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Original Article

Isolation of cDNA clones for genes that are expressed in the tail region of the ascidian tailbud embryo

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ABSTRACT An ascidian tailbud embryo is comprised of the anterior trunk and posterior tail. We constructed cDNA libraries of the tail region and trunk region of the ascidian *Halocynthia roretzi*. The screening of the tail library by tail single-stranded cDNA minus the trunk library RNA as a probe, yielded cDNA clones for genes that are expressed in the tail epidermis, visceral ganglion, trunk lateral cells, muscle cells, and certain regions of the tail. Among them, a cDNA clone for a gene designated *HrPost-1* is described in detail. *HrPost-1* encodes a novel, possible secreted protein of 238 amino acids. The expression of the gene is zygotic. *HrPost-1* transcript was first evident in the posterior B-line blastomeres including muscle cells and endodermal strand cells of the gastrula-stage embryo, and the expression in these regions disappeared by the early tailbud stage. Around neurulation, the *HrPost-1* transcript appeared in epidermal cells of the posterior-most region of the embryo. As development proceeded, the gene expression spread anteriorly in the epidermal cells of the neurula and tailbud embryo, and thus at the early-to-mid tailbud stage, *HrPost-1* expression appeared to define the boundary between the trunk and tail epidermis. These results suggest that, in addition to tissue-specific genes, the activities of a set of region-specific genes are associated with tail formation in the ascidian embryo.

KEY WORDS: *Ascidians, tail-specific genes, HrPost-1, tail epidermis*

Introduction

Ascidians are a primitive group of chordates, and their fertilized eggs develop rather quickly into tadpole larvae with motile tails. The ascidian tadpole is composed of about 2,600 cells, which constitute a small number of, but definite types of tissues including the epidermis, central nervous system (CNS) with two sensory organs, endoderm and mesenchyme in the trunk region, and spinal (nerve) cord, notochord, muscle, and endodermal strand in the tail region (reviewed by Satoh, 1994). The lineage of these embryonic cells is well described (Conklin, 1905; Nishida, 1987), and by taking advantage of the lineage, the patterns of embryonic cell specification have been intensively studied (reviewed by Satoh, 1994). The ascidian embryo has thus been one of the most informative experimental systems for the investigation of the cellular and molecular mechanisms underlying embryonic cell specification.

To identify the molecular basis of ascidian embryogenesis, recent studies have attempted the isolation of cDNA clones for genes that are expressed in cells of specific type of tissues (reviewed by Satoh *et al.*, 1996). In addition, cDNA clones have been isolated and characterized for genes that encode transcription factors, which are involved in differentiation, lineage specification, axis formation and regionalization in the embryo (Satoh *et al.*, 1996). Other studies have elucidated novel maternal genes that are responsible for the pattern formation of the ascidian embryos, which include *Manx* (Swalla and Jeffery, 1996) and *pem* (*posterior end mark*; Yoshida *et al.*, 1996). Despite the accumulating information in this area, our knowledge of ascidian developmental genes remains insufficient.

To elucidate the gene activity during ascidian embryogenesis, in the present study, we attempted the isolation of cDNA clones for genes that are expressed in the tail region of the tailbud embryo. An ascidian egg is enclosed by a rather tough chorion or vitelline membrane, within which embryogenesis proceeds (Fig. 1A). During the chemical dechorionation of early-to-mid tailbud embryos of *Halocynthia roretzi* (Fig. 1B), we noticed that dechorionated tailbud embryos are frequently divided into two pieces of the trunk (Fig. 1C) and tail regions (Fig. 1D). Taking advantage of this phenomenon, we constructed cDNA libraries of the tail region and trunk region. We made a probe of tail single-stranded cDNA minus the trunk library RNA, and the tail cDNA library was screened with this probe. By determining the localization of corresponding mRNA with the *in situ* hybridization of whole-mount specimens, we isolated several cDNA clones for genes that are specifically expressed in the tail region of *H. roretzi*.

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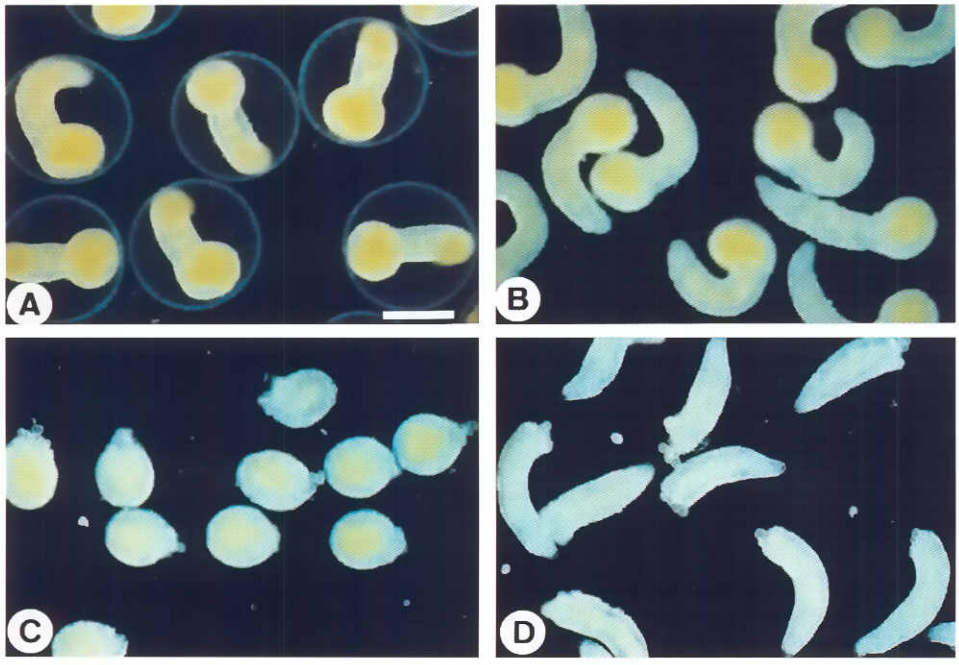

Fig. 1. Separation of the trunk and tail regions of tailbud embryos of the ascidian *Halocynthia roretzi*. **(A)** *Embryos at the mid-tailbud stage within the chorion and* **(B)** *those deprived of the chorion. A gentle pipetting of the naked embryos separated them into two pieces,* **(C)** *the trunk region and* **(D)** *tail region. Scale bar, 250 m.*

Results

Isolation of cDNA clones for genes that are expressed in the tail region of ascidian embryos

From the tail library, positive clones were selected and their nucleotide sequences were partially determined from the 3 end to obtain sequence information and to prevent the further analysis of the same clones. Each clone was then examined for the localization of corresponding mRNA in the tailbud embryos by *in situ* hybridization of whole-mount specimens. Three rounds of screening revealed that the library contains cDNA clones for genes that are expressed in a regional-specific and/or regional-enriched manner. Most of the genes isolated are listed in Table 1.

Three clones (1n20, 2n12 and 3n04) corresponded to genes that are primarily expressed in the epidermis of the tail region of the tailbud embryos (Fig. 2A, B). The expression of the 1n20 gene is described in detail below . The 2n12 gene was expressed in the tip of the tail (Fig. 2B). The 3n04 gene was expressed in certain epidermal cells along the midline of both the ventral and dorsal ectoderm of the tailbud embryo (data not shown). The pattern of the 3n04 expression resembles that of *HrBMPa*, an ascidian homolog of *BMP-5/8* (Miya *et al.*, 1996).

The clone 1n06 represented a gene which is primarily expressed in cells of the visceral ganglion (Fig. 2C, D). The 1n06 gene was first expressed in epidermal cells of the neurula. The 1n06 expression in the epidermal cells was evident at the early tailbud stage but became undetectable by the mid tailbud stage. Around the tailbud embryo formation, the 1n06 transcripts appeared in cells of the visceral ganglion (Fig. 2C), and at the mid-to-late tailbud stage, the gene expression was specific to the visceral ganglion (Fig. 2D). It has been shown that *Hrlim*, an ascidian LIM class homeobox gene, is expressed in the cells of the visceral ganglion (Wada *et al.*, 1995).

Although the library was constructed from mRNAs of the tail region, we were able to isolate two cDNA clones (1n19 and 2n13) for genes of which transcripts are specific to or enriched in the trunk lateral cells (TLCs) (Fig. 2E, F). TLCs are derivatives of A7.6 cells of the 64-cell *Halocynthia* embryo (Nishida, 1987) and are distinguishable by their position within the early tailbud embryo (Fig. 2E, F). Although a TLC-specific monoclonal antibody was established (Nishide *et al.*, 1989), this is the first isolation of cDNA clones for TLC-specific genes. Because TLC is a precursor of coelomic cells or blood cells of the adult, the further characterization of the cDNA clones might contribute to our understanding of the nature of TLCs.

The clone 2n05 represented a gene which is expressed in the tail muscle, spinal chord and dorsal brain (Fig. 2G, H). The expression pattern of 2n05 resembles that of the *Ciona intestinalis* homolog of *Drosophila snail* (Corbo *et al.*, 1997). The *Ciona sail* is also expressed in the dorsal brain region (Corbo *et al.*, 1997). The determination of complete nucleotide sequences may disclose whether 2n05 encodes *Halocynthia snail*.

In addition, we isolated cDNA clones for several muscle-specific genes (Table 1). These include genes for tropomyosin, myosin heavy chain, myosin light chain, two muscle actins, and creatine kinase B. In addition, cDNA clones for two unknown genes were obtained. The expression of one of the clones, 1n27, is shown in Fig. 2I, J.

Expression of the HrPost-1 gene

A gene represented by the 1n20 clone appeared to be expressed primarily in the posterior side of the tailbud embryo. We therefore named the corresponding gene *HrPost-1* (*Halocynthia*

roretzi Posterior gene 1), and the cDNA clone was further characterized.

Sequence analysis

The nucleotide and predicted amino acid sequences of the *HrPost-1* gene are shown in Fig. 3A. The insert of the clone consisted of 1,050 nucleotides including 21 adenylyl residues (Fig. 3A). The clone contained a single open reading frame (ORF) that predicted 237 amino acids. The calculated molecular mass (Mr) of the *HrPost-1*-encoded protein (HrPOST-1) was 23.5 kDa. A Northern blot showing a transcript of about 1.1 kb (Fig. 4A) suggests that the clone contains all the coding sequences and is close to full-length.

We found no similarities in the amino acid sequences between HrPOST-1 and reported proteins. The HrPOST-1 has no sequence motif shared by transcriptional factors, transmembrane domain, nuclear localization signals, or growth factor proteins. However, as shown in Fig. 3B, the mean hydropathy profile of HrPOST-1 showed that the N-terminus was highly hydrophobic. A predicted cleavage site of the signal peptide was evident behind the Ala (position 17). This sequence feature strongly suggests that HrPOST-1 is a secretory protein, with a probability of 78% according to the PSORT program (Netscape: PSORT WWW, Server http://psort.nibb.ac.jp). In addition, the sequence suggests three N-linked glycosylation sites (Fig. 3A).

TABLE 1.

ISOLATION OF CDNA CLONES FOR GENES THAT ARE EXPRESSED IN THE TAIL REGION OF *HALOCYNTHIA RORETZI* TAILBUD EMBRYOS

cDNA clones	Regions with expression	predicted polypeptides	Figure
1n20	epidermis + tip of the tail + muscle	no*	Fig. 2A
2n12	tip of the tail	ND**	Fig. 2B
3n04	epidermis	ND	
1n06	visceral ganglion + epidermis	ND	Fig. 2C, D
1n19	trunk lateral cells	ND	
2n13	trunk lateral cells	ND	Fig. 2E, F
2n05	muscle + CNS	ND	Fig. 2G, H
1n18	notochord + CNS	SH2 domain	
1n14	muscle	Tropomyosin	
1n56	muscle	myosin HC	
1n48	muscle	myosin LC	
1n38	muscle	creatin kinase B	
1n27	muscle	ND	Fig. 2I, J
1n08	muscle	ND	

* no, no similarity; ** ND, not determined.

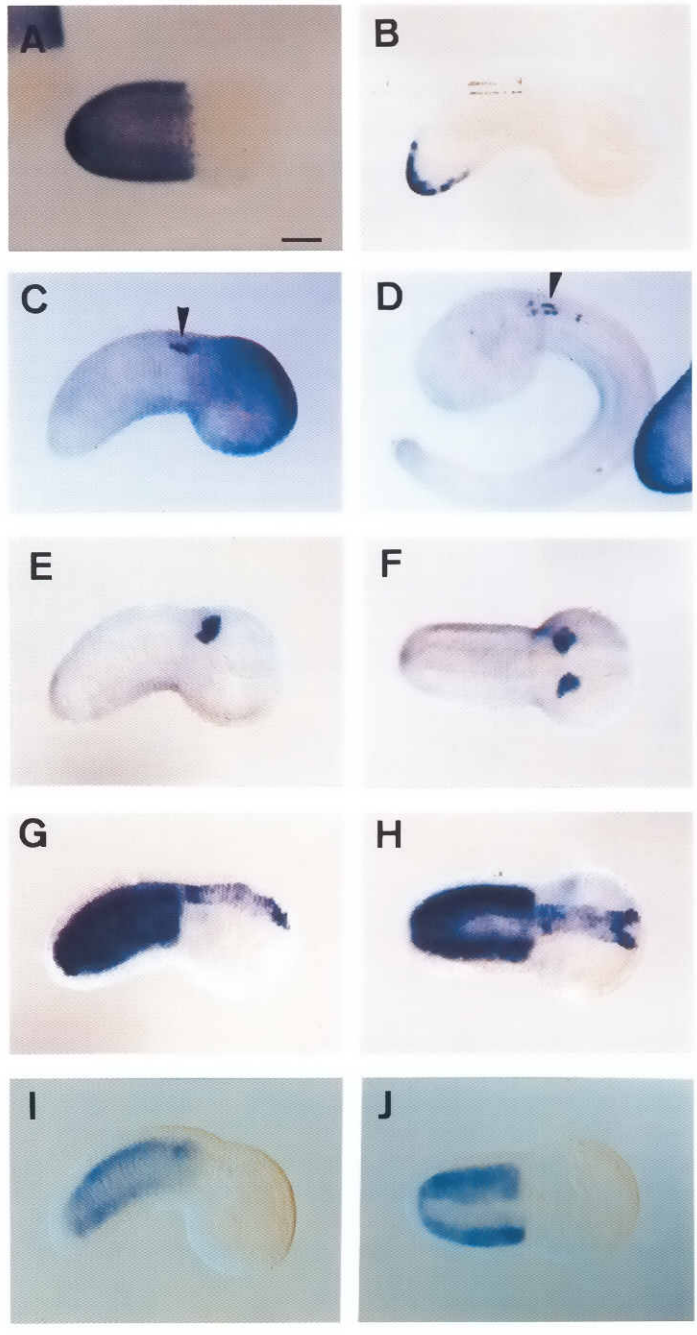

Fig. 2. Isolation of cDNA clones for genes that are expressed in the tail region of *H. roretzi* tailbud embryos as revealed by whole mount *in situ* hybridization with a digoxigenin-labeled antisense probe. (A) *The clone 1n20 for HrPost-1 that is expressed in the tail epidermis. Scale bar, 50 m for all panels.* **(B)** *The clone 2n12 for a gene that is expressed in the epidermis of the tip of the tail.* **(C, D)** *The clone 1n06 for a gene that is expressed in cells of the visceral ganglion (arrow) at the early* **(C)** *and mid-to-late tailbud embryo* **(D). (E, F)** *The clone 2n13 for a gene that is expressed in the trunk lateral cells. An early tailbud embryo; lateral view* **(E)** *and dorsal view* **(F). (G, H)** *The clone 2n05 for a gene that is expressed in the CNS and the tail muscle. An early tailbud embryo; lateral view* **(G)** *and dorsal view* **(H). (I, J)** *The clone 1n27 for a gene that is expressed in the muscle. An early tailbud embryo; lateral view* **(I)** *and dorsal view* **(J).**

A

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   1  CAGAGAAGGATAATACCAATCTTTAAGGTTTTTTTAAGAAAATCCAGAGATCAAAATGAA  60
                                                          M  K   2
  61  GATTGCAGTCATCATCGCCTTATCATTCTGTGGAATCGTGTTCGCCCAGGATACCAACGA 120
       I  A  V  I  I  A  L  S  F  C  G  I  V  F  A▲Q  D  T  N  D  22
 121  TAATATCCGCTCTGTCATAACCTGGCCACAACACGAATACAGCTTAGGAGGGGCTTATGT 180
       N  I  R  S  V  I  T  W  P  Q  H  E  Y  S  L  G  G  A  Y  V  42
 181  TCTAATGGATTATGAAAATTTGGGATCAGGAAGTTGTATCGTTAGTTTACCAAAAATGGT 240
       L  M  D  Y  E  N  L  G  S  G  S  C  I  V  S  L  P  K  M  V  62
 241  GAACTTATTCCATGCCACGAACGGACACATCGTACCCAGCCTTCAAAGCAATCCCTTGGA 300
       N  L  F  H  A  T  N  G  H  I  V  P  S  L  Q  S  N  P  L  E  82
 301  AGGCCAATACAAGATTGCAGCTCCAGCTAGCTGGGAATTAATAAATGGATCTTTCGCTGT 360
       G  Q  Y  K  I  A  A  P  A  S  W  E  L  I  Ⓝ  G  S  F  A  V  102
 361  ATTGATAGTTTTCTCCAAAAACAACATATTCAGCATTTCCGAAATCACAATGATGTGCGA 420
       L  I  V  F  S  K  N  N  I  F  S  I  S  E  I  T  M  M  C  D  122
 421  CCAAACTGATGTTGCAAACATCTCTGTCTACTCCTTCCCCCAAAACAATTTGATCCAACA 480
       Q  T  D  V  A  Ⓝ  I  S  V  Y  S  F  P  Q  N  N  L  I  Q  Q  142
 481  AGCAAGTTCTAACATCTTCTACAGACCAGGATTCCACATGGGAGATGTCCTGACCATCAC 540
       A  S  S  N  I  F  Y  R  P  G  F  H  M  G  D  V  L  T  I  T  162
 541  TCTTCCTAATTCAGTTGCCAGATTTAACTTTACAACTGGTGGCCTTGTTGTTGCGTCGTC 600
       L  P  N  S  V  A  R  F  Ⓝ  F  T  T  G  G  L  V  V  A  S  S  182
 601  GGATGATATGACTGTTCACACAGCAAGCGGCTTCACAAATGAGCCTGGCATCAATGATAT 660
       D  D  M  T  V  H  T  A  S  G  F  T  N  E  P  G  I  N  D  M  202
 661  GAAGGAGGTTGCATTCGAATTGGTATTCAACAATGAAGATGAAACTAACTCTGAATGGTT 720
       K  E  V  A  F  E  L  V  F  N  N  E  D  E  T  N  S  E  W  F  222
 721  CTCCGCCGCAGAAATCACTGTGAGCATTCAAAATATTCAAAACAATATTTAAATAATTTT 780
       S  A  A  E  I  T  V  S  I  Q  N  I  Q  N  N  I  *           238
 781  TCGAGTTGTGAAGCTGTTTCAGGATATGAGATTTTATTTTTCTACAATGTTTTCGTAAAT 840
 841  TAATTTTATAAACGTGCATTCTTATATCATTGCTTACTGGTTACTGTACTGCAATACCGT 900
 901  GATATTTTCTTGCTGTTTACAATATAGCTAACACTTTTTCTCGGATACACATCAATGCAT 960
 961  GTATAGTTACAACCGGGTGTCGCATATTATCAAATCAAAATATTCAAATAAATGATTATT 1020
1021  ATGTAATGTAAAAAAAAAAAAAAAAAAAAA                              1050
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B

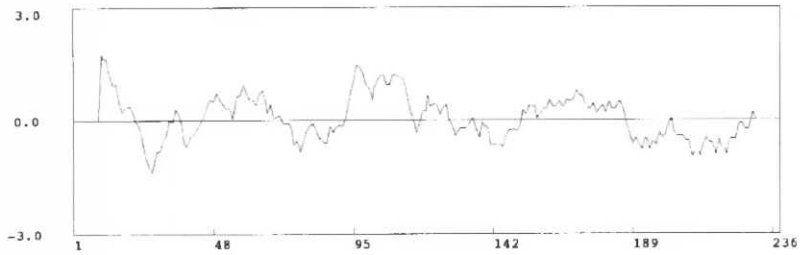

Fig. 3. The nucleotide and predicted amino acid sequences of a cDNA clone for *HrPost-1*. **(A)** *The cDNA clone consists of 1,050 bp, with a single ORF that encodes a polypeptide of 238 amino acids. The asterisk indicates the termination codon. The potential signal sequence for polyadenylation is underlined. The arrowhead indicates a predicted cleavage site. Putative N-linked glycosylation sites are shown by circles. The accession number for the sequence of HrPost-1 is AB005753 in the DDBJ, EMBL and GenBank nucleotide sequence databases.* **(B)** *Mean hydropathy index of the HrPost-1 gene product calculated across a window of 19 residues according to the method of Kyte and Doolittle (1982).*

Genomic Southern blot analysis

We determined the number of different sequences that correspond to *HrPost-1* in the ascidian genome by genomic Southern hybridization. As shown in Fig. 4B, only one major band was detected in the lanes of *Bam*HI (about 8.0 kb), *Eco*RI (about 16 kb), and *Pst*I (about 28 kb). Although we did not detect distinct bands in the lane of *Hin*dIII digestion, these results suggest that *HrPost-1* is present as a single copy per haploid genome of *H. roretzi*.

Northern blot analysis

The temporal expression of *HrPost-1* was examined by Northern blot analysis. As is evident in Fig. 4A, no hybridization signal was detected in unfertilized eggs and early embryos up to the 64-cell stage. The transcript was first detected at the gastrula stage, although the band was faint. The amount of the mRNAs increased markedly at the neurula and tailbud stages. The band intensity was retained by larvae. This result suggests that the expression of *HrPost-1* is zygotic.

We also examined the expression of *HrPost-1* in the tissues and organs of the adult *H. roretzi*. Northern blot analysis failed to detect any signals in the adult organs including the endostyle, pharyngeal gill, body-wall muscle, digestive gland, intestine, and gonads (data not shown). This suggests that *HrPost-1* is expressed exclusively during embryogenesis.

Spatial expression of HrPost-1 examined by whole-mount in situ hybridization

In many cases of zygotic expression of ascidian genes, the detection of mRNA by *in situ* hybridization of whole-mount specimens is more sensitive than that by Northern hybridization. This is because *in situ* hybridization can detect signals first in the nucleus of certain cells which frequently develop in a lineage-specific and/or region-specific manner (Yasuo and Satoh, 1993; Satou *et al.*, 1995). This was the case for the *HrPost-1* signal.

The *in situ* hybridization demonstrated that the first distinct signal was detected at the early-to-mid gastrula stage (Fig. 5A). At this stage, the hybridization signal was evident in nuclei of seven pairs of the B-line (derivatives of B4.1 blastomeres of the 8-cell embryo) primordial muscle cells and a pair of B-line endodermal strand cells (Fig. 5A). During gastrulation and neurulation, these *HrPost-1*-expressing cells moved inside the embryo. The signal in the muscle and endoderm strand cells was retained by the neurula stage (Fig. 5C, D), but became undetectable at the tailbud stage (Fig. 5E, F).

At the neurula stage, the signal appeared in epidermal cells of the posterior-most region of the embryo (Fig. 5C, D). This signal was retained by the cells of the tip of the tail (Fig. 5C-F), and the larva showed the signal in the posterior-most tail region (data not shown). Soon after the occurrence of the signal in this region, the

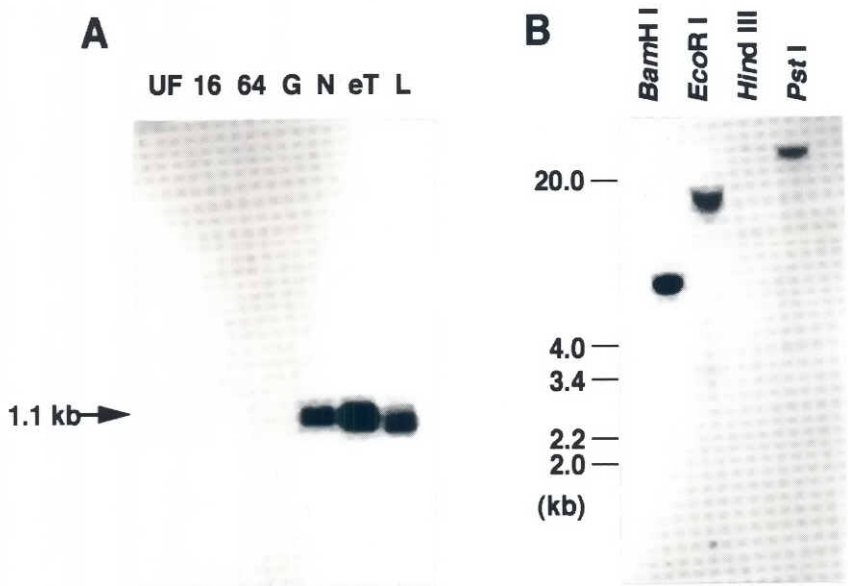

Fig. 4. Northern and Southern blot analyses of *HrPost-1*. **(A)** *Occurrence of HrPost-1 transcript during embryogenesis of H. roretzi. Northern blots of poly(A)⁺ RNA prepared from unfertilized eggs (UF), 16-cell stage embryos (16), 64-cell stage embryos (64), gastrulae (G), neurulae (N), early tailbud embryos (eT) and swimming larvae (L) were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the membrane was washed under high-stringency conditions. The HrPost-1 transcript was first detected in gastrulae, and the transcripts accumulated as development proceeded. Each lane was loaded with 1 g of poly(A)⁺ RNA.* **(B)** *Genomic Southern blot analysis of the HrPost-1 gene. Genomic DNA isolated from a single adult and aliquots were digested separately with BamHI, EcoRI, HindIII and PstI. The blots were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the filter was washed under high-stringency conditions. Ten g of digested genomic DNA were loaded per lane. The numbers indicate sizes (in kb) of signals.*

signal also became evident in the epidermal cells of the posterior region of the embryo (Fig. 5D). The *HrPost-1* expression was first detected in the posterior-most region and then appeared to spread anteriorly. As shown in Fig. 5E, the *HrPost-1* expression at the early tailbud stage was restricted to the posterior region or the tail region of the embryo, so that the gene expression defined the boundary between the epidermis of the trunk region and that of the tail region. This did not always correspond to the boundary between the a-line [derivatives of a4.2 (the anterior animal) blastomeres of the 8-cell embryo] and b-line [derivatives of b4.2 (the posterior animal) blastomeres of the 8-cell embryo] epidermis. As development proceeded, the signal became evident in some epidermal cells of the trunk region (Fig. 5F). However, in the larva, signals were found only in the posterior region of the tail (data not shown).

To explore the putative function of *HrPost-1*, we prepared synthetic capped *HrPost-1* RNA. This synthetic RNA was microinjected into fertilized eggs and injected eggs were allowed to develop into tadpole larvae to determine effects of overexpression of HrPOST-1 protein. When, as a control, synthetic RNA of *As-T* (the ascidian homologue of mouse *Brachyury*) was injected into fertilized eggs, the induction of notochord cell differentiation in the endoderm region was evident in the resultant embryos. However, injection of *HrPost-1* RNA did not cause any detectable morphological changes in the resultant embryos. Therefore, we could not determine the possible function of *HrPost-1* by this method.

Discussion

Isolation of cDNA clones for genes that are expressed in the tail region of ascidian embryos

In the present study, we isolated cDNA clones for genes that are expressed in the tail region of the *H. roretzi* tailbud embryo. Some of the genes are expressed in a cell-type-specific manner, such as the TLC-specific 2n13 and 1n19 and the muscle-specific 1n27 and 1n08, while several other genes including 1n20 and 1n18 are expressed in a region-specific (or restricted) manner and not in a cell-type-specific manner. Recent clonings of ascidian homologs of *Drosophila* or vertebrate developmental genes demonstrated that ascidian homeobox genes or genes for transcriptional factors are expressed in the region-specific manner. For example, *Hrlim* is expressed primarily in the region of endoderm, notochord and spinal chord of the blastula, and later in the visceral ganglion (Wada *et al.*, 1995). An ascidian *fork head/HNF-3b* gene is expressed in the endoderm, notochord, and the ventral row of spinal cord cells (Corbo *et al.*, 1997; Shimauchi *et al.*, 1997). Such genes with region-specific expression should be studied further to clarify the molecular mechanisms underlying ascidian development.

The 2n12 gene is expressed in the epidermis of the tip of the tail (Fig. 2B). At the structural level, the tip of the ascidian embryonic tail is not always homologous to the tailbud of vertebrate embryos (e.g., De Robertis *et al.*, 1994), because the ascidian tailbud consists of a layer of epidermis while the vertebrate tailbud constitutes a kind of organizer that eventually forms components of all of the three germ layers. However, *As-T2*, the second T-box gene of *H. roretzi*, is expressed in tip of the tail (Yasuo *et al.*, 1996), similarly to the expression of the vertebrate *Brachyury* (*T*) in the tailbud (e.g., Smith *et al.*, 1991). This suggests a homology between these two structures at the level of expression of genes with a similar function. In this respect, the 2n12 gene is interesting, and the further characterization of 2n12 might clarify the nature of the tip of the tail of the ascidian tailbud embryos.

In the present study, three rounds of screening resulted in the isolation of twenty cDNA clones for genes that are expressed in the tail region. Further screening might demonstrate cDNA clones for genes with interesting expression patterns. In addition, screening of the trunk cDNA library might enable the isolation of cDNA clones for genes that are expressed in the trunk region.

Expression of the HrPost-1 gene

During embryogenesis of the ascidian *Halocynthia roretzi*, exactly eight-hundred epidermal cells are formed in the larva, and the lineage of the cells has been almost completely described (Nishida,

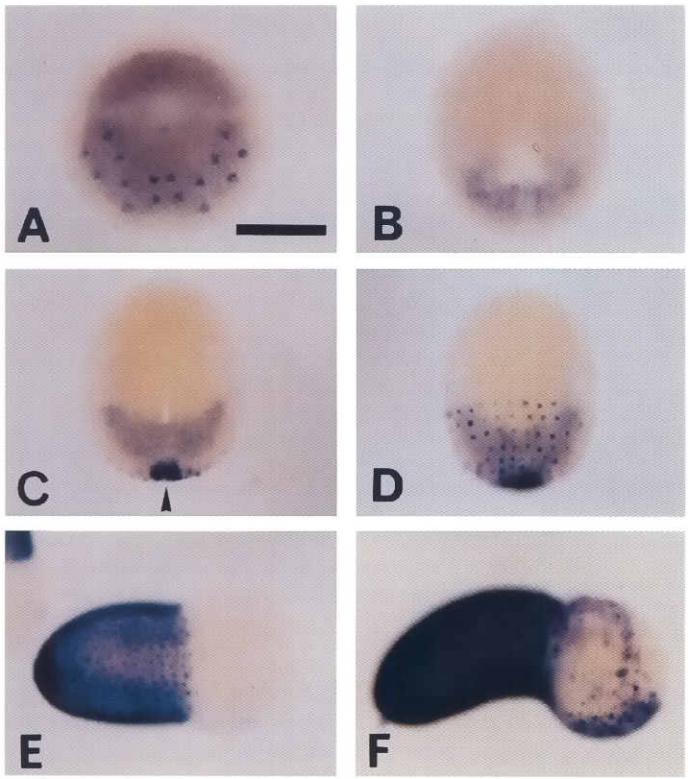

Fig. 5. Spatial distribution of *HrPost-1* transcripts, as revealed by whole-mount *in situ* hybridization. **(A)** *A mid-gastrula viewed from the vegetal pole (future dorsal side of the embryo). Hybridization signals are evident in the nuclei of seven pairs of the primordial B-line muscle cells and a pair of the primordial B-line endoderm and endodermal strand cells. bp, blastopore. Scale bar represents 100 m for all panels. Anterior is up for panels A-C and right for panels D-F.* **(B)** *A neural-plate stage embryo viewed from the vegetal pole. Signals are found in the primordial muscle and endodermal strand cells which have migrated inside the embryo.* **(C)** *A neurula, dorsal side view. In addition to the muscle and endodermal strand cells, the signal became evident in epidermal cells of the posterior-most region of the embryo (arrowhead).* **(D)** *The same neurula, ventral side view focusing on the surface of the embryo. Epidermal cells of the posterior region of the embryo show the hybridization signal.* **(E)** *An early tailbud embryo showing the signal in epidermal cells of the posterior region of the embryo. The expression pattern appears to define the boundary between the trunk and tail region. The transcript disappears from muscle and endoderm strand cells.* **(F)** *A mid-tailbud stage embryo showing the signal in the epidermal cells of the tail region of the embryo, although at this stage some epidermal cells of the trunk region also show the signal.*

1987). The epidermis of the trunk region develops into the epidermis of the adult, whereas that of the tail region is degenerated during metamorphosis, suggesting morphological and/or functional differences in the epidermis between the two regions (e.g., Satoh, 1994).

In previous studies (Ueki *et al.*, 1991, 1994; Ishida *et al.*, 1996), we isolated cDNA clones for eight different epidermis-specific genes and examined the pattern of spatial expression of these genes. Based on their spatial expression patterns at the early-tailbud stage, the eight genes can be divided into six groups (cf., Fig. 6 of Ishida *et al.*, 1996). Interestingly, most of the epidermis-specific genes are not expressed in the cells of the dorsal midline or the anterior-most region of the early-tailbud embryo. This may reflect the fact that these regions without the epidermis-specific gene expression are associated with the formation of the nervous system. Miya *et al.* (1996) showed that the *HrBMPa* gene is expressed in the adhesive organ, midline of the anterior dorsal neuroectoderm and midline of both the ventral and dorsal ectoderm, as it compensates for the expression of epidermis-specific genes.

However, none of the epidermis-specific genes show an expression pattern like that of *HrPost-1*. In addition, it is evident that the boundary of the trunk and tail epidermis defined by the *HrPost-1* expression (Fig. 5E) is not always identical to the boundary between the a4.2-line and b4.2-line epidermis. That is, the anterior half of b8.20-derived epidermal cells did not express the *HrPost-1* gene. Therefore, the *HrPost-1* gene expression in the tail epidermis is not dependent on their lineage-associated determination mechanisms.

The pattern of *HrPost-1* gene expression overlaps somewhat with that of the *As-T2* gene (Yasuo *et al.*, 1996). The *As-T2* gene is first expressed in the blastomeres of endodermal and muscle lineages in embryos at the 64-cell stage. Then, at the neural plate stage, the *As-T2* gene expression begins in cells that give rise to the tip of the tail. In the early-to-mid tailbud embryo, *As-T2* is expressed in the tip of the tail and muscle cells. It is therefore possible that one of the *As-T2* targets is *HrPost-1*. This should be determined in future studies. However, because *HrPost-1* is expressed in the tail epidermis, *HrPost-1* gene expression must be regulated by other upstream genes as well.

In conclusion, the present isolation of cDNA clones for genes that are expressed in the tail region suggests that a complex set of region-specific genes are essential for the formation of the ascidian embryonic tail. The detailed mechanisms of the control of this gene expression will be the focus of future studies.

Materials and Methods

Biological materials

Naturally spawned eggs of *Halocynthia roretzi* were fertilized with a suspension of non-self sperm, and fertilized eggs were cultured in filtered seawater at about 12°C. Embryogenesis proceeded with a high degree of synchrony in different batches of eggs. At this temperature, the first cleavage took place about 2 h after insemination, and first several divisions occurred at approximately hourly intervals. They developed into gastrulae, neurulae, and early-tailbud stage embryos about 12, 17, and 24 h after fertilization, respectively. Tadpole larvae hatched about 40 h after fertilization.

Embryos at appropriate stages were collected by low speed centrifugation, and were frozen with chilled ethanol for Northern blot analysis or fixed for *in situ* hybridization as whole-mount specimens. Tissues and organs of adult specimens were also quickly frozen with chilled ethanol for Northern blot analysis.

Isolation of the trunk and tail regions of tailbud embryos

Embryos at the early to mid-tailbud stages (Fig. 1A) were immersed in seawater that contained 1% sodium thioglycolate (Wako Pure Chem. Ind., Ltd., Osaka, Japan) and 0.05% actinase E (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan). After 30-40 min, the chorion was disintegrated so that embryos became naked (Fig. 1B). After washing several times with Millipore-filtered (pore size, 0.2 μm) seawater, gentle pipetting of the embryos led to break of them into two pieces, the trunk (Fig. 1C) and tail regions (Fig. 1D). The trunk or tail pieces were separately collected and frozen quickly for RNA isolation. When these embryonic pieces were cultured in agar-coated plastic dishes, they showed tissue differentiation.

Construction of cDNA libraries and isolation of cDNA clones for tail-specific genes

Total RNA was extracted from frozen samples of the trunk or tail region by the acid guanidinium-thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNAs were purified by use of Oligotex dT30 beads (Roche Japan, Tokyo, Japan). Complementary DNA was synthesized from the poly(A)$^+$ RNA with a ZAP cDNA kit (Stratagene, La Jolla, CA, USA).

Double-stranded cDNA was size-fractionated on a column of Sephacryl S-500 (Pharmacia Biotech., Sweden), and fractions that contained fragments of more than 300bp in length were collected. Double-stranded cDNA prepared from the trunk region mRNA or from tail region mRNA was cloned directly into the *Eco*RI-*Xho*I site of a Uni-ZAP-II vector (Stratagene).

Subtracted hybridization and library screening were performed essentially according to the method described by Swalla *et al.* (1993). PCR amplification of cDNA libraries were performed in 50 μl volumes using Taq polymerase [Bethesda Research Laboratories (BRL), Bethesda, MD, USA] and a Perkin-Elmer Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA). The trunk or tail cDNA library was boiled in water and added PCR reaction included 50 pmol of T3 primer and T7 primer for the tail library or SK primer and T7 primer for the trunk library. Amplification used 20 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C. After the final cycle, the sample was incubated at 72°C for 15 min, then chilled to 4°C. To synthesize RNA from the amplified libraries, template DNA from each library was amplified as described above, treated with 0.1 mg/ml proteinase K (Sigma), 1% SDS for 30 min at 37°C then extracted with phenol and precipitated with ethanol, and 1 μg DNA was dissolved in 50 μl of RNA synthesis mixture according to Van Gelder *et al.* (1990). 100 units of T7 RNA polymerase (BRL) was used to synthesize antisense RNA from the trunk and tail cDNA libraries. After incubation for 2 h at 37°C, 1 unit of RQ1 DNase (Promega, Madison, WI, USA) was added to digest the DNA template. The tail library RNA was converted into cDNA using SK primer, and RNA was subsequently hydolyzed in 1 N NaOH, 0.5% SDS. The trunk library RNA was labeled with photoactivatible biotin (Clontech Laboratories, Inc., Palo Alto, CA, USA). Subtractive hybridization, in which 2.6 μg of the tail single-stranded cDNA and 22 μg of the trunk library RNA were mixed, was performed according to the procedure of Sive and St. John (1988), three rounds of hybridization were done. After hybridization, 5 μg of streptavidin was added and the mixture was phenol extracted twice to remove the biotin-labeled transcripts. The subtracted single-stranded cDNA (0.2 μg) was then labeled with [^{32}P]-dCTP by random primer labeling (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) and used as a probe to screen the tail cDNA library.

The clones that screened positive with subtracted tail cDNA probes were partially sequenced from poly(A) tail in order not to analyze the same clones in further. After partial sequencing, each clone was examined localization of corresponding mRNA by whole-mount *in situ* hybridization using digoxigenin-labeled antisense RNA probes. Tailbud embryos were used as specimens for *in situ* hybridization screening. cDNA clones exhibiting restricted appearance of corresponding mRNAs were selected for further analyses.

Nucleotide sequences were determined for both strands with dye primer cycle sequencing FS ready reaction kit and ABI PRISM 377 DNA sequencer (Perkin Elmer).

In situ hybridization

Digoxigenin (DIG)-labeled sense and antisense probes were synthesized following the instructions from the suppliers of the kit (DIG RNA Labeling kit; Boehringer Mannheim). Their final sizes were reduced to approximately 150 nucleotides by alkaline hydrolysis.

Whole-mount specimens were hybridized *in situ* using the probes essentially according to the method described by Ishida *et al.* (1996). Briefly, specimens were fixed in 4% paraformaldehyde in 0.1M MOPS buffer (pH 7.5), 0.5 M NaCl. After being thoroughly washed with PBT (phosphate-buffered saline containing 0.1% Tween 20), the specimens were treated with 2 g/ml proteinase K (Sigma) in PBT for 30 min at 37°C, then post-fixed with 4% paraformaldehyde in PBT for 1 h at room temperature. After a 1-h period of prehybridization at 42°C, the specimens were allowed to hybridize with the DIG-labeled probes at a concentration of 1 μg/ml for at least 16 h at 42°C. After hybridization, the hybridization solution was gradually replaced by PBT, then the specimens were digested with 20 μg/ml RNase A (Sigma). Thereafter, the samples were incubated for 1 h with 500 μl anti-DIG-alkaline phosphate conjugate, and color was developed according to the Boehringer protocol.

After dehydration, some of the specimens were cleared by placing them in a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate.

Northern hybridization

Northern blot hybridization was carried out by the standard procedure (Sambrook *et al.*, 1989). DNA probes for blot hybridizations were labeled with [^{32}P]-dCTP using a random primed labeling kit (Boehringer Mannheim). Filters were washed at high stringency conditions.

Genomic Southern hybridization

High-molecular weight genomic DNA was extracted from a single adult by the standard procedure (Sambrook *et al.*, 1989). After exhaustive digestion with *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I, we electrophoresed on a 0.7% agarose gel, then the DNA fragments were blotted onto Hybond-N+ nylon membrane (Amersham). The blots were hybridized with random-primed [^{32}P]-labeled DNA probes at 42°C for 16 h and washed under high-stringency conditions.

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