role in the initial induction of Hemps in PAT. In addition, progression through the first 30 hours of metamorphosis appears to require the presence of this factor, since addition of the anti-Hemps antibody into postlarval

cultures at various times post-initiation inhibits further development (data not shown).

Further substantiation for a regulatory role of Hemps in *H. curvata* metamorphosis was provided by the observation that Hemps-GST recombinant fusion protein induced metamorphosis in competent larvae to similar levels as artificial and natural inducers, while GST and bovine serum albumin had no effect. Interestingly, pre-competent larvae could not be induced to settle and metamorphose with Hemps-GST. Pre-competent *H. curvata* larvae also do not metamorphose when subjected to 40 mM KCl-elevated FSW (Degnan et al., 1997) or live ectoproct (unpublished), but become habituated to these factors so that the onset of competence is retarded in most larvae. Together these data suggest that the acquisition of competence is dependent upon the development or activation of a receptor or signal transducer downstream of Hemps. This situation contrasts with that in molluscs where the availability of chemosensory receptors regulates larval habituation (Trapido-Rosenthal and Morse, 1986).

Thyroid hormone induces a series of morphological and physiological changes during metamorphosis in the amphibian tadpole including tail resorption. It is now evident that this hormone activates a genetic program to induce tail resorption which involves upregulation and downregulation of approximately 30 genes (Brown et al., 1996; Stolow et al., 1996; Furlow et al., 1997). These genes include transcription factors, extracellular matrix and membrane proteins and proteinases. In *Drosophila* replacement of the larval form by a reproductive adult during metamorphosis is dependent upon the hormone ecdysone (Gilbert and Frieden, 1981). Ecdysone induces the DHR3 orphan receptor gene at the onset of *Drosophila* metamorphosis which is sufficient to repress the early puff genes, *Broad-Complex* (BR-C) and E74, and transcriptionally activate beta FTZ-FI competence factor (Von Kalm et al., 1994; Lam et al., 1997; Karin and Thurnel, 1992). Our preliminary data using DDRT-PCR suggest that Hemps plays a similar role to the thyroid hormones in activating a genetic program for metamorphosis. We have shown that anti-Hemps antibody prevents the morphological changes associated with metamorphosis in *H. curvata* and in addition interferes with the expression of only a subset of transcripts expressed as part of this genetic program.

Based on the *Drosophila* and *Xenopus* models it is likely that a cascade of genes involved in metamorphosis will be induced in *H. curvata* by Hemps. Most planktonic marine invertebrate larvae, including ascidian larvae, are less than 1 mm in length, composed of a few hundred to a few thousand cells and intimately associated with surrounding sea water (Davidson et al., 1995). As such, intercellular signaling in these larvae is probably mediated by factors that signal to adjacent cells or can diffuse a short distance in the aqueous extracellular environment rather than the hydrophobic steroid hormones used in amphibians and insects. Immunocytochemical detection of Hemps in the acellular tunic suggests that it is soluble in sea water and regulates ascidian metamorphosis by acting as a paracrine, and possibly an autocrine factor.

While a number of EGF-like proteins are implicated at various stages of the development of other organisms (Davis, 1990, Carpenter, 1993), the Hemps protein described here is the first of this group to have any role in metamorphosis. It is also of interest that when sequence comparison of EGF-related domains is made Hemps can be aligned with cripto and cryptic (murine), nel (chick), FRL-1 (*Xenopus*) and zebrafish one-eyed pinhead (oep) all of which are involved in development (Groenen et al., 1994; Zhang et al., 1998; Shen et al., 1997; Matsuhashi et al., 1995; Kinoshita et al., 1995; Dono et al., 1993; Kinoshita et al., 1995). Since Hemps is the first gene shown to encode a signaling factor that is directly involved in the regulation of metamorphosis in any marine invertebrate, it will be of interest to determine the signaling pathway(s) it controls and whether gene homologs are functionally conserved in other microscopic marine larvae.

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