DEVELOPMENTAL BIOLOGY **192**, 467–481 (1997)
ARTICLE NO. DB978730

etadata, citation and similar papers at core.ac.uk
brought to you by
provided by Elsevier - Publisher C

in the Ascidian Embryo

Yutaka Satou and Noriyuki Satoh

Department of Zoology, Graduate School of Science, Kyoto University,
Sakyo-ku, Kyoto 606-01, Japan

The posterior–vegetal cytoplasm of an ascidian egg contains maternal factors required for pattern formation and cell specification of the embryo. We report here the isolation and characterization of cDNA clones for novel maternal genes, *posterior end mark 2* (*pem-2*), *pem-4*, *pem-5*, and *pem-6*. We obtained these clones from a cDNA library of *Ciona savignyi* fertilized egg mRNAs subtracted with gastrula mRNAs by examining the localization of the corresponding mRNAs of randomly selected clones by whole-mount *in situ* hybridization. As in the case of *pem*, all of these mRNAs were localized in the posterior–vegetal cytoplasm of the egg, and they later marked the posterior end of early embryos. The predicted amino acid sequence suggested that PEM-2 contains a signal for nuclear localization, an src homology 3 (SH3) domain, and a consensus sequence of the CDC24 family guanine nucleotide dissociation stimulators (GDSs). PEM-4 has a signal for nuclear localization and three C2H2-type zinc finger motifs, while PEM-5 and PEM-6 show no similarity to known proteins. These results provide further evidence that the ascidian egg contains maternal messages that are localized in the posterior–vegetal cytoplasm. © 1997 Academic Press

Key Words: **ascidian; posterior–vegetal cytoplasm of eggs; localized maternal mRNA; *pem-2*; *pem-4*; *pem-5*; *pem-6*.**

INTRODUCTION

Ascidian eggs and embryos have provided an appropriate experimental system to understand the molecular nature of localized maternal factors and their roles in cell specification and pattern formation (for recent reviews see Jeffery and Swalla, 1990; Satoh, 1994; Satoh *et al.,* 1996a,b). The fertilized egg develops quickly into a tadpole larva, which consists of a small number of tissues including 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). Since the work of Chabry (1887), which described the first blastomere destruction experiment in the history of embryology, the ascidian egg has been regarded as a typical "mosaic" egg, in which embryonic cells are specified autonomously dependent on prelocalized egg cytoplasmic factors or determinants. Recent studies have provided convincing evidence for determinants responsible for differentiation of muscle (Nishida, 1992; Marikawa *et al.,* 1994), epidermis (Nishida, 1994a), and endoderm (Nishida, 1993), factors for the establishment of the anteroposterior axis of the embryo (Nishida, 1994b), and those for initiation of gastrulation (Jeffery, 1990; Nishida, 1996). In particular, the posterior–vegetal cytoplasm of the fertilized egg or the so-called myoplasm contains muscle determinants, factors for the anteroposterior axis establishment, and those for initiation of gastrulation.

Several studies have been performed to elucidate the molecular nature of such localized factors (Swalla *et al.,* 1993; Swalla and Jeffery, 1995). We also have attempted to isolate cDNA clones for maternal genes with localized messages. In a previous study, we reported isolation and characterization of a novel maternal gene in the ascidian *Ciona savignyi* (Yoshida *et al.,* 1996). The gene was named *posterior end mark (pem),* because the transcript is initially concentrated in the posterior–vegetal cytoplasm of the fertilized egg, and later the distribution of the transcript marks the posterior end of the developing embryos.

Centrifugation of unfertilized *C. savignyi* eggs yielded four types of fragments: a large nucleated red fragment and small enucleated black, clear, and brown fragments. Experiments of fusion of these fragments revealed that maternal factors for muscle and endoderm differentiation and for the

0012-1606/97 $25.00
Copyright © 1997 by Academic Press
All rights of reproduction in any form reserved.

anteroposterior axis establishment are preferentially separated into black fragments (Marikawa *et al.,* 1994). In addition, results of UV irradiation experiments in black fragments suggest that maternal mRNAs are associated with the activities of these factors (Marikawa *et al.,* 1995). Differential screening of cDNA libraries of black and red fragments yielded a cDNA clone for *pem* (Yoshida *et al.,* 1996). Although the amino acid sequence of the *pem* gene product showed no significant homology to known proteins, overexpression of this gene by microinjection of synthesized *pem* mRNA into fertilized eggs resulted in development of tadpole larvae with deficiency of the anterior-most adhesive organ, dorsal brain, and sensory pigment cells. Lineage tracing analysis revealed that the anterior epidermis and dorsal neuronal cells are translocated posteriorly into the tail region, suggesting that overexpression of this gene affects the patterning of the anterior and dorsal structures of the larva.

In the present study, we adopted another approach to isolate cDNA clones for novel maternal messages that are localized. During ascidian development, expression of tissue-specific genes commences much earlier than that of other chordates, reflecting very early fate determination and subsequent specification of embryonic cells (cf. Satoh, 1994). For instance, zygotic expression of a muscle-specific actin gene *HrMA4a* begins at the 32-cell stage (Satou *et al.,* 1995) and an epidermis-specific gene *HrEpiC* at the 76-cell stage (Ishida *et al.,* 1996). This suggests that fertilization recruits maternal mRNAs promptly into processes to perform their functions. We therefore attempted to isolate maternal genes by subtractive hybridization of mRNAs of fertilized eggs with those of gastrulae. Taking advantage of the well-known lineage and segregation pattern of developmental fates as well as *in situ* hybridization of whole-mount specimens, we were able to isolate several cDNA clones of maternal genes with localized mRNAs, which will be described in this report.

MATERIALS AND METHODS

Ascidian Eggs and Embryos

C. savignyi 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 gastrulae by the acid guanidinium thiocyanate–phenol–chloroform method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified using Oligotex beads (Roche Japan, Tokyo, Japan). cDNA libraries of fertilized eggs (FE-library) and gastrulae (G-library) were constructed in Uni-ZAP XR using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA).

From the FE-library, single-stranded DNA was isolated as described by Schweinfest *et al.* (1990) except that the VCSM13 helper phage was used instead of the R408 helper phage. From the G-library, the single-stranded phage was rescued according to the mass excision protocol supplied with the ZAP-cDNA synthesis kit. The single-stranded phage was converted to pBluescript double-stranded phagemid DNA containing cDNA insert by infection into SoloR cells. The phagemid DNA was prepared with QIAGEN Tip 100 (QIAGEN Inc., Santa Clarita, CA). The phagemid DNA was linearized with *Xho*I and used as template DNA for *in vitro* transcription. After *in vitro* transcription with T3 RNA polymerase (Gibco BRL, Gaithersburg, MD), template DNA was digested with RQ1 DNase (Promega, Madison, WI). Fifty micrograms of the synthesized RNA was photobiotinylated with a photobiotin labeling system (Gibco BRL).

Fifty micrograms of the biotinylated RNA was ethanol precipitated together with 5 μg of single-stranded DNA derived from FE-library and 5 μg poly(A) (Pharmacia, Piscataway, NJ), and then they were dissolved in 10 μl hybridization buffer (25 mM Hepes–NaOH, pH 7.5, 0.75 M NaCl, 0.5 mM EDTA, 0.1% SDS). Following incubation at 95°C for 4 min, hybridization was performed at 65°C for 20 h. After hybridization, the mixture was added to 80 μl of buffer containing 50 mM Hepes–NaOH, pH 7.5, and 437 mM NaCl. We separated hybrids from single-stranded DNA using streptavidin (Gibco BRL) as described by Sive and John (1988). The subtracted single-stranded DNA was ethanol precipitated and dissolved in 34 μl TE (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA).

A quarter of the subtracted DNA was converted to double-stranded DNA and used for transformation of XL-1 Blue supercompetent cells (Stratagene) as described by Schweinfest *et al.* (1990).

Screening of the Subtracted cDNA Library and Sequencing

From the library, clones were randomly picked up and partially sequenced from the 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. Fertilized eggs, 8-cell stage and 32-cell stage embryos, were used as specimens for the *in situ* hybridization screening. cDNA clones exhibiting 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 an ABI PRISM 377 DNA sequencer (Perkin–Elmer, Norwalk, CT).

Northern Analysis

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

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

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). Control specimens hybridized with sense probes did not show signals above background.

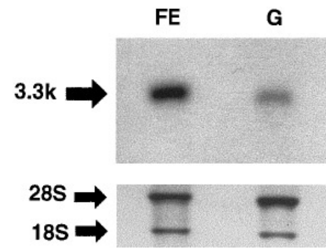


FIG. 1. Northern blots of poly(A)$^+$ RNA of fertilized eggs (FE) and gastrulae (G) showing a predominance of *pem-2* mRNA in fertilized eggs. Each lane was loaded with 1.8 μg of poly(A)$^+$ RNA, and the amount of loaded RNA was shown by ethidium bromide staining of 28S and 18S rRNA (lower photograph).

RESULTS

Isolation of cDNA Clones for Maternal Genes with Localized mRNA

We constructed a cDNA library of fertilized-egg mRNAs subtracted with gastrula mRNAs of *C. savignyi.* The library was estimated to contain about 1000 independent 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. Fertilized eggs and 8-cell- and 32-cell-stage embryos were subjected to *in situ* hybridization to determine the specific localization of the gene transcript. We have examined 102 clones to date and found that among them 7 cDNA clones are of mitochondrial genes, 2 clones are of cytoplasmic actin gene(s), 3 are of α-tubulin gene(s), 1 is of a β-tubulin gene, and 1 is of a TFIIB gene. The localization and segregation patterns of the 7 mitochondrial clones coincided with that of the myoplasm (data not shown), while none of the other clones showed a specific localization pattern.

In addition, we were able to find five independent clones that contained mRNAs that were localized in the posterior end of the 8-cell and 32-cell embryos. Because the localization patterns of these clones resembled that of the *pem* cDNA clone (Yoshida *et al.,* 1996), we tentatively designated these clones as *pem-2, pem-3, pem-4, pem-5,* and *pem-6,* in the order of their isolation. The clones we have examined thus far did not contain *pem* cDNA itself. No cDNA clone has been isolated that exhibits localization in regions other than the posterior–vegetal cytoplasm except the clones for mitochondrial genes. The results of Northern blot analysis as well as *in situ* hybridization suggested that among these localized messages *pem* mRNA is the most abundant, and the amount of mRNA was lessened in the order of *pem-2, pem-3, pem-4, pem-5,* and *pem-6.* Here we describe *pem-2, pem-4, pem-5,* and *pem-6* in detail.

pem-2

Northern blot analysis identified a distinct single *pem-2* transcript of about 3.3 kb in fertilized egg mRNAs, the signal intensity of which was considerably weaker in gastrula mRNAs (Fig. 1). We detected no changes in the size of *pem-2* mRNA during embryogenesis.

Characterization of the pem-2 cDNA clone. The cDNA clone we initially isolated did not contain the entire coding region. Screening of the FE-library yielded two kinds of cDNA clones with the entire *pem-2* open reading frame. One was 3260 bp long and the other 2950 bp long. The nucleotide and deduced amino acid sequences of the longer clone are shown in Fig. 2. The two clones differed from each other in their 3′ UTR sequence length. The 3′ UTR of the longer cDNA clone contained two polyadenylation signals (Fig. 2). The sequence of the shorter clone terminated at nucleotide position 2950 (black-boxed in Fig. 2), which may correspond to the first polyadenylation signal. These results demonstrate the presence of two kinds of *pem-2* transcripts, although Northern hybridization failed to detect the shorter transcripts (Fig. 1).

The *pem-2* cDNA had a single open reading frame encoding a polypeptide of 820 amino acids (Fig. 2). Database searches indicated that the predicted PEM-2 protein contained a signal for nuclear localization (amino acid position 25–42; underlined in Figs. 2 and 3A), the src homology 3 (SH3) domain (amino acid position 238–285; white capitals in Fig. 2), and a consensus sequence of the guanine nucleotide dissociation stimulators (GDSs; also known as guanine nucleotide exchange factors or guanine nucleotide-releasing proteins) that was classified in the CDC24 family (amino acid position 399–552; boxed in Figs. 2 and 3B). The CDC24 family GDSs are thought to be specific to Rho/Rac proteins which are involved in the organization of the cytoskeleton, and the family includes Dbl, Vav, Bcr, rasGRF, and ect2 (reviewed by Boguski and McCormick, 1993). As shown in Fig. 3B, 21 of the 26 diagnostic consensus residues were conserved in PEM-2. This suggests that PEM-2 is a new member of the CDC24 family. Many proteins of this family contain additional functional domains, including pleckstrin homology (PH), src homology 2 (SH2), and SH3 domains. PEM-2 also has an SH3 domain. This finding provides further support that PEM-2 is a member of the CDC24 family.

The localization and segregation of pem-2 mRNA. Simi-

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

```
TGTAGATTGACTTCTTAGACTTCCTGTTTCAATGGCGCTGCTTCGCAAGCTTAACGCTCCCAGGTGAGCCGTGGATTAATGCTTGGTTCA   90
                                                                                  M  L  G  S    4
ATGGCGGCCGAGGAAGCCACGCAAGTTCGGGTCGCCAGAAATGGCGCAAGTGACATCGAAAAGAAGAAGCAGTACAACTCCATATTCAGG  180
M  A  A  E  E  A  T  Q  V  R  V  A  R  N  G  A  S  D  I  E  K  K  K  Q  Y  N  S  I  F  R   34
TCGTTTGTGAAAAGGAACAAATTGCTGTCGCGAGTAGTAGGTTCGGCCAGGTGCGTCTCTACTTCCACCCTCCCATCGCACTCCAAAGGG  270
S  F  V  K  R  N  K  L  L  S  R  V  V  G  S  A  R  C  V  S  T  S  T  L  P  S  H  S  K  G   64
CAACCTGTCGAAATTTCCAACTCTTCTACGTCACAGGCGAACCCTGTTGCCGCGGACAAATGCAATAGAAAAATGTCGCGTGACCTTGTA  360
Q  P  V  E  I  S  N  S  S  T  S  Q  A  N  P  V  A  A  D  K  C  N  R  K  M  S  R  D  L  V   94
TGCCGCCGAAGTTTCGAGGTGCGGAAGACGCCAGCTGGAACGTTCACGCCTCAGTGCACAAGGGCTAACGTTATGCAGCCTCCGCCAGCT  450
C  R  R  S  F  E  V  R  K  T  P  A  G  T  F  T  P  Q  C  T  R  A  N  V  M  Q  P  P  P  A  124
CAGCTCCGCAGCAGGGCTCAGTCATCAAACGTATATTTAGAAATTCAACCTTTCGAAGATGACTACTGTCTTGATGGTGACTGTGACGAT  540
Q  L  R  S  R  A  Q  S  S  N  V  Y  L  E  I  Q  P  F  E  D  D  Y  C  L  D  G  D  C  D  D  154
GATGATGTGTTTGACATTACTCTCACACGCGCATGCAGCAAACCCTTATCCCGGAGCCACACTCACCCAAAGCACAGAACCAAATCCGTC  630
D  D  V  F  D  I  T  L  T  R  A  C  S  K  P  L  S  R  S  H  T  H  P  K  H  R  T  K  S  V  184
TCAAGTGGCTGCATTTCGAGCGAGTCGGGCTACGGGACCATGACCAGGAATGGGCCACGCTTAAGAACACCTGACAACGACGAGGAAGTC  720
S  S  G  C  I  S  S  E  S  G  Y  G  T  M  T  R  N  G  P  R  L  R  T  P  D  N  D  E  E  V  214
TTTGTCAACACTTCAACACAGACAGCCGATGGCACGTCATCAGCCCGCGCGGAGGGAATCTTCGTCGAGGCTCTGTGGGATCACGTGACC  810
F  V  N  T  S  T  Q  T  A  D  G  T  S  S  A  R  A  E  G  I  F  V  E  A  L  W  D  H  V  T  244
ATGGATCCCGATGAGCTCGGATTCAAAGTCGGTGACGTCATCCGCGTGAATGACATGTCGAACGCTGATTGGTGGTGGGGCGAGATTGAC  900
M  D  P  D  E  L  G  F  K  V  G  D  V  I  R  V  N  D  M  S  N  A  D  W  W  W  G  E  I  D  274
TCCGCGGAAGGGTGGTTCCCCGCCACATTTGTTCGGATCCTTGTGAACCAGCAAATTCGGACGGACTCCAATAACAGTGACGTCATTATT  990
S  A  E  G  W  F  P  A  T  F  V  R  I  L  V  N  Q  Q  I  R  T  D  S  N  N  S  D  V  I  I  304
TCCAACTACGCCAGCAGCTCTCAGAGCGAGGATGAGATTTCTGTAACGTCACATGAGTTTTCCCGCGTCACGTCGTTTAACGACTTCTGC 1080
S  N  Y  A  S  S  S  Q  S  E  D  E  I  S  V  T  S  H  E  F  S  R  V  T  S  F  N  D  F  C  334
CAGTGCACCGAGCAGCGATCAACAACCTCAGTTCATTGTAATGTTTGTGGGAGGCCTTTGACGGACGCAACGCCTGCCAGCAGGACGTCC 1170
Q  C  T  E  Q  R  S  T  T  S  V  H  C  N  V  C  G  R  P  L  T  D  A  T  P  A  S  R  T  S  364
ATTGCCCACCCGAGTAAACAACCCGAGATCGTGATAACAAAGCCGCCACCTTCCAATACTTCCCGCAAATCCTCAATCACAAGGGACCAG 1260
I  A  H  P  S  K  Q  P  E  I  V  I  T  K  P  P  P  S  N  T  S  R  K  S  S  I  T  R  D  Q  394
ATACGAACCAATGTCATTCGGGAAATCATCAACTCGGAAAAAGTCTTTGTCGGTCATCTTAAGGACGTGGTACAGGGATACCTCACCCGA 1350
I  R  T  N  V  I  R  E  I  I  N  S  E  K  V  F  V  G  H  L  K  D  V  V  Q  G  Y  L  T  R  424
TGTCGAAACCGGTCGGATATGTTCTCGGACGAGATTCTTAACACGTTGTTTGGAAACATAGAAGACATCTACCTGTTTCAACGAGAATTC 1440
C  R  N  R  S  D  M  F  S  D  E  I  L  N  T  L  F  G  N  I  E  D  I  Y  L  F  Q  R  E  F  454
GCGGCTGAGTTGGAAGCATCACTTGATAATGTGTCTACTCATGCAACGAATATCGGAAACGTCTTCTTAAAACACAAAGACGGCTTCTGC 1530
A  A  E  L  E  A  S  L  D  N  V  S  T  H  A  T  N  I  G  N  V  F  L  K  H  K  D  G  F  C  484
ATTTATTCCGAATACTGCAACAACCACCCGCAGGCTGTGGCCGAACTGGCCCAGCTGCTCACCAACAAAAAGTTCATGCATTTCTTCGAG 1620
I  Y  S  E  Y  C  N  N  H  P  Q  A  V  A  E  L  A  Q  L  L  T  N  K  K  F  M  H  F  F  E  514
GCTTGCCGTTTGCTACAGCGCATGATCGATATCCCTCTAGATGGCTTCTTACTCACGCCGGTTCAGAAGATCTGCAAATACCCCCTGCAG 1710
A  C  R  L  L  Q  R  M  I  D  I  P  L  D  G  F  L  L  T  P  V  Q  K  I  C  K  Y  P  L  Q  544
TTGGCGGAGTTGTTGAAGTATACCCACCCCGGCCACCAAGATTATGAAGCTGTAAAATCTGCTCTGGAAGCGATGAAAGGCGTCGCTCGA 1800
L  A  E  L  L  K  Y  T  H  P  G  H  Q  D  Y  E  A  V  K  S  A  L  E  A  M  K  G  V  A  R  574
ATGATCAACGAAAGAAAACGTAAAATGGAGAATCTTCGCAAGATATCACAGTGGCAGGCCAGCATAGTGAACTGGCAGGGCGAGAGCGTA 1890
M  I  N  E  R  K  R  K  M  E  N  L  R  K  I  S  Q  W  Q  A  S  I  V  N  W  Q  G  E  S  V  604
CTTTCCCGAAGCTGTGAGTTGGTGCACTCAGGTGAAATCCACGCGCTCTCTCAAGTTAAAGGAAAACCAAAACCTCGCGTCGCATTCCTG 1980
L  S  R  S  C  E  L  V  H  S  G  E  I  H  A  L  S  Q  V  K  G  K  P  K  P  R  V  A  F  L  634
TTCGACCATCAGATGATTCTGTGCAAAAAGGATTTGCTCCGTCGTGACCTGCTGTACTACAAAGCCCGCATCGACCTTGACGCCGTCGAG 2070
F  D  H  Q  M  I  L  C  K  K  D  L  L  R  R  D  L  L  Y  Y  K  A  R  I  D  L  D  A  V  E  664
GTTGCACCGATCGACCCAGAGTCGCCCAGGTCGTCCCACTCGGGGTCCGACTTCCATTCCTCATCGAGCTTAGGGTCGTGCCAGGGCTGG 2160
V  A  P  I  D  P  E  S  P  R  S  S  H  S  G  S  D  F  H  S  S  S  S  L  G  S  C  Q  G  W  694
AAGGTGACAGACACCACGACGGGCGAGTCGGGACTCATGTACAGCAAGAACCAGGAGGATTGCGGGAAATGGATGAAAGCGTTCGCCCTG 2250
K  V  T  D  T  T  T  G  E  S  G  L  M  Y  S  K  N  Q  E  D  C  G  K  W  M  K  A  F  A  L  724
GAACGAAAGATAGTTAGAACGGAAGGTACAACATCGCCTCTCCTCAACGAAGCTTTAACGCTGGGCTTGCTACGAAACTCGTTACCAATG 2340
E  R  K  I  V  R  T  E  G  T  T  S  P  L  L  N  E  A  L  T  L  G  L  L  R  N  S  L  P  M  754
TCGCGGAAGCCAAAAGAATCCGGGTCCCAAAGGTTAGCGCCGTCGTACCGCAACCGAATGAAGATGGCGCAGGAACTGCAGGATTACTCC 2430
S  R  K  P  K  E  S  G  S  Q  R  L  A  P  S  Y  R  N  R  M  K  M  A  Q  E  L  Q  D  Y  S  784
AACAAGCAAAACAGCTTCGCGTCCGACAAGCCCAGTTTCTTCAGCAGCGTGGCGGGCAAGTTGACGCCCTTCCGCCGTACCAACCCTCGG 2520
N  K  Q  N  S  F  A  S  D  K  P  S  F  F  S  S  V  A  G  K  L  T  P  F  R  R  T  N  P  R  814
GAAAGCAGCTTCGTATTTTAAGATTACGCGTCGTTTTAAATCGCTATTTTCATTTCCTGTATTTGCACACGTTGCTCTCTTATTGAATTG 2610
E  S  S  F  V  F  *                                                                        820
GTTAACCATTCAAGAAGCTGGCGTTTCCATTTTTGCTGTATTATAAGTGTATAAACTATGGAGAAACATAAAATGATAATCTCCGAAGAT 2700
GTATAAGGTTTTATTGCAACATAACATTGTCCAGGACAGTGCGGACGCTTGCTGTGTATTTTACCACCAATCTGTTTTTATTTCCACTGT 2790
GTTTACCTCACCGCCAAACGTTCCGGTCGTAGCTTTGGCCTCGTGGCCAGCACGACTGTATTATTAATTTTATCGAAGAAGAATGATTCT 2880
TTTGCACGTGCAATTCACCAATACCACCTTTGCTGTTGTTGTTCACAATAAATGCTTACGACACACCACAGCAGACGAGATCACCACTGG 2970
TTTTTATTATACCTGATCAACGTGCTTGCGTCACCCAGCAGACACGACCTTGTCATAGCTTAACGAGACTCACTGGTCCCCTATAAATTT 3060
CCTACATGTATGGTCGCTAGCTATTTGGTAAATAAGGAGTATTTAATAATTTTTTTAAAAATTTTCGCATTGCCAACTGACCTTTTTTA 3150
ACATTTGCAACCGAACAAGAAACCGATTGGAATAAACCAATTTATCCTGCAAAGGCGCCCGATAATAAAAAACAAACTGCAAATATAAAA 3240
AAAAAAAAAAAAAAAAAAAAAAAAA                                                                  3266
```

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

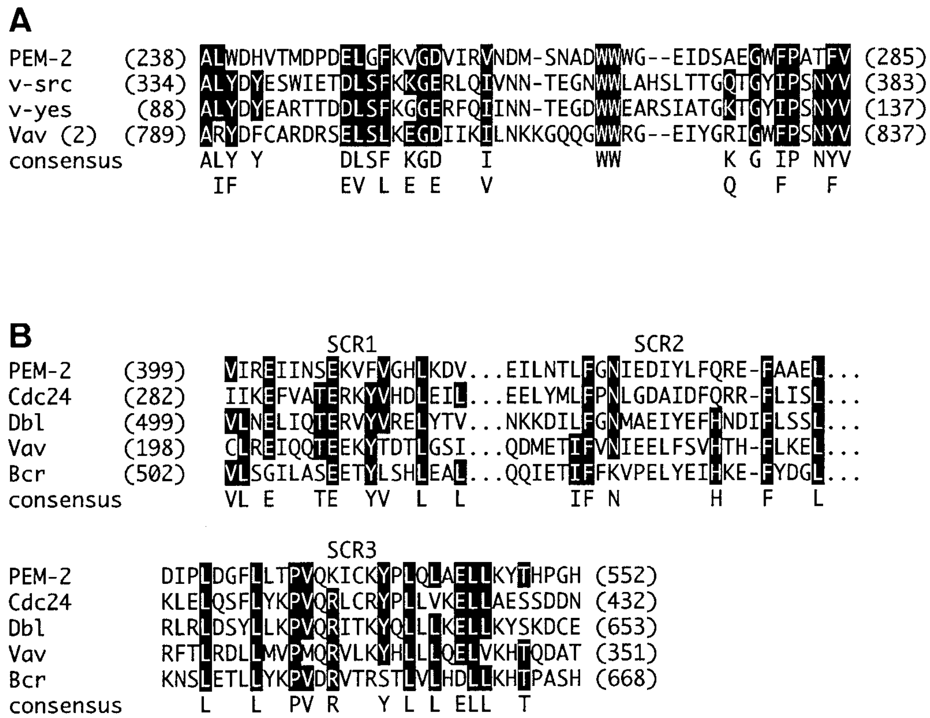
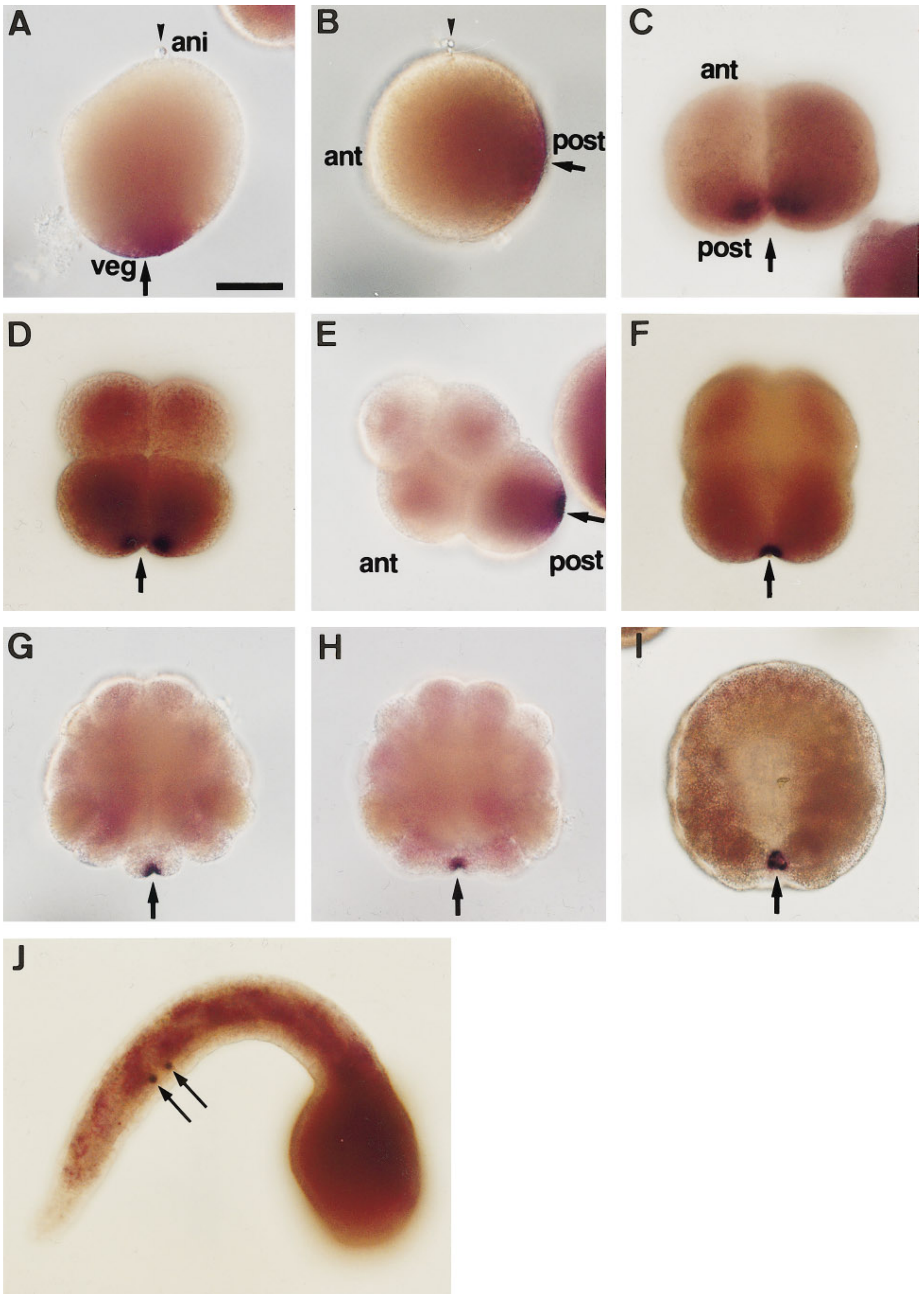


FIG. 3. (A) SH3 domain sequences of PEM-2, v-src (Czernilofsky *et al.*, 1983), v-yes (Kitamura *et al.*, 1982), and Vav (Adams *et al.*, 1992). The consensus sequence indicated below is adapted from Leto *et al.* (1990). Residues which accord with the consensus are enclosed within black boxes. The numbers in the left and right margins represent the amino acid residue locations within the proteins. (B) Structurally conserved regions (SCRs) of the GDS's CDC24 family (Miyamoto *et al.*, 1987; Ron *et al.*, 1988; Adams *et al.*, 1992; Hariharan *et al.*, 1987). The putative GDS domain corresponds to a region of about 180 residues, of which three SCRs are shown. The consensus residues indicated below are adapted from Boguski and McCormick (1993). Residues which accord with the consensus are enclosed within black boxes. The numbers in the left and right margins represent the amino acid residue locations within the proteins.

lar to *pem,* the localization and segregation of *pem-2* mRNA marked the posterior end of developing embryos (Fig. 4). In the unfertilized eggs, the hybridization signal appeared in the peripheral cytoplasm but its localization was not so conspicuous (data not shown). In ascidian eggs, fertilization evokes a dynamic rearrangement of the egg cytoplasm called ooplasmic segregation, yielding the establishment of the dorsoventral and anteroposterior axes of the embryo (reviewed by Satoh, 1994). The first phase of ooplasmic segregation involves rapid movement of the peripheral cytoplasm including the myoplasm (the cytoplasm to be segregated into muscle-lineage blastomeres) to form a transient cap near the vegetal pole of the egg. After the first phase of ooplasmic segregation, the *pem-2* hybridization signal became stronger near the vegetal pole (Fig. 4A). This was assessed by the position of the polar body, which was a landmark of the animal pole region (Figs. 4A and 4B). During the second phase, the myoplasm shifts from the vegetal-pole region to a new position near the subequatorial zone of the egg and forms a crescent, which marks the posterior side of the future embryo. As shown in Fig. 4B, after the second phase of ooplasmic segregation, the *pem-2* transcript moved to the subequatorial region to form a rather broad crescent-like structure. The cleavage of ascidian eggs is bilaterally symmetrical. During the first two cleavages, the *pem-2* transcript was localized to the posterior–vegetal cy-

FIG. 2. Nucleotide and deduced amino acid sequence of the *pem-2* cDNA clone. The 3266-bp insert includes a single open reading frame that encodes a polypeptide of 820 amino acids. The termination codon is shown by an asterisk. Two potential signal sequences for polyadenylation are shown by bold letters with underlines. A black box indicates the last nucleotide of a cDNA clone that has a shorter 3′ UTR sequence. A signal motif for the nuclear localization is underlined (amino acid positions 25–42). The SH3 domain is shown by open letters (amino acid positions 238–285). A putative guanine nucleotide dissociation stimulator (GDS) domain is enclosed by a box (amino acid positions 399–552). Three 6-bp sequences (TTTATT) in the 3′ UTR are shown by dotted underlines.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.


A
ani
veg
B
ant
post
C
ant
post
D
E
ant
post
F
G
H
I
J

toplasmic region of the two-cell (Fig. 4C) and four-cell embryo (Fig. 4D).

During early cleavages, the distribution of the *pem-2* transcript became narrower and narrower (Figs. 4C–4F), and at the 8-cell-stage *pem-2* mRNA was restricted to the very narrow, posterior region of B4.1 cells (a pair of posterior vegetal blastomeres in the bilaterally symmetrical embryo; Figs. 4E and 4F). At the 16-cell stage, the *pem-2* mRNA was found in the posterior-most region of the embryo, only in the posterior cytoplasm of the B5.2 cells (data not shown). Positive hybridization signals for the *pem-2* transcript were detected in the posterior cytoplasm of B6.3 in the 32-cell-stage embryo (Fig. 4G) and then in B7.6 of the 64-cell-stage embryo (Fig. 4H). At the gastrula stage, the *pem-2* transcript was seen in two invaginating posterior cells (Fig. 4I). At the tailbud stage, hybridization signals were detectable in 2 cells of the endodermal strand (Fig. 4J).

Thus, as in the case of *pem* (Yoshida *et al.,* 1996), the distribution of the *pem-2* transcript marks the posterior-most region of early embryos, and the maternal transcripts are destined to two cells of the endodermal strand at the tailbud stage.

pem-4

Characterization of the pem-4 cDNA clone. Northern blot analysis revealed a maternal *pem-4* transcript of about 2.3 kb which became barely detectable at the gastrula stage (data not shown). The cDNA sequence of *pem-4* was 2281 bp long and had a single open reading frame encoding a polypeptide of 525 amino acids (Fig. 5). Database searches indicated that the predicted PEM-4 protein contained a signal for nuclear localization (amino acid position 83–98; underlined in Fig. 5) and three C2H2-type zinc finger motifs in the C-terminal region (amino acid positions 436–458, 466–488, and 496–516; open letters in Fig. 5), suggesting that PEM-4 is a transcriptional factor.

The localization and segregation of pem-4 mRNA. The localization and segregation of *pem-4* mRNA also marked the posterior end of developing embryos (Fig. 6). The *pem* and *pem-2* transcripts appear to be abundant maternal messages, and a dynamic change in their localization and segregation was very clearly revealed by the whole-mount *in situ* hybridization at stages from unfertilized eggs to the midtailbud stage (cf. Fig. 2 of Yoshida *et al.,* 1996 and Fig. 4 of the present study). However, as mentioned above, presumably reflecting a lesser amount of *pem-4* transcript, the distribution pattern of this mRNA was not so evident until the 8-cell stage and was much narrower at later stages than those of *pem* and *pem-2.*

In the unfertilized eggs, the hybridization signal appeared in the peripheral cytoplasm but its localization was not so conspicuous. After the first and second phases of ooplasmic segregation, the *pem-4* hybridization signal became stronger near the vegetal pole and then the posterior side of the egg, but again its localization pattern was not so distinct (data not shown). During the first two cleavages, the *pem-4* transcript was distributed over the posterior–vegetal cytoplasmic region of the embryos, but not so much restricted to the cortical cytoplasm (data not shown).

At the 8-cell stage, however, the distribution of *pem-4* transcript was restricted to the very narrow, posterior region of B4.1 cells (Figs. 6A and 6B). At the 16-cell stage, *pem-4* mRNA was found in the posterior-most region of the embryo, only in the posterior cytoplasm of the B5.2 cells (data not shown). Positive hybridization signals for the *pem-4* transcript were detected in the posterior cytoplasm of B6.3 in 32-cell-stage embryos (Fig. 6C) and then in B7.6 of the 64-cell-stage embryos (data not shown). At the gastrula stage, the *pem-4* transcript was seen in 2 invaginating posterior cells (Fig. 6D). The *pem-4* signals in embryos up to the gastrula stage appeared to be restricted to a region more posterior than that of the *pem-2* signals. However, the hybridization signals were undetectable in the B7.6 descendants at the tailbud stage (data not shown). Thus, the distribution of the *pem-4* transcript also marks the posterior-most region of early embryos, and the maternal transcript was diminished by the tailbud stage.

pem-5

Characterization of the pem-5 cDNA clone. Northern blot analysis revealed a maternal *pem-5* transcript of about 2.7 kb (data not shown). The cDNA sequence of *pem-5* was 2688 bp long (Fig. 7). The cDNA had a single open reading frame encoding a polypeptide of 768 amino acids. The predicted PEM-5 protein did not contain any consensus motif, nor did it show any similarity to known proteins.

FIG. 4. Distribution of *pem-2* maternal mRNA marks the posterior end of developing embryos, as revealed by whole-mount *in situ* hybridization. (A) A fertilized egg after completion of the first phase of ooplasmic segregation, lateral view. *pem-2* maternal mRNA is concentrated at the vegetal pole (arrow). ani, animal pole; veg, vegetal pole of the egg. Arrowhead indicates the polar body. (B) A fertilized egg after completion of the second phase of ooplasmic segregation, lateral view. *pem-2* maternal mRNA is concentrated at the posterior side of the egg (arrow). ant, anterior; post, posterior side of the egg. Arrowhead indicates the polar body. (C) A 2-cell embryo and (D) a 4-cell embryo, animal pole view, showing localization of the *pem-2* mRNA in the posterior side of the embryos (arrow). (E, F) An 8-cell embryo: (E) lateral view and (F) vegetal pole view. The very narrow localization of the *pem-2* mRNA is shown by arrow. (G) A 32-cell embryo, vegetal pole view. (H) A 64-cell embryo, vegetal pole view. (I) A midgastrula, vegetal pole view. (J) A tailbud embryo, side view. The *pem-2* mRNA was detected in 2 cells of the endodermal strand. Scale bar represents 50 μm for all photographs.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

```
GATAGATTGGAGAACGGAGGTTCCTATGAAAGTGGAAATATTAAGTTTATGTTCTTTTACTTCTAATTAAGATTTCTATATATCTAAAAT    90
                                                                                         M     1
GAGATCATTACCAGCATCACCTACAAACTCTACTTCTGATTTTGAGGGAAATTGCTTCCAATCTACGACAATGGAACAGTTTGTCCACCC   180
 R  S  L  P  A  S  P  T  N  S  T  S  D  F  E  G  N  C  F  Q  S  T  T  M  E  Q  F  V  H  P    31
ATGTCATCTACCAGACTTGTCCGACTCTGACTCGAGATCGCGACACAGCAGTGATTCGTGTACCGGCTGTAACGATTGTGAAGCAGCTGA   270
 C  H  L  P  D  L  S  D  S  D  S  R  S  R  H  S  S  D  S  C  T  G  C  N  D  C  E  A  A  D    61
TACCTTGTTATCCCTGTGTGGCCGCCAATCCAGTCCCTGTGTTGCTGCCAATCCAGTTACACAGCGCAAACCGGTCCTGCCCCCAAGACT   360
 T  L  L  S  L  C  G  R  Q  S  S  P  C  V  A  A  N  P  V  T  Q  R  K  P  V  L  P  P  R  L    91
ACGATATCGGAAAAAGTACCGCCAAAATTTACTCAAAGAGCTAGAGGGCATTAATTCAACTCAACAACCAACCAAAGGACCTCTGGGTGT   450
 R  Y  R  K  K  Y  R  Q  N  L  L  K  E  L  E  G  I  N  S  T  Q  Q  P  T  K  G  P  L  G  V   121
AAATACTTCGATGTCCACCCCGCCATATACTCCACCTCCGGTTGTGGATACACAATACAGTACGCACCCAATAAGCGCCGAACCAACAAT   540
 N  T  S  M  S  T  P  P  Y  T  P  P  P  V  V  D  T  Q  Y  S  T  H  P  I  S  A  E  P  T  M   151
GACCATGTCCAGAAAGAAAGACGTAACGACGGTCACTTGTTCCAGACCCAATAACCGCCATCAACACGGTAGCGATGGTATGTTTTCCGA   630
 T  M  S  R  K  K  D  V  T  T  V  T  C  S  R  P  N  N  R  H  Q  H  G  S  D  G  M  F  S  D   181
CACTTGTAGCTGTCCGGATACATCAGATTCCTCAGCGAAGGACTGCATTCTATACGCGCCCCCATCTCTATCTGCAAGCTGCTGTAACTC   720
 T  C  S  C  P  D  T  S  D  S  S  A  K  D  C  I  L  Y  A  P  P  S  L  S  A  S  C  C  N  S   211
GCCTACAATGGAAGACAATGTTGTGACACGGGCGTACAGCCCAACCTTGAACACTCCAGTATATCTAACCCCAGCTGCTTCGCCGGTAAG   810
 P  T  M  E  D  N  V  V  T  R  A  Y  S  P  T  L  N  T  P  V  Y  L  T  P  A  A  S  P  V  S   241
CAATGTTCCCGGCGCGCCGTCCACACCACTCAGCGGTGATTCAGAAAGCACCAAGGATGAGTCTTCCGACATGGAAACCGACGATGTTGA   900
 N  V  P  G  A  P  S  T  P  L  S  G  D  S  E  S  T  K  D  E  S  S  D  M  E  T  D  D  V  E   271
ATCGTGCAAACTTACCGAAAAACCGCATACTCCCCAAATACACAAAGAGGCTCCTTTGCCGTTTGTAACTACATCTTTTATGATGCCCAC   990
 S  C  K  L  T  E  K  P  H  T  P  Q  I  H  K  E  A  P  L  P  F  V  T  T  S  F  M  M  P  T   301
TATATCACAACCAACCACCATCCCCGCTTCGTCCATTCAACCCATAAAGCTCCTACAGAACGGCAGTGCACAATTTCTGCCCATAACACC  1080
 I  S  Q  P  T  T  I  P  A  S  S  I  Q  P  I  K  L  L  Q  N  G  S  A  Q  F  L  P  I  T  P   331
GAAAAGCAATATTCACACCATGCCCATTGTGATCATGGGTAACATGGAGTCGCTACAGCACGCACAAAGCGCAGTGCTGCTTATGGTTAA  1170
 K  S  N  I  H  T  M  P  I  V  I  M  G  N  M  E  S  L  Q  H  A  Q  S  A  V  L  L  M  V  N   361
TCCGAAGATTCACGTACAGCCTTCAACACCCGTTTCTCTCCCGATGGGAGGTAAACCCTCCCAGTTGGCACCGGCGCCTCAAAAACTCTA  1260
 P  K  I  H  V  Q  P  S  T  P  V  S  L  P  M  G  G  K  P  S  Q  L  A  P  A  P  Q  K  L  Y   391
TTCGCTTGCGCCGGCACCGCAGGCATTGCGGGCTACGCCGCCAACATCACCCACTGGCATAATGCAACCATCGAGCACATATTTAACGGG  1350
 S  L  A  P  A  P  Q  A  L  R  A  T  P  P  T  S  P  T  G  I  M  Q  P  S  S  T  Y  L  T  G   421
CGGTAATCGCTCCAATGAAACAAACCGACGTCGTACCCACATCTGCCCCTACGAAAACTGCGGAAAGACGTATTTCAAGAGCAGTCACCT  1440
 G  N  R  S  N  E  T  N  R  R  R  T  H  I  C  P  Y  E  N  C  G  K  T  Y  F  K  S  S  H  L   451
TAAAGCTCATCTGCGGACACACACCGGCGAGAAGCCCTTTAAATGTCAGTGGGAAAGTTGTGGGAAATGTTTTGCAAGGTCAGACGAACT  1530
 K  A  H  L  R  T  H  T  G  E  K  P  F  K  C  Q  W  E  S  C  G  K  C  F  A  R  S  D  E  L   481
CTCCCGACACAGACGAACCCACACCGGTGAAAAGCGATTCGTATGTCCGACATGTGATCGGCGGTTCATGCGAAGCGACCACCTCACAAA  1620
 S  R  H  R  R  T  H  T  G  E  K  R  F  V  C  P  T  C  D  R  R  F  M  R  S  D  H  L  T  K   511
GCACATGAAGCGGCACAGCGGAAACCGGAAAATTCCCAACTGGTAGAAGGAAATAAACAAGCTGAAGGCAACTTCAGCACCTGTTGAACA  1710
 H  M  K  R  H  S  G  N  R  K  I  P  N  W  *                                                525
ATCGCCCAACAGCCCAAAAATTATTTCGACATCACTAACCTCGATTTTAACTCCAATCACATCGAACGGATCGCGAAACCAAATCAAAAT  1800
CGCACCTAAAATGAGTCCAACTCAGTCACATCAGGGTTTTCAATACGTCAGCCTTCCAAGCACGACGACTTGCCGGTTACCTTTGATGGC  1890
TGTTTCAACCTCGTAAATCAGCTTTTATGTTTATTTTACATAACATTTTGACCACCTAGCTTGAAGCACTATTCTTTCTTTCATTTTGTC  1980
TCACACCGTTGAGTCAAAAACCGCATTCACTCTGTATGTTACTTTACTGCAGCTAAGCGCATGTGTGATGATTGAGTGCAATACGGTGTG  2070
GAGTCAGTCCTTCGAAAGTGCCCTGGTTGTATTGTTTGCAACATGAACGGTCTTTCAGAATGCTGCACTATATTTTCGTTGTTCAGAAAC  2160
CTCTTTTTGTACACTTCCTTTTTTTAAGTGTTTTGTAATACGTGTACAATGTACATGATCCACGTTCCCTTTAGTATCACGTTATTAATA  2250
TACGCGCCATACCAAAAAAAAAAAAAAAAAA                                                            2281
```

FIG. 5. Nucleotide and deduced amino acid sequence of the *pem-4* cDNA clone. The 2281-bp insert includes a single open reading frame that encodes a polypeptide of 525 amino acids. The termination codon is shown by an asterisk. A predicted signal for nuclear localization (amino acid positions 83–98) is underlined. Three predicted C2H2-type zinc finger motifs in the C-terminal region are shown by open letters (amino acid positions 436–458, 466–488, and 496–516). The 6-bp sequence (TTTATT) in the 3′ UTR is shown by a dotted underline.

The localization and segregation of pem-5 mRNA. The localization and segregation of *pem-5* mRNA also marked the posterior end of developing embryos (Fig. 8). However, as in the case of the *pem-4* transcript, the *pem-5* distribution pattern was not so evident until the 8-cell stage and was much narrower at later stages than that of *pem-2.* At the 8-cell stage, the *pem-5* transcript was found to be distributed in the very narrow, posterior region of B4.1 cells (Figs. 8A and 8B). At the 16-cell stage, *pem-5* mRNA was restricted to the posterior cytoplasm of the B5.2 cells (data not shown). Then, positive hybridization signals for the *pem-5* transcript were detected in the posterior cytoplasm of B6.3 in 32-cell-stage embryos (Fig. 8C) and then in B7.6 of the 64-cell-stage embryos (data not shown). At the gastrula stage,

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

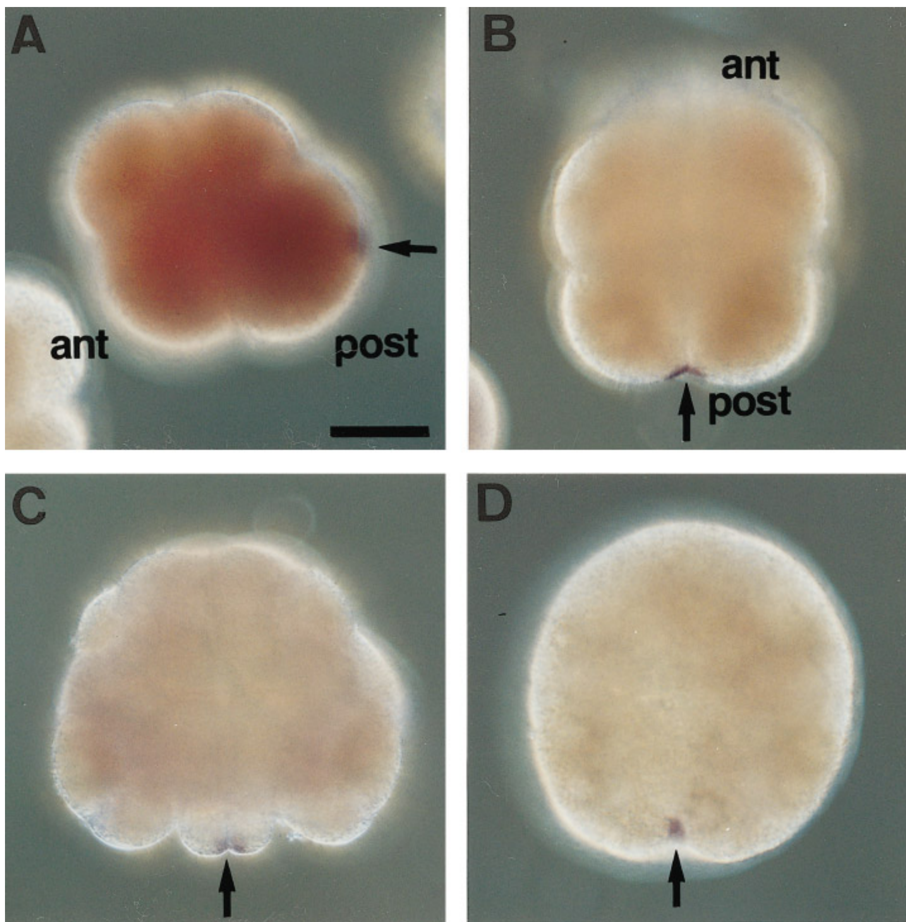


FIG. 6. Distribution of *pem-4* maternal mRNA as revealed by whole-mount *in situ* hybridization. (A, B) An 8-cell embryo: (A) lateral view and (B) vegetal pole view. ant, anterior; post, posterior side of the embryo. The very narrow localization of *pem-4* mRNA in the posterior side of the embryo is shown by arrows. (C) A 32-cell embryo, vegetal pole view. (D) A midgastrula, vegetal pole view. *pem-4* mRNA marks the posterior end of the embryo (arrow). Scale bar represents 50 μm for all photographs.

the *pem-5* transcript was seen in 2 invaginating posterior cells (Fig. 8D). Thus, the *pem-5* signals in embryos up to the gastrula stage appeared to be restricted to a region more posterior than that of the *pem-2* signals. However, the hybridization signals were undetectable in the B7.6 descendants at the tailbud stage (data not shown). Thus, the distribution of the *pem-5* transcript also marks the posterior-most region of early embryos, and the maternal transcript was diminished by the tailbud stage.

pem-6

Characterization of the pem-6 cDNA clone. Northern blot analysis also revealed a maternal transcript of *pem-6,* which was about 1.7 kb in length. The cDNA sequence of *pem-6* was 1646 bp long (Fig. 9). The cDNA had a single open reading frame encoding a polypeptide of 202 amino acids. Database searches indicated that none of the reported proteins had similarity to the predicted PEM-6 protein.

The localization and segregation of pem-6 mRNA. The localization and segregation of *pem-6* mRNA also marked the posterior end of developing embryos (Fig. 10). However, as in the case of the *pem-4* and *pem-5* transcripts, the *pem-6* distribution pattern was not so evident until the 8-cell stage and was much narrower at later stages than that of *pem-2.* At the 8-cell stage, the distribution of the *pem-6* transcript was restricted to the very narrow, posterior region of B4.1 cells (Figs. 10A and 10B). At the 16-cell stage, *pem-6* mRNA was found in the posterior-most region of the embryo, only in the posterior cytoplasm of the B5.2 cells (data not shown). Positive hybrid-

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

```
CGTATTTATTTTAGCATGGTGGACTCAAAATACTCGGTCGCTTGTGCCTAACTGAGAAAAGGATGAATTTAAAGGAGATAGTTGAATGTT   90
                                                   M  N  L  K  E  I  V  E  C  F   10
TTAATGCTCCCATCAAAGAAGAACAAGCTTGGGCAGTATGTCACCAGTGCGGAAAATACCTCAAAAAGCAAGAGTCGGAGAACATCGATC  180
 N  A  P  I  K  E  E  Q  A  W  A  V  C  H  Q  C  G  K  Y  L  K  K  Q  E  S  E  N  I  D  L   40
TATCTAAGCCAAGGTACACTGCCCCAGAGGGATTACATGCAGCTTATTTCGGTGAAAATGGACGCATAGATTTGCTAGGAGAACCCACAG  270
 S  K  P  R  Y  T  A  P  E  G  L  H  A  A  Y  F  G  E  N  G  R  I  D  L  L  G  E  P  T  E   70
AATTCAGTGAAGAGATCACCCTCGGTCGAGTTGGACTCGTCGTATTTTCCGCTCTCAACTACGGTCTGGGAGCTCACCAGGAACAAGAGA  360
 F  S  E  E  I  T  L  G  R  V  G  L  V  V  F  S  A  L  N  Y  G  L  G  A  H  Q  E  Q  E  I  100
TCTCTCTCTCGTTGGATGACGTAATTTCACGGATGACTGCGCATCTTCAGACGGGGGCGGACGAGGAATACCACGACGAGGGTTACGACG  450
 S  L  S  L  D  D  V  I  S  R  M  T  A  H  L  Q  T  G  A  D  E  E  Y  H  D  E  G  Y  D  G  130
GCGATAAACCGTCCACCGACACCGGGTATCAGTCATGTACGATCGACGAATTATTAACCTTTTGCGAGAGCCGATTGTCGCAGAGATGGG  540
 D  K  P  S  T  D  T  G  Y  Q  S  C  T  I  D  E  L  L  T  F  C  E  S  R  L  S  Q  R  W  E  160
AAGCGGACGTGCACTATACCGCAGTGTGTTCGGCGATGCTGCGTGAGGCCCAGGAACTCAAGATTTTCCTCAGCAAAGTAGAATCCGCAA  630
 A  D  V  H  Y  T  A  V  C  S  A  M  L  R  E  A  Q  E  L  K  I  F  L  S  K  V  E  S  A  N  190
ACCAGAGTTTGCGGAAACTTACGCAAGTCACGTCATCGTCACGTGATCCACACTGTGAATCTCCTGATGAAATACCGAACTTGGGATTTG  720
 Q  S  L  R  K  L  T  Q  V  T  S  S  S  R  D  P  H  C  E  S  P  D  E  I  P  N  L  G  F  A  220
CTGATTGGGCCCACCTGTGGATGCAAGTGTTGGACCAGCTACGAAAGGGGGTGACCCGACTGAAAAAGGTCGAGCAACAAACCCGGAATT  810
 D  W  A  H  L  W  M  Q  V  L  D  Q  L  R  K  G  V  T  R  L  K  K  V  E  Q  Q  T  R  N  S  250
CGGTGGAGTACGAACTGACTCCATACGAAATCCTAATGGAAGACATTAGACAGAAGAAGTACACTCTGAAAAAGGTGACGCAGTTCGAGC  900
 V  E  Y  E  L  T  P  Y  E  I  L  M  E  D  I  R  Q  K  K  Y  T  L  K  K  V  T  Q  F  E  L  280
TGCTACCGCCTCAAGTGAAGAAAAGTGCTCATGACGTCATACTGGAGTTTATTCGTTCACGACCCAACCTGTCGCCGGCCCACCAGCGAA  990
 L  P  P  Q  V  K  K  S  A  H  D  V  I  L  E  F  I  R  S  R  P  N  L  S  P  A  H  Q  R  I  310
TACTAGCCCCCACACCCCCAAGGTCAAAGTCCCTGCACGAACAACTAATCGAGCAGATACACGAGCAAGCGGTGCCTCTTCGTTCAATCG 1080
 L  A  P  T  P  P  R  S  K  S  L  H  E  Q  L  I  E  Q  I  H  E  Q  A  V  P  L  R  S  I  D  340
ACAACCTGTCAATTAAACCTTCGAGAACTTTCGACGTCGGTAAAAGACCAGACACCGAGTCGATCGTCGGAATGACGTCATCAAAATCCT 1170
 N  L  S  I  K  P  S  R  T  F  D  V  G  K  R  P  D  T  E  S  I  V  G  M  T  S  S  K  S  C  370
GCCGACGAATTTTGAAGCTGGAAAATCTGAGCGACGACGAAAACGACTCGTTCACAGAGTCGGATTCGGATTATGACGTAACAAGCGGCC 1260
 R  R  I  L  K  L  E  N  L  S  D  D  E  N  D  S  F  T  E  S  D  S  D  Y  D  V  T  S  G  H  400
ACAATGACGTCAATACGACCGAGGACCTGGACCAATCGTACTCGCCACTTCGACCTTTCAACAGATCTACGACTTCCTCTGCCCGATCGT 1350
 N  D  V  N  T  T  E  D  L  D  Q  S  Y  S  P  L  R  P  F  N  R  S  T  T  S  S  A  R  S  S  430
CGGGAACTTCTGGTGTGTCGTCTTCCAGCCACGGCGACGCCAGCGAGCCCACTACCGCCACCCACCGACCATTGTCGATACGATTTTCCG 1440
 G  T  S  G  V  S  S  S  S  H  G  D  A  S  E  P  T  T  A  T  H  R  P  L  S  I  R  F  S  D  470
ATTCAGTTCGTCGAAAACGACTTCTACCGCTCGAGCTGCCGCCCAGTCCGGCCAAAACTCCGACCGACTCCCCTGCTGGCCACCTCCCGT 1530
 S  V  R  R  K  R  L  L  P  L  E  L  P  P  S  P  A  K  T  P  T  D  S  P  A  G  H  L  P  S  500
CCTTCGTCCCATCTTTTCTAAGCAAGCTGCCCTCGCGCGCCCTTCGCCGGTCGCAGAGCGTCGCCCTTCGTTCCAACCACACACCGCGGC 1620
 F  V  P  S  F  L  S  K  L  P  S  R  A  L  R  R  S  Q  S  V  A  L  R  S  N  H  T  P  R  P  530
CAAGTTTGTTGGATCGCAAAATCACCTGGCTGGACCCTTTGTCGCCAGAAAGCCCCACGAGAAATGACGTCATGCTTCCGCCTCCTCCGC 1710
 S  L  L  D  R  K  I  T  W  L  D  P  L  S  P  E  S  P  T  R  N  D  V  M  L  P  P  P  P  R  560
GACCCCCAGTGACAGCGGAAGAAAAGCGACAATACTTCAGACGCACAAAGTCTGAGCGGAAACGAAATACCACTGTGGATTGTTCCGATT 1800
 P  P  V  T  A  E  E  K  R  Q  Y  F  R  R  T  K  S  E  R  K  R  N  T  T  V  D  C  S  D  W  590
GGCAGCAACCAGTCGCGAGTCTGTCACTGACAGTGGAAGAAGTAATGCACATCAGGCAGGTACTTACCAGGGCTGAGCTGGAGAAGTTTC 1890
 Q  Q  P  V  A  S  L  S  L  T  V  E  E  V  M  H  I  R  Q  V  L  T  R  A  E  L  E  K  F  Q  620
AATCCGACAAGGAGCTCTACAGGGCGCTTAAAAACGAAAAGGTGTGTTTCACATGCCGAACGAAGAAATTTACCGTATTTTCGTGGCCCA 1980
 S  D  K  E  L  Y  R  A  L  K  N  E  K  V  C  F  T  C  R  T  K  K  F  T  V  F  S  W  P  N  650
ACACCTGTGAGATTTGCAAGATGAAAATTTGCTCCAAATGTACGAGAAAGGTACGGGCACCCACCAATAAATACCTCGAAACACCAGTGT 2070
 T  C  E  I  C  K  M  K  I  C  S  K  C  T  R  K  V  R  A  P  T  N  K  Y  L  E  T  P  V  F  680
TCACGTTAAGTCCTTCGATGCATAAAATGTCCGGCGCCCACTCGGTCTACTCCTTTAACAGGTTTGACAGTAAACACCAACCAGTGTCTC 2160
 T  L  S  P  S  M  H  K  M  S  G  A  H  S  V  Y  S  F  N  R  F  D  S  K  H  Q  P  V  S  P  710
CGATCAACTTATTACGTGAGCAGTACCAACGTGACTCCGGCGAATCGATAACCTCGCAAATTTGGAACCGCTCCCGAAAAATGGACGTTT 2250
 I  N  L  L  R  E  Q  Y  Q  R  D  S  G  E  S  I  T  S  Q  I  W  N  R  S  R  K  M  D  V  C  740
GTTGCGAGTGCCACCTACTTTTGTCCGGCGTCGTAAATAGCAGCCGCGAGGTGGCGCGCCAACACATCTACACCAGTAACGCCGCTTAGC 2340
 C  E  C  H  L  L  L  S  G  V  V  N  S  S  R  E  V  A  R  Q  H  I  Y  T  S  N  A  A  *     768
CGTGATTTTGTAGATTTCCAGCACAGTTTTATTAATTTATTCACTATATGTTAACCAAGAGTTTAAATTGCACATTCCTACCAAAGTATC 2430
TTTTGTCTGTATAAAGTGTGATAAAGGTTTTCTTCCATATTTCGTTGTGTGTTTTTCGAGAGCAGATATCTACGTTTACCGAACCCAACT 2520
TCGTTTAAAAGTCTTCTGTTTTAACGCTCCCATGTTCCCGCAACCTTAGGTGGCGTGTGAATCTATTTACGAGTATCCCGTTTGCTGCAA 2610
TATGTTGTTTGCTTTACTGTGCTTGGTGCAAATACACAAACGTCCTCCACGTGAAAAAAAAAAAAAAAAAAAAAAAAA            2688
```

FIG. 7. Nucleotide and deduced amino acid sequence of the *pem-5* cDNA clone. The 2688-bp insert includes a single open reading frame that encodes a polypeptide of 768 amino acids. The termination codon is shown by an asterisk. The predicted amino acid sequence shows no similarity to known proteins. The 6-bp sequences (TTTATT) in the 3′ UTR are shown by dotted underlines.

ization signals for the *pem-6* transcript were detected in the posterior cytoplasm of B6.3 in the 32-cell-stage embryos (Fig. 10C) and then in B7.6 of the 64-cell-stage embryos (data not shown). At the gastrula stage, the *pem-6* transcript was also seen in 2 invaginating posterior cells (Fig. 10D). However, hybridization signals were undetect-

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

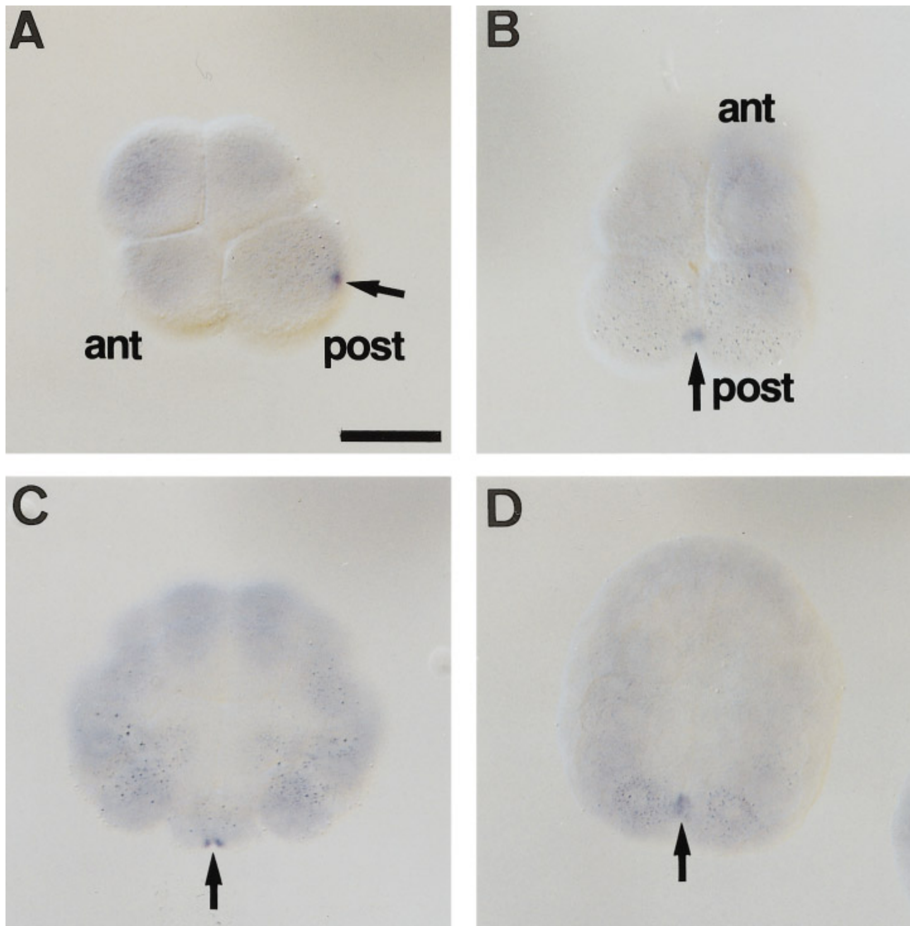


FIG. 8. Distribution of *pem-5* maternal mRNA marks the posterior end of developing embryos, as revealed by whole-mount *in situ* hybridization. (A, B) An 8-cell embryo: (A) lateral view and (B) vegetal pole view. ant, anterior; post, posterior side of the embryo. The very narrow localization of *pem5* mRNA in the posterior side of the embryo is shown by arrows. (C) A 32-cell embryo, vegetal pole view. (D) A midgastrula, vegetal pole view. *pem-5* mRNA marks the posterior end of the embryo (arrows). Scale bar represents 50 μm for all photographs.

able in the B7.6 descendants at the tailbud stage (data not shown). Thus, the distribution of the *pem-6* transcript also marks the posterior-most region of early embryos, and the maternal transcripts are diminished by the tailbud stage.

DISCUSSION

Maternal factors localized in the posterior–vegetal cytoplasm of an ascidian egg are essential for cell specification and pattern formation of the embryo (reviewed by Satoh, 1994). The molecular identification of these localized factors and the elucidation of the machinery associated with the localization are therefore key research subjects. The *pem* cDNA clone was originally obtained by differential screening of a black fragment cDNA library with a red fragment cDNA library of *C. savignyi* unfertilized eggs (Yoshida *et al.,* 1996). In the present study, we attempted the isolation of maternal genes with localized mRNA by another approach. We constructed a cDNA library of fertilized egg mRNAs subtracted with gastrula mRNAs. The library was estimated to contain about 1000 independent clones. Thus far, we have examined 102 clones that were randomly selected from the library. The determination of localization of the corresponding mRNAs by whole-mount *in situ* hybridization revealed that 5 independent clones showed localization of the corresponding mRNAs.

Interestingly, all of the six clones including *pem* showed localization in the posterior–vegetal cytoplasm

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

```
CCAACACTAGCAAAATAAAAGGAAACCTAATTATTAAAACTGGTGATAAGTGACGATTTTCATCTGGTATATAAACATATCCACTACTAA   90
GTCACAGCCTAGTGTAGAAGTTGATTATCGGTACCAACACTACTTGTACCCCACGAGCTATTTCTGAGACATTTTGAAACCGCTAACATG  180
                                                                                      M     1
GAAGGAGCTAGTCAGTCGAGTCAAGCACAGTTGTGTCGGAGAAACGGATGCGGATTTTATGGAAATTCAAAATTTGAGGGAATGTGCTCG  270
E  G  A  S  Q  S  S  Q  A  Q  L  C  R  R  N  G  C  G  F  Y  G  N  S  K  F  E  G  M  C  S    31
ATGTGCTATAAGGACACTGTTCAGAAAAAGAACAACTCCGGCCGAAAATCGCCAGCTGTTTCCATCTCATCCAAGTCACCAGAACCCCCA  360
M  C  Y  K  D  T  V  Q  K  K  N  N  S  G  R  K  S  P  A  V  S  I  S  S  K  S  P  E  P  P    61
AGTTTGAATGAGAAACAAGACGGATCGGTAGCAAATGCAATGGCCTCTCTTGCAACAAACGATGGCCCCAATACAACGATAAGTGCAACA  450
S  L  N  E  K  Q  D  G  S  V  A  N  A  M  A  S  L  A  T  N  D  G  P  N  T  T  I  S  A  T    91
GCTTCACCTACTGGAAGCACCCCCATTTCGATCCCCTCAGCCACGAATCCAACTACTTCTCTCACCTCCCCTTCCCCCAAGAACTCAAGT  540
A  S  P  T  G  S  T  P  I  S  I  P  S  A  T  N  P  T  T  S  L  T  S  P  S  P  K  N  S  S   121
TTCGACGAAGCAAGCACGTCGTTTGACAGCCAATCCTCCCCTTCAAAACCCAAGAAAAATCGATGTGCCAGCTGCAGGAAACGCCTTGGC  630
F  D  E  A  S  T  S  F  D  S  Q  S  S  P  S  K  P  K  K  N  R  C  A  S  C  R  K  R  L  G   151
CTTACTGGCTTCTACTGTAGATGTGGCCAGATTTTCTGCAGCCTGCATCGATATTCCGACCAACATTCGTGCGACTTTGACTACAAGGCG  720
L  T  G  F  Y  C  R  C  G  Q  I  F  C  S  L  H  R  Y  S  D  Q  H  S  C  D  F  D  Y  K  A   181
GACGCTCAGGCGAAAATCCGAAAAGAAAACCCTGTCATTGTCGGCGAAAAGATCAACAAAATCTAAAGAAAAAAACAACTTCTTTCACGC  810
D  A  Q  A  K  I  R  K  E  N  P  V  I  V  G  E  K  I  N  K  I  *                           202
CACCCGCGCGCGATCATTTCCCCTCCTGTGTAGAAACTTTTTTGTTTTCGTCATTTTACTTTATACTAGGCCCAGTAATAAACATATGTA  900
TATTATTAAACAGGACTTTCCTTTCACAAACACTAGTAATTCATTGGTCATATACCTATTTTCAAAAATCGCGCCGAGAACATTTGTCTT  990
GATGGGGCTTTTGGCCGGCCATGTTTTCTTGAAATCCCGTATAATACAATTTCTTGTGTTCTTACCATAATAACGTCACTTGTATAGTTT 1080
GCTGCCCTTTTAATCACACTTACTTAGCTAATATTCCTTTAAGATGGGCGCCCTGTGCGCGTTGTGTCTTAAAGGATAATTGATTTCTTA 1170
TTTATTCTGCGTTGTTGCATATTTTATCAGCGGTAAGGACCCCCCCGCATGGCATTTTAATTTCCTTTCCACGAAAATACCAGTAGGCTT 1260
TTTTCTCCTTTCCTCCATTAAAACTAGTTTTTTTTTGTTTCCTTGCATATCTAGTATAGCGTTAATTGCATGTGTGGATTTTAGAGGGTG 1350
TTTTGTTTTTCCAACGCTTGGTGTCGTTTTCGTGCCCCGGAAATTTGTCCCCGGGTGTTTTCTGGCCCCGTTGTTTATGCCTCAAGGAGC 1440
GCCACTTTTTCAAGCATAATTGTTGTCCAGTCCGTCACCACTCCACACGGCTGCATTTTAATCTCGCAGCCACGCTGATCGAATCCACAG 1530
TATCGAATTGTACGCATGCTTCTGTGCTCATTGCTACATTGCAACACGTGTACAGTGAGTTATAAGATGCTGAATAAACCATTATCTGTA 1620
GAAAAAAAAAAAAAAAAAAAAAAAAA                                                                 1646
```

FIG. 9. Nucleotide and deduced amino acid sequence of the *pem-6* cDNA clone. The 1646-bp insert includes a single open reading frame that encodes a polypeptide of 202 amino acids. The termination codon is shown by an asterisk. The potential signal sequence for polyadenylation is underlined. The predicted amino acid sequence contains no consensus motifs. The 6-bp sequence (TTTATT) in the 3′ UTR is shown by a dotted underline.

of fertilized eggs and early embryos. In other words, our previous (Yoshida *et al.,* 1996) and present studies did not isolate cDNA clones that exhibit localization in regions other than the posterior–vegetal cytoplasm, except clones of the seven mitochondrial genes that showed a localization/segregation pattern similar to that of mitochondria or the myoplasm (cf. Fujiwara and Satoh, 1990). The cDNA clones for maternal genes with localized mRNA, other than the *pem* group, were isolated from *Styela clava* by Swalla and Jeffery (1995, 1996). They reported that *yellow crescent (YC)* RNA and *PCNA* mRNA are localized or enriched in the myoplasm and the ectoplasm, respectively. Despite of their previous findings, our studies suggest that a considerable part of the localized mRNAs are restricted to the posterior-most region of the early ascidian embryo. We examined only 10% of the subtractive library clones and were able to obtain five clones with a *pem*-like expression pattern. Many other clones with a *pem*-like expression pattern are expected to be isolated by further characterization. It seems surprising that about 5% of the library clones showed localization in the posterior–vegetal cytoplasm. However, this may be partially due to the method we adopted. Our library was constructed with fertilized egg mRNAs that are selectively concentrated by subtractive hybridization with gastrula mRNAs. It is expected that there are many maternal mRNAs without localization. If these mRNAs are retained by the gastrula stage (cf. Beach and Jeffery, 1990; Miya and Satoh, 1997), then the subtractive procedure failed to pick up these mRNAs for the objectives. On the other hand, if localized mRNAs have an inclination to diminish or decrease dramatically by the gastrula stage [see Fig. 1 for *pem-2,* and similar results were obtained in the other *pem*s (data not shown)], the subtractive procedure might preferentially select these mRNAs as the objectives. Thus, the ratio of cDNA clones for localized mRNAs might rise within the library.

The localization of *pem*s to the posterior–vegetal cytoplasm suggests that all of these mRNAs are anchored to this restricted region by some mechanisms. Is the localization of all of these mRNAs controlled by a common mechanism or is their localization regulated by independent mechanisms? It has been shown that the 3′ untranslated regions (3′ UTRs) of mRNAs often contain the information for their localization (reviewed by Ding and Lipshitz, 1993). One possible mechanism is therefore that the 3′ UTRs of the six *pem*s contain a common structural motif that might mediate their subcellular localization. We therefore examined whether the 3′ UTRs of these mRNAs contain common motifs. We found that they share a six-base motif ''UUAUUU,'' which are shown by dotted underlines in Figs. 2, 5, 7, and 9. It should be determined in future studies whether this motif is critical for the localization of the six *pem*s. In addition, it is possible that three-dimensional

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

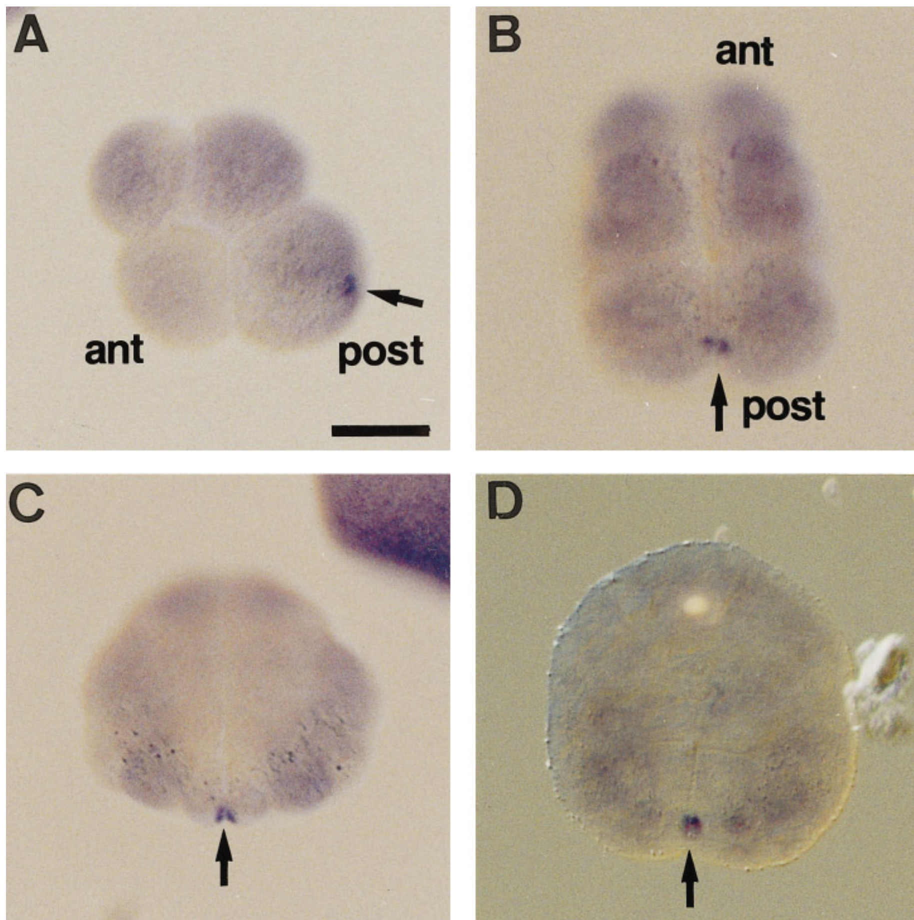


FIG. 10. Distribution of *pem-6* maternal mRNA marks the posterior end of developing embryos, as revealed by whole-mount *in situ* hybridization. (A, B) An 8-cell embryo: (A) lateral view and (B) vegetal pole view. The very narrow localization of *pem-6* mRNA is shown by arrows. ant, anterior; post, posterior side of the embryo. (C) A 32-cell embryo, vegetal pole view. (D) A midgastrula, vegetal pole view. *pem-6* mRNA also marks the posterior end of the embryo (arrow). Scale bar represents 50 μm for all photographs.

structures of these mRNAs, rather than the six-base motif, are critical for their localization.

The pattern of localization and segregation of *pem* and *pem-2* mRNAs in eggs and embryos up to the eight-cell stage closely resembles that of the so-called myoplasm. The myoplasm of ascidian eggs contains muscle determinants (Nishida, 1992; Marikawa *et al.,* 1995), factors for the anteroposterior axis formation (Nishida, 1994b), and factors for the initiation of gastrulation (Jeffery, 1990; Nishida, 1996). These factors may be present as localized maternal mRNAs or proteins. The myoplasm is a unique cytoskeletal domain (reviewed by Jeffery and Swalla, 1990) which consists of several cytoskeletal proteins (Swalla *et al.,* 1991; Nishikata and Wada., 1996). Therefore, it is likely that the localization and segregation of *pem* mRNAs in eggs and embryos up to the eight-cell stage are dependent on the myoplasmic cytoskeletal domain. Experiments with several reagents which disturb the myoplasm cytoskeletal components indicated that the cytoskeletal components required for the localization of the myoplasm are also required for the localization of *pem* mRNAs during the ooplasmic segregation (Yoshida *et al.,* unpublished). However, as shown in our studies, after the eight-cell stage, the *pem* mRNAs are not segregated with the myoplasm but are restricted to the posterior end of the embryo. This suggests the presence of another anchoring mechanism in the posterior-most region of the embryo. The identification of such anchoring mechanisms is an intriguing future research subject.

PEM-2 protein is expected to function in some signaling pathway, since it has a SH3 domain and a GDS domain (Boguski and McCormick, 1993). PEM-4 protein is expected to function as a transcription factor, since it has three C2H2-type zinc fingers and a nuclear localization signal. PEM-5 and PEM-6 proteins do not show any known motif.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

The functions of the PEMs are under investigation. Overexpression of *pem* by microinjection of synthesized *pem* RNA into fertilized eggs resulted in development of tadpole larvae with deficiency of the adhesive organ, brain, and sensory pigment cells (Yoshida *et al.,* 1996). However, overexpression of *pem-2, pem-4, pem-5,* and *pem-6* by microinjection of synthesized RNAs did not show any effects on development. Microinjection of *pem-2* mRNA without the SH3 domain or GDS domain showed no effects on development. Neither *pem-4* mRNA without the zinc finger motif affected the morphogenesis of the embryo. Therefore, at present, functions of these localized maternal messages are unclear.

At the 32-cell stage, a pair of very small blastomeres are formed at the posterior-most region of the vegetal hemisphere (cf. Figs. 4G, 6C, 8C, and 10C). This blastomere is named B6.3 and contains the developmental fates to give rise to muscle, endodermal strand, and mesenchyme. All of the *pem*-group mRNAs are localized in the posterior cytoplasm of B6.3 blastomeres. However, destruction of the blastomere by a fine glass needle at this stage did not affect the development, and morphologically normal tadpole larvae developed (Yoshida *et al.,* 1997). This result suggests that PEMs have completed their function before the 32-cell stage.

In summary, we have isolated four cDNA clones encoding mRNAs that are localized in the posterior–vegetal cytoplasm of the ascidian egg and early embryo. Because our subtractive library contains a high percentage of cDNA clones encoding localized mRNAs, we will be able to isolate such cDNA clones further. The characterization of such localized maternal messages might disclose the molecular nature and localization mechanisms of the developmentally important egg-cytoplasmic information.

ACKNOWLEDGMENTS

We thank Mr. Kouichi Morita and all staffs of the Otsuchi Marine Research Center for their hospitality and their help in collecting ascidians. Y.S. was supported by a Predoctoral Fellowship from the Japan Society for the Promotion of Science for Japanese Junior Scientists with Research Grant 8-6806. This research was supported by a Grant-in-Aid for Specially Promoted Research (07102012) from the Ministry of Education, Science, Sports and Culture, Japan to N.S. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases with Accession Nos. AB001770 for *pem-2,* AB001771 for *pem-4,* AB001772 for *pem-5,* and AB001773 for *pem-6.*

REFERENCES

Adams, J. M., Houston, H., Allen, J., Lints, T., and Harvey, R. (1992). The hematopoietically expressed vav proto-oncogene shares homology with the dbl GDP-GTP exchange factor, the bcr gene and yeast gene (CDC24) involved in cytoskeletal organization. *Oncogene* **7,** 611–618.

Beach, R. L., and Jeffery, W. R. (1990). Temporal and spatial expression of a cytoskeletal actin gene in the ascidian *Styela clava. Dev. Genet.* **11,** 2–14.

Boguski, M. S., and McCormick, F. (1993). Proteins regulating Ras and its relatives. *Nature* **366,** 643–654.

Chabry, L. (1887). Contribution a l'embryologie normale et teratologique des Ascidies simples. *J. Anat. Physiol. (Paris)* **23,** 167–319.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidium thiocyanate–phenol–chloroform extraction. *Anal. Biochem.* **162,** 156–159.

Conklin, E. G. (1905). The organization and cell lineage of the ascidian egg. *J. Acad. Nat. Sci. (Philadelphia)* **13,** 1–119.

Czernilofsky, A. P., Levinson, A. D., Varmus, H. E., Tischer, E., and Goodman, H. (1983). Correction to the nucleotide sequence of the src gene of Rous sarcoma virus. *Nature* **301,** 736–738.

Ding, D., and Lipshitz, H. D. (1993). Localized RNAs and their functions. *BioEssays* **15,** 651–658.

Fujiwara, S., and Satoh, N. (1990). Pattern of segregation of mitochondria into muscle lineage cells during embryogenesis of the ascidian *Halocynthia roretzi. Dev. Growth Differ.* **32,** 531–539.

Hariharan, I. K., and Adams, J. M. (1987). cDNA sequence for human bcr, the gene that translocates to the abl oncogene in chronic myeloid leukaemia. *EMBO J.* **6,** 115–119.

Ishida, K., Ueki, T., and Satoh, N. (1996). Spatio-temporal expression patterns of eight epidermis-specific genes in the ascidian embryo. *Zool. Sci.* **13,** 699–709.

Jeffery, W. R. (1990). Ultraviolet irradiation during ooplasmic segregation prevents gastrulation, sensory cell induction, and axis formation in the ascidian embryo. *Dev. Biol.* **140,** 388–400.

Jeffery, W. R., and Swalla, B. J. (1990). The myoplasm of ascidian eggs: A localized cytoskeletal domain with multiple roles in embryonic development. *Semin. Dev. Biol.* **1,** 373–381.

Kitamura, N., Kitamura, A., Toyoshima, K., Hirayama, Y., and Yoshida, M. (1982). Avian sarcoma virus Y73 genome sequence and structural similarity of its transforming gene product to that of Rous sarcoma virus. *Nature* **297,** 205–208.

Leto, T. L., Lomax, K. J., Volpp, B. D., Nunoi, H., Sechler, J. M. G., Nauseef, W. M., Clark, R. A., Gallin, J. I., and Malech, H. L. (1990). Cloning of a 67-kD neutrophil oxidase factor with similarity to a noncatalytic region of p60c-src. *Science* **248,** 727–729.

Marikawa, Y., Yoshida, S., and Satoh, N. (1994). Development of egg fragments of the ascidian *Ciona savignyi*: The cytoplasmic factors responsible for muscle differentiation are separated into a specific fragment. *Dev. Biol.* **162,** 134–142.

Marikawa, Y., Yoshida, S., and Satoh, N. (1995). Muscle determinants in the ascidian egg are inactivated by UV irradiation and the inactivation is partially rescued by injection of maternal mRNAs. *Roux's Arch. Dev. Biol.* **204,** 180–186.

Miya, T., and Satoh, N. (1997). Isolation and characterization of cDNA clones for β-tubulin genes as a molecular marker for neural cell differentiation in the ascidian embryo. *Int. J. Dev. Biol.* **41,** 551–557.

Miyamoto, S., Ohya, Y., Ohsumi, Y., and Anraku, Y. (1987). Nucleotide sequence of the CLS4 (CDC24) gene of *Saccharomyces cerevisiae. Gene* **54,** 125–132.

Nishida, H. (1987). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue restricted stage. *Dev. Biol.* **121,** 526–541.

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.

Nishida, H. (1992). Regionality of egg cytoplasm that promotes muscle differentiation in embryo of the ascidian, *Halocynthia roretzi. Development* **116,** 521–529.
Nishida, H. (1993). Localized regions of egg cytoplasm that promote expression of endoderm-specific alkaline phosphatase in embryos of the ascidian *Halocynthia roretzi. Development* **118,** 1–7.
Nishida, H. (1994a). Localization of egg cytoplasm that promotes differentiation to epidermis in embryos of the ascidian *Halocynthia roretzi. Development* **120,** 235–243.
Nishida, H. (1994b). Localization of determinants for formation of the anterior–posterior axis in eggs of the ascidian *Halocynthia roretzi. Development* **120,** 3093–3104.
Nishida, H. (1996). Vegetal egg cytoplasm promotes gastrulation and is responsible for specification of vegetal blastomeres in embryos of the ascidian *Halocynthia roretzi. Development* **122,** 1271–1279.
Nishikata, T., and Wada, M. (1996). Molecular characterization of myoplasmin-C1: A cytoskeletal component localized in the myoplasm of the ascidian egg. *Dev. Genes Evol.* **206,** 72–79.
Ron, D., Tronick, S. R., Aaronson, S. A., and Eva, A. (1988). Molecular cloning and characterization of the human dbl proto-oncogene: Evidence that its overexpression is sufficient to transform NIH/3T3 cells. *EMBO J.* **7,** 2465–2473.
Satoh, N. (1994). ''Developmental Biology of Ascidians.'' Cambridge Univ. Press, New York.
Satoh, N., Makabe, K. W., Katsuyama, Y., Wada, S., and Saiga, H. (1996a). The ascidian embryo: An experimental system for studying genetic circuitry for embryonic cell specification and morphogenesis. *Dev. Growth Differ.* **38,** 325–340.
Satoh, N., Araki, I., and Satou, Y. (1996b). An intrinsic genetic program for autonomous differentiation of muscle cells in the ascidian embryo. *Proc. Natl. Acad. Sci. USA* **93,** 9315–9321.
Satou, Y., Kusakabe, T., Araki, I., and Satoh, N. (1995). Timing of initiation of muscle-specific gene expression in the ascidian embryo precedes that of developmental fate restriction in lineage cells. *Dev. Growth Differ.* **37,** 319–327.
Schweinfest, C. W., Henderson, K. W., Gu, J., Kottaridis, S. D., Besbeas, S., Panotopoulou, E., and Papas, T. S. (1990). Subtraction hybridization cDNA libraries from colon carcinoma and hepatic cancer. *Genet. Anal. Technol. Appl.* **7,** 64–70.
Sive, H. L., and John, T. S. (1988). A simple subtractive hybridization technique employing photoactivatable biotin and phenol extraction. *Nucleic Acids Res.* **16,** 10937.
Swalla, B. J., Badgett, M. R., and Jeffery, W. R. (1991). Identification of a cytoskeletal protein localized in the myoplasm of ascidian eggs: Localization is modified during anural development. *Development* **111,** 425–436.
Swalla, B. J., Makabe, K. W., Satoh, N., and Jeffery, W. R. (1993). Novel genes expressed differentially in ascidians with alternate modes of development. *Development* **119,** 307–318.
Swalla, B. J., and Jeffery, W. R. (1995). A maternal RNA localized in the yellow crescent is segregated to the larval muscle cells during ascidian development. *Dev. Biol.* **170,** 353–364.
Swalla, B. J., and Jeffery, W. R. (1996). PCNA mRNA has a 3′ UTR antisense to yellow crescent RNA and is localized in ascidian eggs and embryos. *Dev. Biol.* **178,** 23–34.
Yoshida, S., Marikawa, Y., and Satoh, N. (1996). *posterior end mark*: A novel maternal gene for localized factors. *Development* **122,** 2005–2012.
Yoshida, S., Satou, Y., and Satoh, N. (1997). Maternal genes with localized mRNA and pattern formation of the ascidian embryo. *Cold Spring Harbor Symp. Quant. Biol.* **62,** in press.

Received for publication April 9, 1997
Accepted September 4, 1997

Copyright © 1997 by Academic Press. All rights of reproduction in any form reserved.