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Isolation and Characterization of cDNA Clones for Epidermis-Specific and Muscle-Specific Genes in *Ciona savignyi* Embryos

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ABSTRACT—Ascidian eggs and embryos have provided an appropriate experimental system to explore the cellular and molecular mechanisms involved in the embryonic cell specification and pattern formation of the embryo. In Japan, most of the studies of ascidian embryology have been carried out with the large eggs of *Halocynthia roretzi*. However, for future studies, *Ciona* species may provide a better experimental system, in particular with respect to the incorporation of genetic approaches. In order to establish *Ciona* as an experimental system, molecular markers with which to examine cellular differentiation are required. In the present study, we isolated and characterized cDNA clones for two epidermis-specific genes (*CsEpi-1* and *CsEpi-2*) and for two muscle-specific genes (*CsMA-1* and *CsMu-1*). *CsEpi-1* encodes a polypeptide with three trefoil domains, while *CsMA-1* encodes a muscle-type actin from *C. savignyi*. Although *CsEpi-2* and *CsMu-1* transcripts seem to have a poly(A) tail at the 3' end, we could not find a distinct open reading frame in the sequences. Probes for *CsEpi-1*, *CsMA-1* and *CsMu-1* cross-reacted with *C. intestinalis* embryos. These cDNAs are useful as molecular markers for the specification of epidermis and muscle of *Ciona* embryos.

INTRODUCTION

Ascidian eggs and embryos have provided an appropriate experimental system to explore the molecular nature of localized maternal factors and their roles in cell specification and pattern formation (for reviews see Satoh, 1994; Satoh *et al*., 1996). The fertilized egg develops quickly into a tadpole larva, which consists of a small number of tissues including the epidermis, central nervous system with two sensory organs, nerve cord, endoderm, mesenchyme, notochord and muscle. The lineage of these embryonic cells is completely described up to the gastrula stage (Conklin, 1905; Nishida, 1987).

Recent molecular embryological studies have isolated and characterized cDNA clones for genes that are expressed in a tissue-specific manner, genes encoding transcriptional factors, and genes encoding signal molecules (reviewed by Chiba and Nishikata, 1998). In addition, recent studies have succeeded in the characterization of maternal genes with localized mRNAs, including *posterior end mark* (*pem*; Yoshida *et al*., 1996), *pem-2*, *pem-4*, *pem-5*, and *pem-6* (Satou and Satoh, 1997), and *HrWnt-5* (Sasakura *et al*., 1998). One of the difficulties in ascidian molecular embryology is the need for techniques that deduce the function of these developmentally important genes. In some of the genes, the overexpression of the proteins produced by a microinjection of synthetic mRNA (Yoshida *et al*., 1996; Yasuo and Satoh, 1998) and the inhibition of the mRNA function by treatment with antisense oligonucleotides (Swalla and Jeffery, 1996; Olsen and Jeffery, 1997) resulted in distinct effects, providing cues to infer the gene functions. However, these techniques are not always successful.

We have speculated that genetic approaches such as those used for *Drosophila, C. elegans* and zebrafish could be applied to ascidians to identify genes with developmentally important functions. In Japan, most of the studies of ascidian embryology have been carried out with the large and transparent eggs of *Halocynthia roretzi*. However, *H. roretzi* may not be an appropriate system for future studies with genetic approaches. The spawning season is limited to winter, and the generation time may be more than two years. We propose *Ciona* eggs and embryos as an experimental system for further studies, because their spawning season is basically all year-round, and their generation time appears to be about 3 months (Kano and Amemiya, personal communication).

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Ciona savignyi or *C. intestinalis* could thus be useful as an experimental system for future studies, including those using genetic approaches.

In the present study, we therefore attempted to isolate cDNA clones for genes that are useful as molecular markers for the specification of embryonic cells. With such an aim, we used the subtractive hybridization of mRNAs of tailbud embryos with those of fertilized eggs. Taking advantage of the well-known lineage and segregation pattern of developmental fates as well as the *in situ* hybridization of whole-mount specimens, we were able to isolate cDNA clones for two epidermis-specific and two muscle-specific genes.

MATERIALS AND METHODS

Ascidian eggs and embryos

Ciona savignyi and *C. intestinalis* adults were collected near the Otsuchi Marine Research Center, Ocean Research Institute of the University of Tokyo, Iwate, Japan, and maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at about 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate.

RNA isolation and cDNA library construction

Total RNA was isolated from fertilized eggs or tailbud embryos by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified using Oligotex beads (Roche Japan, Tokyo). cDNA libraries of fertilized eggs (FE-library) and tailbud embryos (TB-library) were constructed in Uni-ZAP XR using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA, USA). A tailbud-mRNA concentrated subtractive library was constructed from TB- and FE-libraries as described by Satou and Satoh (1997).

Screening of the subtracted cDNA library and sequencing

From the library, clones were randomly picked up and partially sequenced from poly(A) tail to avoid analyzing the same clones any further. After partial sequencing, each clone was examined for the localization of corresponding mRNA by whole-mount *in situ* hybridization using digoxigenin-labeled antisense RNA probes. Gastrulae and tailbud embryos were used as specimens for the *in situ* hybridization screening. cDNA clones exhibiting the localization of corresponding mRNAs were selected for further analyses.

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

Northern analysis

Poly(A)$^+$ RNA was isolated as described above and fractionated by agarose gel electrophoresis, and transferred to a Hybond-N(+) membrane (Amersham, Buckinghamshire, UK). Blots were hybridized with ^{32}P-random-labeled DNA probes in 6 X SSPE, 0.5% SDS, 5 X Denhardt's solution, 100 μg/ml salmon sperm DNA, and 50% formamide. The filter was washed twice in 2 X SSC/0.1% SDS, and twice in 0.2 X SSC/0.1% SDS at 65°C, and exposed to X-ray film.

Whole-mount *in situ* hybridization

RNA probes were prepared with a DIG RNA labeling kit (Boehringer Mannheim, Heidelberg, Germany). Whole-mount *in situ* hybridization was performed as described previously (Satou *et al.*, 1995). The control specimens hybridized with sense probes did not show signals above the background.

RESULTS AND DISCUSSION

Isolation of cDNA clones for tissue-specific genes in *C. savignyi* embryos

In order to obtain cDNA clones for genes that are expressed in a tissue-specific manner, we constructed a cDNA library of tailbud-embryo mRNAs subtracted with the fertilized-egg mRNAs of *C. savignyi*. The library was estimated to contain about 90,000 clones. From the library, clones were randomly selected and their nucleotide sequences were determined from the 3' end to prevent the further analysis of the same clones. Each clone was then examined for the localization of corresponding mRNA by whole-mount *in situ* hybridization. Gastrulae and tailbud embryos were subjected to *in situ* hybridization to determine the specific expression of the genes. We have examined 100 clones to date and were able to find cDNA clones for two epidermis-specific genes (*CsEpi-1* and *CsEpi-2*) and two muscle-specific genes (*CsMA-1* and *CsMu-1*) of *C. savignyi* embryos, which are described below.

Expression of the *CsEpi-1* gene

Sequence analysis. The nucleotide and predicted amino acid sequences of a cDNA clone for *CsEpi-1* are shown in Fig. 1. The insert of the clone consisted of 2,653 nucleotides. The clone contained a single open reading frame (ORF) that predicted 741 amino acids. The calculated molecular mass (Mr) of the *CsEpi-1*-encoded protein (CsEpi-1) was 81.9 k. A Northern blot showing a transcript of about 3.0 kb (Fig. 3) suggested that the clone contains all the coding sequences and is close to full-length.

As shown in Fig. 1, the sequence motif search using the Block Searcher (http://www.blocks.fhcrc.org/blocks-search.html) suggested that CsEpi-1 contains three P-type trefoil domains in the C-terminal half. Thim (1989) pointed out that four peptides present in completely different biological sources have been shown to exhibit a large degree of structural similarity. The peptides include the breast cancer-associated pS2 peptide isolated from human gastric juice and culture media of the human breast cancer cell line MCF-7 (Jakowlew *et al.*, 1984), the pancreatic spasmolytic polypeptide (PSP) isolated from porcine pancreas (Tomasetto *et al.*, 1990), and the peptide predicted from a cDNA isolated from the skin of *Xenopus laevis* (Hoffmann, 1988). The domain contain 6 cysteine residues in nearly the same positions, and these 6 residues are linked by 3 disulphide bonds to form a characteristic "trefoil" disulphide loop structure, as shown in Fig. 2b. Several studies have shown the presence of the trefoil domain in peptides abundantly produced at the mucousal surface of various animals (e.g., Hauser *et al.*, 1992; Podolsky *et al.*, 1993). Figure 2a shows a comparison of the amino acid sequence of the trefoil domain of CsEpi-1 with those of human intestinal trefoil hITF (Podolsky *et al.*, 1993), human pS2 (Jakowlew *et al.*, 1984), PSP (Tomasetto *et al.*, 1990), the *Xenopus laevis* skin protein FIM-A.1 (Hoffmann, 1988), and another *Xenopus laevis* skin protein xP2 (Hauser *et al.*, 1992). These domains shared the consensus sequences (Hoffmann

and Hauser, 1993).

The present report may be the first report of a trefoil family protein from invertebrates, although another trefoil family protein has been isolated from a colonial ascidian (Dr. Kazuo Kawamura, personal communication). During ascidian embryogenesis, epidermal cells produce larval and adult tunics. The tunic consists of mucus substances. *CsEpi-1* may be a component of such mucus substances.

Spatial expression of CsEpi-1. The *in situ* hybridization of whole-mount specimens demonstrated that no signal was detected by the early gastrula stage (Fig. 4a) and that the first distinct signal was detected at the neurula stage (Fig. 4b). At this stage, the hybridization signal was evident in the nuclei of almost all of the epidermal cells. This signal was retained by the epidermal cells of the early tailbud embryos (Fig. 4c). A cross-section of hybridized embryos clearly showed that the *CsEpi-1* expression was restricted to epidermal cells (Fig. 4d).

Cross-reactivity with Ciona intestinalis embryos. When we examined whether the *CsEpi-1* antisense probe cross-reacts with *C. intestinalis* embryos, it became clear that the probe cross-reacted with *C. intestinalis* embryos (Fig. 5a). Thus, this gene is a useful molecular marker for epidermal cell differentiation in embryos of both *Ciona* species.

Expression of the *CsEpi-2* gene

Sequence analysis. Nucleotide sequence of a cDNA clone for *CsEpi-2* is shown in Fig. 6. The insert of the clone consisted of 1,618 nucleotides. There was a putative signal sequence for polyadenylation. In addition, the sequence in-

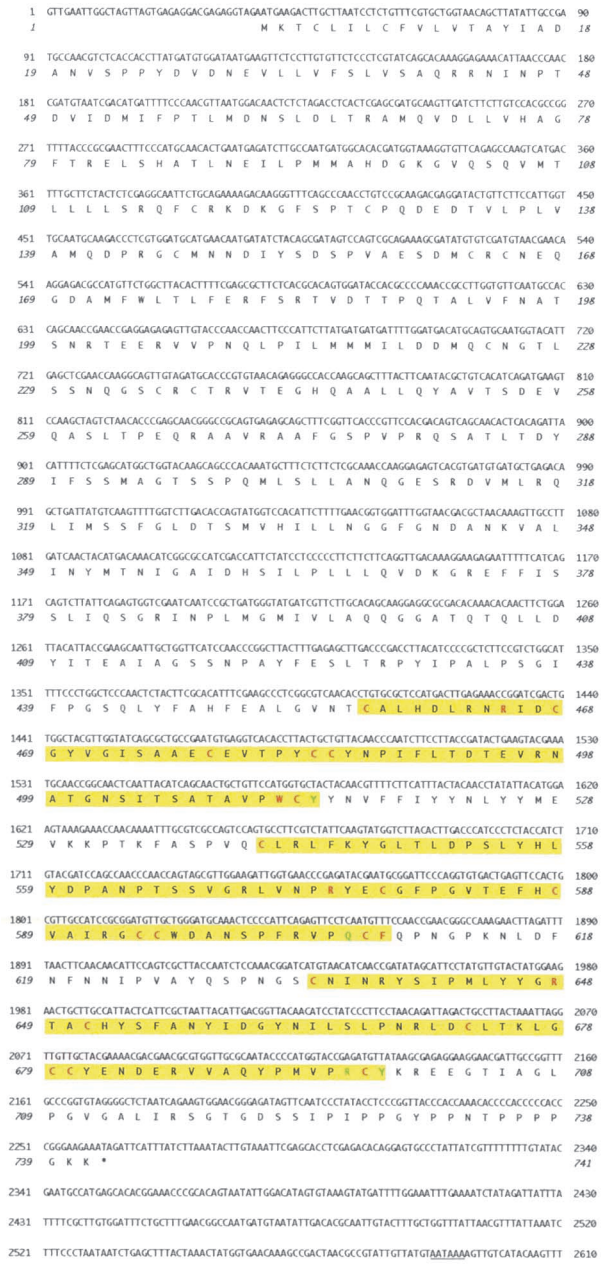

Fig. 1. Nucleotide and predicted amino acid sequences of a cDNA clone for *CsEpi-1*. The insert of the cDNA clone consists of 2,653 bp, with a single ORF that encodes a polypeptide of 741 amino acids. The asterisk indicates the termination codon. Two potential signal sequences for polyadenylation are underlined. The putative P-type trefoil domains are enclosed by yellow boxes. The amino acid residues that are conserved to form the P-type trefoil domain (see Fig. 2) are shown with red or green capitals. The accession number for the sequence of *CsEpi-1* is AB008818 in the DDBJ, EMBL and GenBank nucleotide sequence databases.

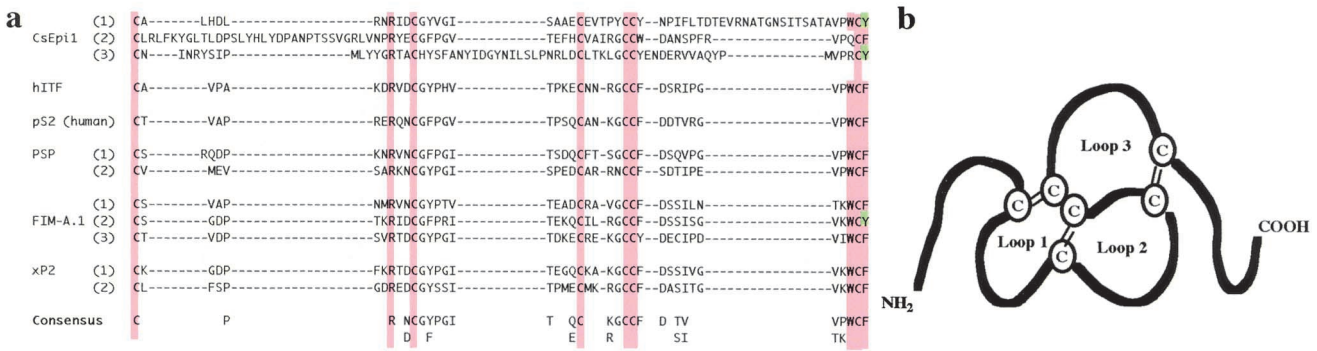

Fig. 2. (**a**) Comparison of amino acid sequences of the putative P-type trefoil domains of CsEpi-1 with those of other trefoil family members. Dashes indicate amino-acid residues without conservation. Sources: hITF (human, L08044; Podolsky *et al.*, 1993), pS2 (human, X00474; Jakowlew *et al.*, 1984), PSP (pig, X51696; Tomasetto *et al.*, 1990), FIM-A.1 (*Xenopus*, M19971; Hoffmann, 1988), and xP2 (*Xenopus*, M90095; Hauser *et al.*, 1992). The consensus was adopted from Hoffmann and Hauser (1993). (**b**) The predicted secondary structure of trefoil family proteins, adopted from Thim (1989).

cluded 31 adenylyl residues at the 3' end, suggesting that the transcript has a poly(A) tail (Fig. 6). However, we could not detect any distinct ORF in the cDNA (Fig. 6). A Northern blot analysis, shown in Fig. 3, demonstrated that *CsEpi-2* was not expressed in fertilized eggs but the transcript of about 1.7 kb was evident in the tailbud embryos. In addition, as described below, an *in situ* hybridization showed that the *CsEpi-2* transcript was evident in the nuclei of the 8-cell embryos. All of these data suggest that *CsEpi-2* is expressed zygotically in *C. savignyi* embryos. We repeated the isolation and sequence determination of three independent clones corresponding to *CsEpi-2*, which showed sequence identity with a few differences.

As mentioned above, the *CsEpi-2* transcript has no distinct ORF. We therefore examined possible secondary structures of *CsEpi-2* transcript by calculation with the version 2.3 of Mfold (Zuker, 1989; Zuker and Jacobson, 1995). The predicted secondary structures of the *CsEpi-2* transcript are shown in Fig. 7a.

Spatial expression of CsEpi-2. In most 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 cer-

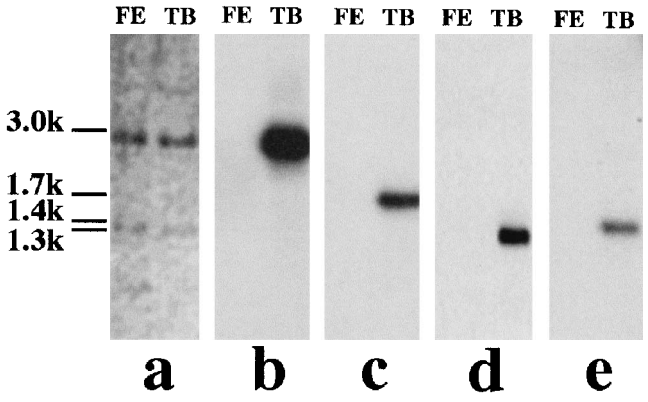

Fig. 3. Occurrence of transcripts of (**b**) *CsEpi-1*, (**c**) *CsEpi-2*, (**d**) *CsMA-1* and (**e**) *CsMu-1* in *C. savignyi* tailbud embryos. (**a**) Control gel. Northern blots of poly(A)$^+$ RNA prepared from fertilized eggs (left lanes, FE) and tailbud embryos (right lanes, TB) were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the membranes were washed under high-stringency conditions. Each lane was loaded with 1.5 μg of poly(A)$^+$ RNA.

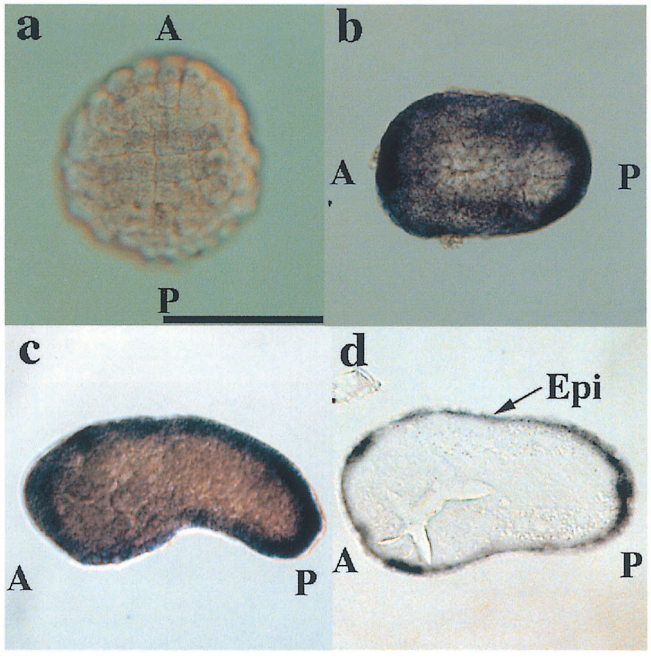

Fig. 4. Spatial expression of *CsEpi-1*, as revealed by whole-mount *in situ* hybridization. (**a**) An embryo at about the 110-cell stage viewed from the animal pole (future dorsal side of the embryo). No hybridization signal is detected at this stage. A, anterior; P, posterior. Scale bar represents 100 μm for all panels. (**b**) A neurula, dorsal side view showing distinct signal in the epidermal cells. (**c**) An early tailbud embryo showing the signal in epidermal cells. (**d**) A sagittal section of the hybridized embryo showing that the signal is restricted to epidermis (Epi).

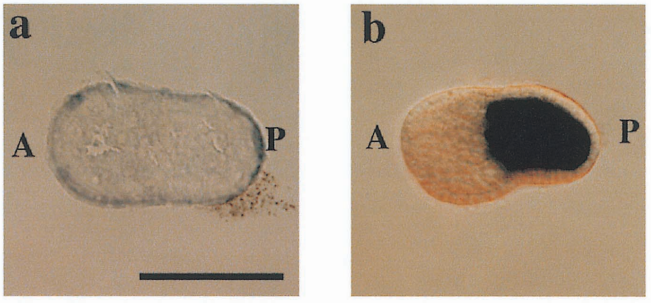

Fig. 5. Cross-reactivity of *C. savignyi CsEpi-1* (**a**) and *CsMA-1* (**b**) probes with *C. intestinalis* embryos, as revealed by whole-mount *in situ* hybridization. (**a**) The *CsEpi-1* probe identified epidermal cells of *C. intestinalis* embryo (section), and (**b**) the *CsMA-1* probe identified muscle cells of *C. intestinalis* embryo (whole).

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   1  CAGAACTGTGTTTCCAAATCCACAGTCTGTATAGCCTAGGCTCTGGTGCTCTCGAGGGCGGGGAATACGCTTAACGTACCCTACAGCCAGT  90
  91  CCCCACTTATAAAACTACGTAGCCTGTTATGCTTATATGCAATATTGTAATTGTACCGTTGCGCTTATCGGAGTCAAACTGTAATAATAA  180
 181  AGCTAAACTGCTACCGTCTACCTTGTAAAGTCAAACCAGTTATTATATTAAATAAGCAGCCGCTACAATTGGTGAATACCCGTAAACGTG  270
 271  GTTGAACTCAGCAACCTTCCGATGCCTAACGTTACCTATGTCCTGCTTATTCGGACACCTTAATATTATTTGTTTTCAGTATTTATCTCC  360
 361  AAAAGAAACAGCTTCACGATCCTCTACGTCAACGACGGAAATAAACACCGGCGTCATGTCGTGGAATCGAAGCGTTTTGTCAaaGCCAAG  450
 451  CCCAAAATCCGCGTCAACCAAACCAGGTGTGTCTATTTCAAAAGTACCGTCGGACCACGGTCACCTTAACGCGACCTTAGCAAGAAAAGG  540
 541  CGCTAACTAACTGTTGGAGCGAACTACGCATAAACAACCAACAGATTTACACCCCCCACTCATGTCACTGGAGGGAGGTCCAATTTAACC  630
 631  TGGTATTCGCGGATGGCTGGCAGACACAAATTGAACGGAACATCAATCTCAACAACCCTCTGAAATTTCTACAAGAACAACTCCCGGAAG  720
 721  TTCACTGCTGGTCGGACGAAGGAGGGCGGTTCAATCGATAAGAGTACAACAGGACTGTCTATATACACCCAGGTGCGCCTAAGTCTACAA  810
 811  GTCTGTTTTATATTGCCTGGGCCAATTATCGAAGGCCTGGATTACACGGCGTCCTCTGCTACCCGAGCAAGAAGCTCTCCGAGGCGCGAC  900
 901  TGTTGGTGCTTCAATAAGCATAAAATCCAACAGGAATTTTGTCTCCATCCGACCACGATGGAGACCGTCTAATTTTCGTCGTCCAAGGAA  990
 991  CGATGGACGAATATTACACCTGGGTGCTCGAAACGAACTGTTGACTCTTTATCGGGAAGGCCGAACTTCAAAAGTATCCACCGGGCGAAC 1080
1081  TTTAATTCAAATTATTCAAAACCCGAAAAAAAGGAGGTATGTCGTATACCGACATTGGCTGTTAGCGAAATCGTTCCAAGAAGACTACAA 1170
1171  GGAACAAAGGAGTCTCTAAATGACGAAGAACGATCTAAATTCACGATGCGATCAACGCAACTTCTAAATTTAACGTGCGGAGATAATCCA 1260
1261  TTGTATCGAGCGCAAAAATAATCTATTGTGCGTTTTTAGATATTCTGCATCGCATTTTTTCCCTTGGAGCCAAATCGCATATAAAAGACA 1350
1351  TCATTTCATCAGAGAGAGACACAGACAGAGATGTCTAGGCCTACAGAACTGTTGTTTCCAAATCCACAGTCTGTATAGCCTAGGCTCTGGT 1440
1441  GCTCTCGAGGGCGGGAATACGCTTAACGTACCCTACAGCCCGTCCCCACTTATAAAACTACGTAGCCGGTTATGCTTATATCCATTATTG 1530
1531  TATTTGCACCGTTGCGCTTATCGGACCCAATATAATAAAGCAAAACTGCTACCCGTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA    1618
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Fig. 6. Nucleotide sequence of a cDNA clone for *CsEpi-2*. The insert of the cDNA clone consists of 1,618 bp. The potential signal sequence for polyadenylation is underlined. The accession number for the sequence of *CsEpi-2* is AB008820 in the DDBJ, EMBL and GenBank nucleotide sequence databases.

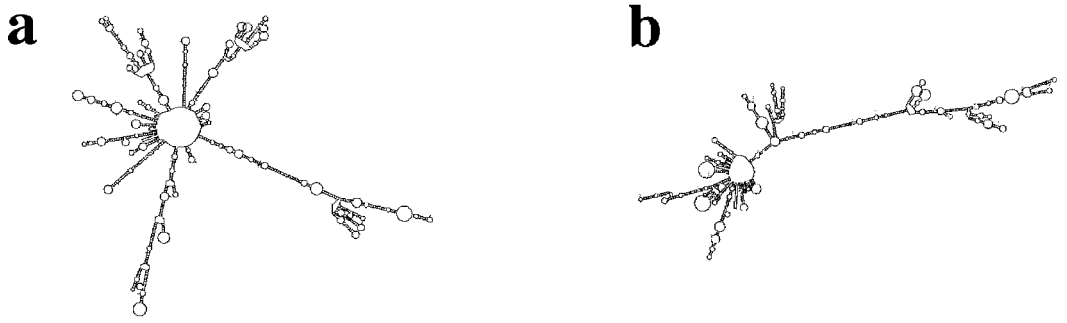

Fig. 7. Predicted secondary structures of (**a**) *CsEpi-2* and (**b**) *CsMu-1* transcripts.

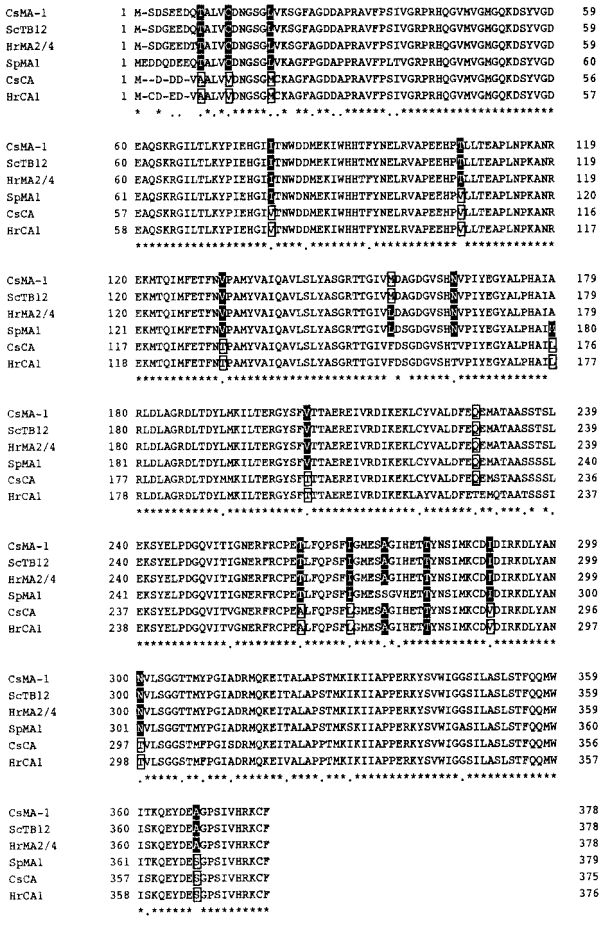

Fig. 9. The predicted amino acid sequences of a polypeptide encoded by a cDNA clone for *CsMA-1*, and comparison of the sequence with those of muscle-type (shown by dark boxes) and cytoplasmic-type actins (shown by white boxes) of ascidians. The accession number for the sequence of *CsMA-1* is AB008819 in the DDBJ, EMBL and GenBank nucleotide sequence databases. Sources: ScTB12 (*Styela clava*, muscle actin; Beach and Jeffery, 1992), HrMA2/4 (*Halocynthia roretzi*, muscle actin; Kusakabe *et al*., 1991), SpMA1 (*Styela plicata*, muscle actin; Kovilur *et al*., 1993), CsCA (*Ciona savignyi*, cytoplasmic actin; Y. Satou, unpublished data), and HrCA1 (*H. roretzi*, cytoplasmic actin; Araki *et al*., 1996).

tain 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 *CsEpi-2* gene.

Fig. 11. Nucleotide sequence of a cDNA clone for *CsMu-1*. The cDNA clone consists of 1,364 bp. The potential signal sequence for polyadenylation is underlined. The accession number for the sequence of *CsMu-1* is AB008821 in the DDBJ, EMBL and GenBank nucleotide sequence databases.

The *in situ* hybridization demonstrated that the first distinct signal was detected as early as the 8-cell stage (Fig. 8). At this stage, the hybridization signal was evident in the nuclei of pairs of the a- and b-line primordial epidermal cells (Fig. 8a). During gastrulation and neurulation, the *CsEpi-2* expression was retained only by epidermal cells (Fig. 8b). This signal was evident in the epidermal cells of the early tailbud embryos (Fig. 8c). The cross-section of hybridized embryos clearly showed that the *CsEpi-1* expression was restricted to epidermal cells (Fig. 8d).

In order to deduce the gene function, we treated embryos with *CsEpi-2* antisense oligos, but we did not obtain any meaningful results. The *CsEpi-2* antisense probe did not cross-react with *C. intestinalis* embryos (data not shown).

The initiation of the appearance of *CsEpi-2* was as early as the 8-cell stage. The first detection of a zygotic expression of ascidian genes was of the *forkhead/HNF-3* gene which was reported to be at the 16-cell stage (Corbo *et al*., 1997; Olsen and Jeffery, 1997; Shimauchi *et al*., 1997). The *CsEpi-2* gene may therefore represent the first zygotic expression of ascidians.

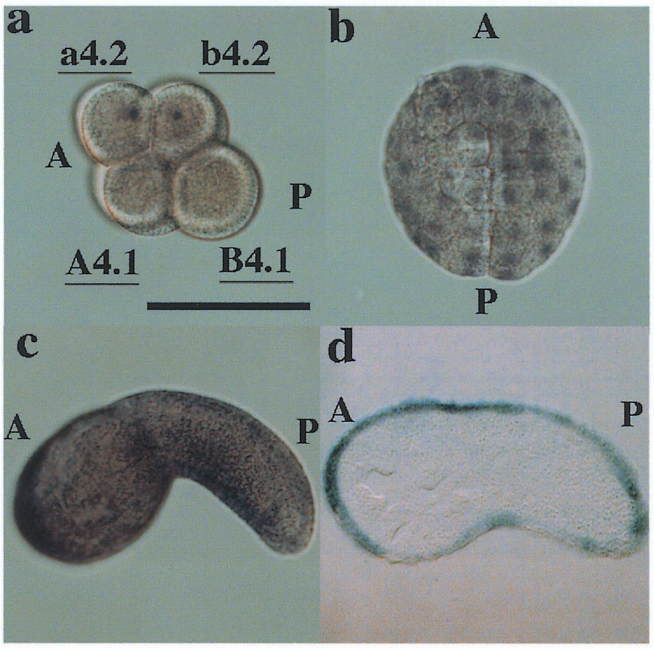

Fig. 8. Spatial distribution of *CsEpi-2* transcript, as revealed by whole-mount *in situ* hybridization. (**a**) An 8-cell embryo, side view, showing that the hybridization signal is evident in the nuclei of a4.2 and b4.2 blastomeres, presumptive epidermal cells. A, anterior; P, posterior. Scale bar represents 100 μm for all panels. (**b**) A gastrula viewed from the animal pole. Signal is found in the primordial epidermal cells. (**c**) An early tailbud embryo showing the signal in epidermal cells. (**d**) A sagittal section of the hybridized embryo showing that the signal is restricted to epidermis.

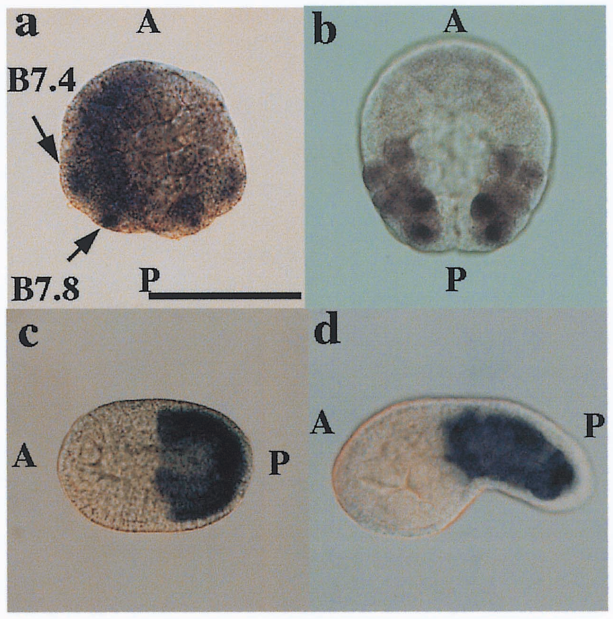

Fig. 10. Spatial expression of *CsMA-1*, as revealed by whole-mount *in situ* hybridization. (**a**) A 64-cell embryo viewed from the vegetal pole (future dorsal side of the embryo). Hybridization signal is evident in the nuclei of B7.4 and B7.8 , the primordial B-line muscle cells. A, anterior; P, posterior. Scale bar represents 100 μm for all panels. (**b**) A gastrula viewed from the vegetal pole showing the signal in the primordial muscle cells. (**c**) A neurula, dorsal side view, showing the signal in the primordial muscle cells. (**d**) An early tailbud embryo showing the signal in muscle cells of the tail region of the embryo.

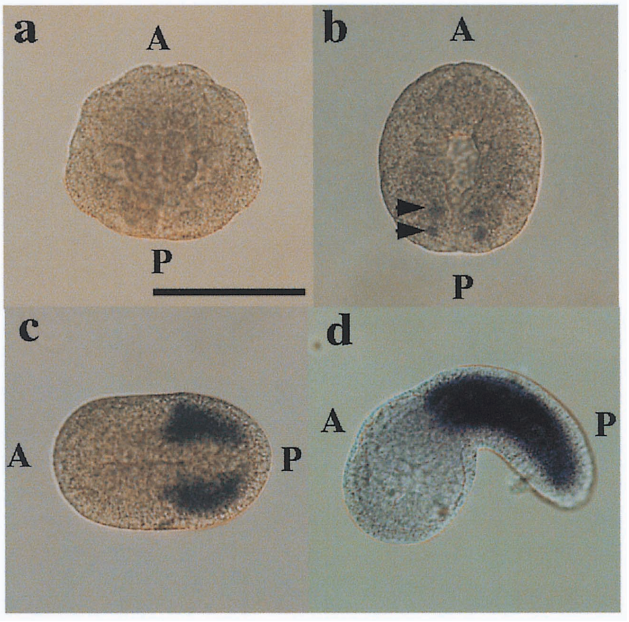

Fig. 12. Spatial distribution of *CsMu-1* transcript, as revealed by whole-mount *in situ* hybridization. (**a**) A 64-cell embryo viewed from the vegetal pole showing no hybridization signal. A, anterior; P, posterior. Scale bar represents 100 μm for all panels. (**b**) A gastrula viewed from the vegetal pole showing the signal in two pairs of primordial muscle cells (arrowheads). (**c**) A late neurula, dorsal side view, showing the signal in the primordial muscle cells. (**d**) An early tailbud embryo showing the signal in muscle cells of the tail region of the embryo.

Expression of a muscle-type actin gene *CsMA-1*

Sequence analysis. The nucleotide sequence of the cDNA for *CsMA-1* will appear under the accession number AB008817 in the DDBJ/EMBL/GenBank database. The insert of the clone consisted of 1,300 nucleotides including 21 adenylyl residues. The clone contained a single ORF that predicted 378 amino acids. Since (as shown below) the clone encodes a muscle actin, we designated this gene *CsMA-1*. The calculated molecular mass (Mr) of the *CsMA-1*-encoded protein (CsMA-1) was 42.1 k.

Most animals exhibit multiple actin isoforms which are encoded by a small gene family. In mammals, there are four muscle isoforms (α-skeletal, α-cardiac, α-vascular, and γ-enteric) and two nonmuscle isoforms (β- and γ-cytoplasmic) (Vandekerckhove and Weber, 1979) The mammalian α-skeletal muscle actin is distinguishable from the β-cytoplasmic actin by about 20 diagnostic amino acid positions (Vandekerckhove and Weber, 1978, 1979). Figure 9 shows the comparison of the amino acid sequence of CsMA-1 with those of muscle-type and cytoplasmic-type actin genes of ascidians. The comparison of the amino acid residues at the diagnostic positions indicated that the CsMA-1 is a muscle actin, while CsCA-1 is a cytoplasmic actin (Y. Satou, unpublished data).

Spatial expression of CsMA-1. The *in situ* hybridization demonstrated that the first distinct signal was detected at the 64-cell stage (Fig. 10a). The signals are evident in the nuclei of B7.4 and B7.8 , the primordial B-line muscle cells. During gastrulation, signals became evident in B- (Fig. 10b), A-, and

b-line presumptive muscle cells, and the neurulae showed signals in the primordial muscle cells (Fig. 10c). An early tailbud embryo showed distinct signal in muscle cells of the tail region of the embryo (Fig. 10d).

Cross-reactivity with Ciona intestinalis embryos. We confirmed that the *CsMA-1* antisense probe cross-reacts with *C. intestinalis* embryos (Fig. 5b), and thus is useful as a molecular marker in that embryo.

The isolation of a *C. savignyi* muscle actin may provide material for future studies. We have already isolated a genomic clone of *CsMA-1* and characterized the cis-regulatory elements required for the muscle-specific expression of *CsMA-1*.

Expression of the *CsMu-1* gene

Sequence analysis. The nucleotide sequence of the cDNA clone of the *CsMu-1* gene is shown in Fig. 11. The insert of the clone consisted of 1,364 nucleotides. There was a putative signal sequence for polyadenylation. In addition, the sequence included 51 adenylyl residues at the 3' end, suggesting that the transcript has a poly(A) tail (Fig. 11). However, as in the case of *CsEpi-2*, we did not detect any distinct ORF in the *CsMu-1* cDNA (Fig. 11). The Northern blot analysis shown in Fig. 3 demonstrated that *CsMu-1* is not expressed in fertilized eggs, but the transcript of about 1.4 kb is evident in the tailbud embryos. In addition, the *in situ* hybridization showed that the *CsMu-1* transcript is evident in the nuclei of the gastrula. Therefore, it is highly likely that *CsMu-1* is expressed zygotically in *C. savignyi* embryos. We examined four independent clones corresponding to *CsMu-1*, which showed sequence identity with a few differences.

Similarity to the case of the *CsEpi-2* transcript, we inferred possible secondary structures of the *CsMu-1* transcript by calculation with the 2.3 version of Mfold. The predicted secondary structures of the *CsMu-1* transcript are shown in Fig. 7b.

Spatial expression of CsMu-1. The *in situ* hybridization demonstrated that the first distinct signal was detected at the late gastrula stage (Fig. 12a, b). At this stage, the hybridization signal was evident in the nuclei of pairs of primordial muscle cells (Fig. 12b). During neurulation, the *CsMu-1* expression expanded (Fig. 12c). An early tailbud embryo showed distinct signal in muscle cells of the tail region of the embryo (Fig. 12d). In order to deduce the gene function, we treated embryos with *CsMu-1* antisense oligos, but we did not obtain any meaningful results.

Cross-reactivity with Ciona intestinalis embryos. We examined whether the *CsMu-1* antisense probe cross-reacts with *C. intestinalis* embryos. The probe identified muscle cells of *C. intestinalis* tailbud embryos (data not shown), and thus is useful as a molecular marker in that embryo.

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