posterior end mark, a novel maternal gene encoding a localized factor in the ascidian embryo

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SUMMARY

Ascidian embryogenesis is regarded as a typical 'mosaic' type. Recent studies have provided convincing evidence that components of the posterior-vegetal cytoplasm of fertilized eggs are responsible for establishment of the antero-posterior axis of the embryo. We report here isolation and characterization of a novel maternal gene, *posterior end mark* (*pem*). After fertilization, the *pem* transcript is concentrated in the posterior-vegetal cytoplasm of the egg and later marks the posterior end of developing ascidian embryos. Despite its conspicuous localization pattern, the predicted PEM protein shows no significant homology to known proteins. Overexpression of this gene by microinjection of synthesized *pem* mRNA into fertilized eggs results in development of tadpole larvae with deficiency of the anteriormost adhesive organ, dorsal brain and sensory pigment-cells. Lineage tracing analysis revealed that the anterior epidermis and dorsal neuronal cells were translocated posteriorly into the tail region, suggesting that this gene plays a role in establishment of anterior and dorsal patterning of the embryo. The ascidian tadpole is regarded as a prototype of vertebrates, implying a similar function of *pem* in vertebrate embryogenesis.

Key words: ascidian, cytoplasmic factors, maternal messenger RNA, localization, patterning, *posterior end mark*

INTRODUCTION

Animal body plan is established by a series of complex elementary processes, usually initiated by maternal information confined to a particular region of the egg cytoplasm (Wilson, 1925; Davidson, 1986). In *Drosophila*, there is a great deal of information on the genetic program responsible for establishment of the body plan (reviewed by Levine and Harding, 1989; St. Johnston and Nüsslein-Volhard, 1992), while many maternal factors have been clarified for *C. elegans* early embryonic cell specification (reviewed by Bowerman et al., 1993; Priess, 1994; Bowerman, 1995). However, our knowledge of maternal genes responsible for the formation of the vertebrate body plan is incomplete (reviewed by Gurdon, 1992; Davidson, 1994), although maternal *Vg-1* (Weeks and Melton, 1987) and *noggin* (Smith and Harland, 1992) have been implicated in embryonic axis formation during amphibian development.

The fertilized ascidian egg develops quickly into a tadpole larva, which comprises a small number of tissues including epidermis, central nervous system with two sensory organs, nerve cord, endoderm, mesenchyme, notochord and muscle (reviewed by Satoh, 1994). The lineages of these embryonic cells have been 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), and factors for the establishment of the embryonic anteroposterior axis (Nishida, 1994b) and for initiation of gastrulation (Jeffery, 1990). Several studies have been performed to elucidate the molecular nature of these determinants. For example, using two closely-related species, *Molgula oculata* and *M. occulta*, the former produces tailed (urodele) larvae while the latter produces tailless (anural) larvae, Swalla et al. (1993) isolated two maternal genes that are expressed exclusively in eggs and embryos of the tailed ascidian species. In addition, Swalla and Jeffery (1995) recently isolated cDNA clones for maternal RNA that is abundant in the myoplasm in *Styela* eggs. However, little is known about the molecular nature or mode of action of the localized factors in ascidian eggs.

We report here isolation and characterization of a novel maternal gene expressed in the ascidian egg. This gene was named *posterior end mark* (*pem*), because the transcript initially concentrates to the posterior-vegetal cytoplasm of the fertilized egg and later the distribution of the transcript marks the posterior end of developing ascidian embryos. The amino acid sequence of the *pem* gene product showed no significant

homology to known proteins and contains no motifs specific for transcriptional factors, integral membrane proteins or signaling molecules. However, overexpression of this gene by microinjection of synthesized *pem* mRNA into fertilized eggs resulted in development of tadpole larvae with deficiency of the anteriormost adhesive organ, dorsal brain and sensory pigment-cells. Lineage tracing analysis revealed that the anterior epidermis and dorsal neuronal cells were translocated posteriorly into the tail region, suggesting that overexpression of this gene affects the patterning of the anterior and dorsal structures of the larva.

MATERIALS AND METHODS

Ascidian eggs and production of egg fragments

Ciona savignyi adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at 18°C in Millipore-filtered seawater (MFSW) containing 50 μg/ml streptomycin sulfate. The first cleavage occurred about 1 hour after insemination and tadpole larvae hatched at about 18 hours of development. Dechori-onation of unfertilized eggs and production of egg fragments were performed as described previously (Marikawa et al., 1994; summa-rized in Fig.1A). Dechorionated eggs were immersed for 10 minutes in MFSW containing 10 μg/ml cytochalasin B (Aldrich, Milwaukee, WI, USA). This suspension of eggs was placed on a discontinuous gradient of 42% and 50% Percoll (Sigma, St. Louis, MO, USA) in MFSW. Centrifugation at 1,500 *g* for 20 minutes yielded egg fragments from nearly 100% of the eggs. Black and clear fragments were found above the 42% Percoll layer and brown and red fragments were seen below this layer.

Isolation of *pem* cDNA clone

Total RNA was isolated from egg fragments by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Poly(A)+ RNA was purified using Oligotex-dT30 Latex beads (Roche Japan, Tokyo, Japan). cDNA libraries of black and red fragments were constructed in uni-ZAP XR and λZAP II (Stratagene, La Jolla, CA, USA), respectively. Duplicate filters of the libraries were hybridized with ^{32}P-labeled total cDNA probes prepared from 5 μg of poly(A)+ RNAs of fragments. Plaques that showed positive hybridization with the probes from black fragments but were negative with the probes from red fragments were selected and isolated by two rounds of screening. Sequences of both strands of the clones were determined by the dideoxy chain termination method with Sequenase ver. 2.0 (USB; United States Biochemical Corp., Cleveland, OH, USA).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed using digoxigenin-labeled antisense probes as described previously (Yasuo and Satoh, 1994). Control embryos hybridized with a sense probe did not show signals above background.

Injection of synthetic capped mRNA

Pem cDNA was subcloned into the *Eco*RI site of pBluescript-RN3 vector (Lemaire et al., 1995). Control frame-shifted *pem* cDNA was made by digesting *pem* cDNA with *Bgl*II, followed by filling-in pro-truding ends with T4 DNA polymerase and religating the cDNA. The cDNA was subcloned into the *Eco*RI site of the pBluescript-RN3 vector.

As another control, a plasmid encoding β-galactosidase was also constructed in the pBluescript-RN3 vector (Yasuo and Satoh, unpub-lished data). Synthetic capped mRNA was prepared using the pBlue-script-RN3 vector according to the method described by Lemaire et al. (1995) using an RNA cap structure analog (New England Biolabs, Inc., Beverly, MA, USA). To obtain a high proportion of capped mRNA, the concentration of GTP in the reaction was lowered 10-fold and the cap analog 7mGpppG was added at a final concentration of 0.5 mM. After DNase I digestion of the template DNA, the mRNA was purified by phenol-chloroform extraction and ethanol and lithium precipitation.

The synthesized mRNA was injected into fertilized eggs as described previously (Marikawa et al., 1995), using a micromanipu-lator (Model MP-1; Narishige Sci. Instr. Lab., Tokyo, Japan). The volume injected was estimated to be about 1/100 of the volume of an egg by measuring the quantity of coinjected marker dye. After injection, eggs were allowed to develop and the effects of mRNA microinjection were examined.

Injection of lineage tracer

Normal and experimental eggs injected with synthetic *pem* mRNA were allowed to develop until the 8-cell stage, then either right <u>a4.2</u> or left a4.2 of the embryo was injected with lysinated fluorescein-dextran. After injection, embryos were allowed to develop into tadpole larvae and the distribution of the lineage tracer was examined.

RESULTS AND DISCUSSION

Isolation of cDNA clones for maternal genes with developmental functions

To isolate maternal genes with developmentally important functions, we developed an experimental system consisting of egg fragmentation and fusion of the fragments (Marikawa et al., 1994). As shown in Fig. 1A, centrifugation of unfertilized eggs of the ascidian *Ciona savignyi* yielded four types of fragments: a large nucleated red fragment, and small enucle-ated black, clear and brown fragments. When fertilized, only red fragments cleave and develop into so-called permanent blastulae in which only epidermal cell differentiation is evident. However, when red fragments are fused with black fragments and the fusion products are fertilized, nearly all of the fusion products develop muscle and endoderm cells, and sometimes morphologically normal tadpole larvae are formed (Marikawa et al., 1994). Clear and brown fragments have no such abilities. Therefore, these maternal factors appear to be preferentially separated into black fragments. In addition, results of u.v. irradiation experiments with black fragments suggest that maternal mRNAs are associated with the activi-ties of these factors (Marikawa et al., 1995).

Differential screening of cDNA libraries of black fragments yielded three cDNA clones specific to or enriched in black fragments (Fig. 1B). Sequencing of these clones revealed two to be mitochondrial genes. Since, as shown below, the tran-script of the third clone marks the posterior end of the devel-oping embryo, we designated the corresponding gene as *posterior end mark* (*pem*). Nucleotide and predicted amino acid sequences of *pem* gene are shown in Fig. 1C, and the hydropathy profile of the predicted gene product in Fig. 1D. The sequence predicts a protein of 374 amino acids with a cal-culated molecular mass of 41 kDa. The amino acid sequence, however, showed no significant homology to known proteins, and contained no motifs specific for transcriptional factors, integral membrane proteins or signaling molecules. Despite the mRNA localization described below, the *pem* cDNA sequence contains no localization signal sequence motif in the 3′
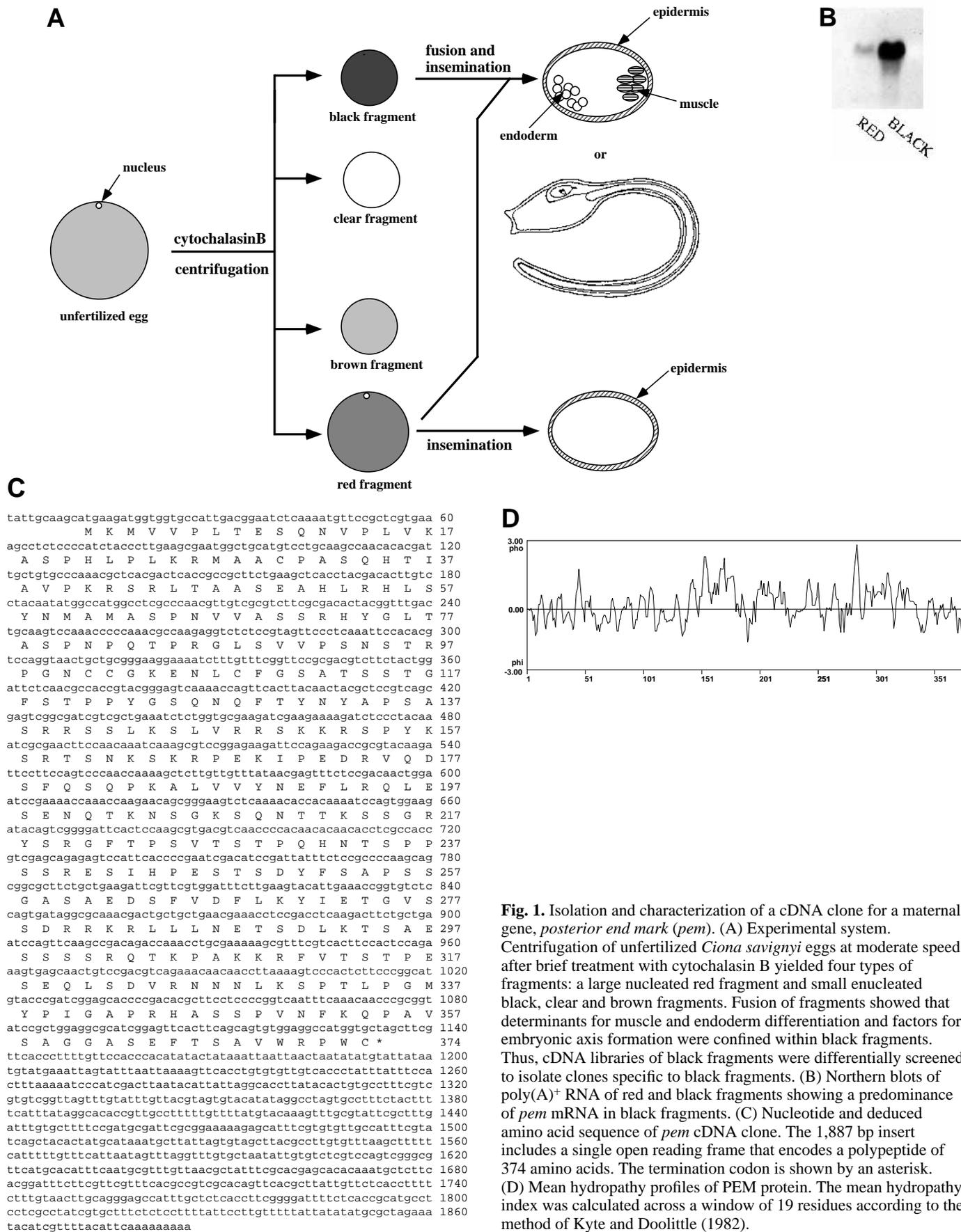
A

B

C

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tattgcaagcatgaagatggtggtgccattgacggaatctcaaaatgttccgctcgtgaa  60
           M  K  M  V  V  P  L  T  E  S  Q  N  V  P  L  V  K  17
agcctctccccatctacccttgaagcgaatggctgcatgtcctgcaagccaacacacgat  120
  A  S  P  H  L  P  L  K  R  M  A  A  C  P  A  S  Q  H  T  I  37
tgctgtgcccaaacgctcacgactcaccgccgcttctgaagctcacctacgacacttgtc  180
  A  V  P  K  R  S  R  L  T  A  A  S  E  A  H  L  R  H  L  S  57
ctacaatatggccatggcctcgcccaacgttgtcgcgtcttcgcgacactacggtttgac  240
  Y  N  M  A  M  A  S  P  N  V  V  A  S  S  R  H  Y  G  L  T  77
tgcaagtccaaacccccaaacgccaagaggtctctccgtagttccctcaaattccacacg  300
  A  S  P  N  P  Q  T  P  R  G  L  S  V  V  P  S  N  S  T  R  97
tccaggtaactgctgcgggaaggaaaatctttgtttcggttccgcgacgtcttctactgg  360
  P  G  N  C  C  G  K  E  N  L  C  F  G  S  A  T  S  S  T  G  117
attctcaacgccaccgtacgggagtcaaaaccagttcacttacaactacgctccgtcagc  420
  F  S  T  P  P  Y  G  S  Q  N  Q  F  T  Y  N  Y  A  P  S  A  137
gagtcggcgatcgtcgctgaaatctctggtgcgaagatcgaagaaaagatctccctacaa  480
  S  R  R  S  S  L  K  S  L  V  R  R  S  K  K  R  S  P  Y  K  157
atcgcgaacttccaacaaatcaaagcgtccggagaagattccagaagaccgcgtacaaga  540
  S  R  T  S  N  K  S  K  R  P  E  K  I  P  E  D  R  V  Q  D  177
ttccttccagtcccaaccaaaagctcttgttgtgtttataacgagtttctccgacaactgga  600
  S  F  Q  S  Q  P  K  A  L  V  V  Y  N  E  F  L  R  Q  L  E  197
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  Y  S  R  G  F  T  P  S  V  T  S  T  P  Q  H  N  T  S  P  P  237
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  G  A  S  A  E  D  S  F  V  D  F  L  K  Y  I  E  T  G  V  S  277
cagtgataggcgcaaacgactgctgctgaacgaaacctccgacctcaagacttctgctga  900
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aagtgagcaactgtccgacgtcagaaacaacaaccttaaaagtcccactcttcccggcat  1020
  S  E  Q  L  S  D  V  R  N  N  N  L  K  S  P  T  L  P  G  M  337
gtacccgatcggagcaccccgacacgcttcctccccggtcaatttcaaacaacccgcggt  1080
  Y  P  I  G  A  P  R  H  A  S  S  P  V  N  F  K  Q  P  A  V  357
atccgctggaggcgcatcggagttcacttcagcagtgtggaggccatggtgctagcttcg  1140
  S  A  G  G  A  S  E  F  T  S  A  V  W  R  P  W  C  *        374
ttcacccttttgttccacccacatatactataaattaattaactaatatatgtattataa  1200
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tacatcgtttttacattcaaaaaaaaa
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D

Fig. 1. Isolation and characterization of a cDNA clone for a maternal gene, *posterior end mark* (*pem*). (A) Experimental system. Centrifugation of unfertilized *Ciona savignyi* eggs at moderate speed after brief treatment with cytochalasin B yielded four types of fragments: a large nucleated red fragment and small enucleated black, clear and brown fragments. Fusion of fragments showed that determinants for muscle and endoderm differentiation and factors for embryonic axis formation were confined within black fragments. Thus, cDNA libraries of black fragments were differentially screened to isolate clones specific to black fragments. (B) Northern blots of poly(A)$^+$ RNA of red and black fragments showing a predominance of *pem* mRNA in black fragments. (C) Nucleotide and deduced amino acid sequence of *pem* cDNA clone. The 1,887 bp insert includes a single open reading frame that encodes a polypeptide of 374 amino acids. The termination codon is shown by an asterisk. (D) Mean hydropathy profiles of PEM protein. The mean hydropathy index was calculated across a window of 19 residues according to the method of Kyte and Doolittle (1982).
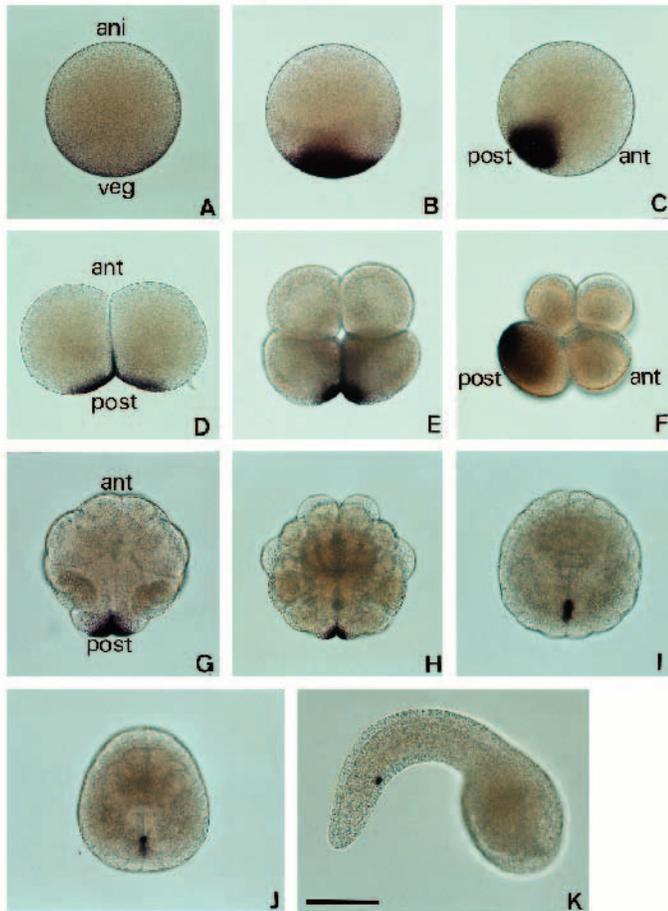
Fig. 2. Distribution of *pem* maternal mRNA marks the posterior end of developing embryos, as revealed by whole-mount in situ hybridization. (A) An unfertilized egg, lateral view. ani, animal pole; veg, vegetal pole of the egg. (B) A fertilized egg after completion of the first phase of ooplasmic segregation, lateral view. (C) A fertilized egg after completion of the second phase of ooplasmic segregation, lateral view. ant, anterior; post, posterior side of the egg. (D) A 2-cell embryo, animal pole view. (E) A 4-cell embryo, animal pole view. (F) An 8-cell embryo, lateral view. (G) A 16-cell embryo, vegetal pole view. (H) A 32-cell embryo, vegetal pole view. (I) An early gastrula, vegetal pole view. (J) An embryo at the neural plate stage, vegetal pole view. (K) A tailbud embryo, side view. Scale bar represents 100 μm for all photographs.

decreased during embryogenesis (data not shown). We detected no changes in the size of *pem* mRNA or any signals of zygotic *pem* mRNAs, suggesting that *pem* is maternal.

The localization of *pem* mRNA marks the posterior end of developing embryos

In unfertilized eggs, the mRNA was detected in the peripheral cytoplasm except for the animal pole region (Fig. 2A) in which the nucleus is present. In ascidian eggs, fertilization evokes 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). Ooplasmic segregation consists of two distinct phases, the first of which 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, *pem* transcript became concentrated near the vegetal pole (Fig. 2B). 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 is a landmark of the posterior side of the future embryo. As shown in Fig. 2C, after the second phase of ooplasmic segregation, the *pem* transcript moved to the subequatorial region to form a crescent-like structure. Cleavage of ascidian eggs is bilaterally symmetrical. The animal pole view of the 2-cell-stage

untranslated region (3′UTR) reported in some other localized messages (reviewed by Ding and Lipshitz, 1993). The myoplasm-abundant mRNAs in *Styela* eggs also lack the specific localization signal sequences in the 3′UTR (Swalla and Jeffery, 1995).

Northern blot analysis identified a distinct single transcript of about 2.0 kb. The amount of *pem* mRNA gradually
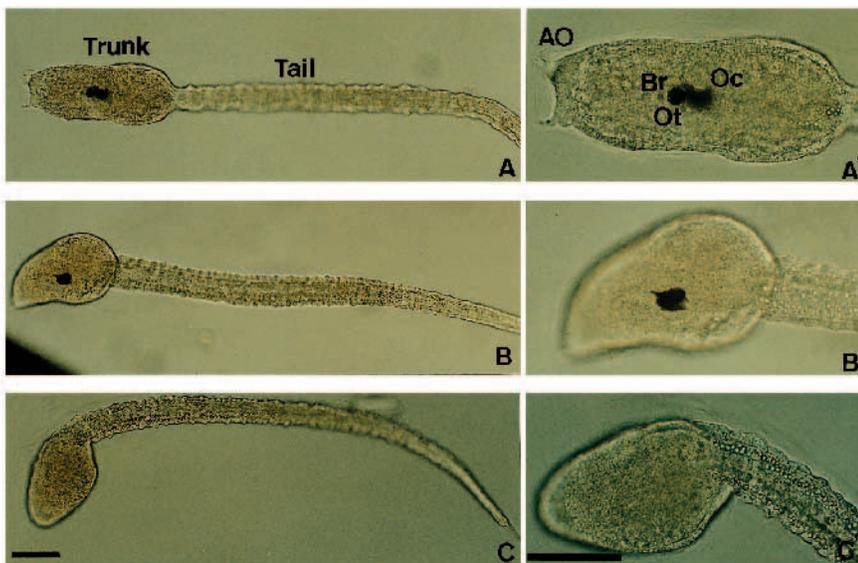

Fig. 3. Overexpression of *pem* affects the formation of the anterior and dorsal structures of tadpole larvae. Synthetic *pem* mRNA was microinjected into fertilized eggs and injected eggs were allowed to develop into tadpole larvae. (A, A′) A control larva derived from an egg injected with lacZ-RN3 mRNA, showing normal larval morphology. (A′) Higher magnification of the trunk region of the larva with the adhesive organ (AO), otolith (Ot) and ocellus (Oc). Br, brain. (B, B′) An experimental larva derived from an egg injected with *pem* mRNA showing lack of the adhesive organ and disturbance of sensory pigment cells. (C, C′) An experimental larva lacking the adhesive organ, sensory pigment cells and brain vesicle. Scale bar represents 100 μm.
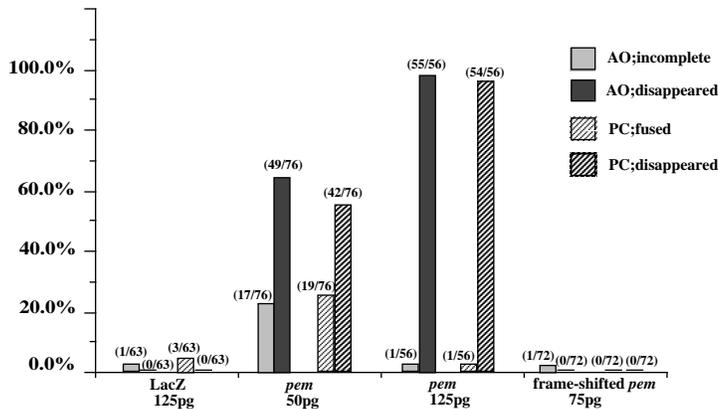
Fig. 4. Effects of *pem* overexpression on formation of anterior and dorsal larval structures. Larvae with abnormal adhesive organ (AO) and sensory pigment cells (PC) and those lacking these anterior and dorsal structures were scored. As controls, eggs were injected with lacZ mRNA or frame-shifted *pem* mRNA.

embryo shown in Fig. 2D indicates that *pem* mRNA was localized in the rather narrow peripheral cytoplasm of the posterior region of the blastomeres. The second cleavage furrow is at a right angle to the first. At the 4-cell stage, *pem* transcript was restricted to the posterior blastomere, near the posteriormost region and also along the second cleavage furrow (Fig. 2E). The third cleavage is latitudinal and results in slightly unequal division. In the 8-cell-stage embryo, the distribution of *pem* transcript was restricted to the posterior region of B4.1 cells (a pair of posterior vegetal blastomeres in the bilaterally symmetrical embryo; Fig. 2F).

The region with positive hybridization signals becomes narrower as development proceeded. At the 16-cell stage, the myoplasm becomes distributed to both blastomeres (B5.1 and B5.2 cells) at the posterior-vegetal region of the embryo. In contrast, *pem* mRNA was found in the posteriormost region of the embryo, the posterior cytoplasm of the B5.2 cells (Fig. 2G). Positive hybridization signals for *pem* transcript were detected in the posterior cytoplasm of B6.3 in the 32-cell-stage embryo (Fig. 2H) then in B7.6 of the 64-cell-stage embryo (data not shown). At the gastrula stage, *pem* transcript was seen in a few invaginating posterior cells (Fig. 2I), then in a very narrow region of the embryo at the neural plate stage (Fig. 2J). Finally, positive hybridization signals were found in a few cells of the ventral side of the tail region of tailbud embryos, presumably endodermal strand cells (Fig. 2K).

Thus, the distribution of *pem* transcript marks the posteriormost region of early embryos, and its final destination was the endodermal strand. This is consistent with the results of cell lineage analysis in which it was revealed that the developmental fate of posteriormost blastomeres is restricted at the 64-cell stage to give rise to the endodermal strand only. Previous in situ hybridization experiments of whole-mount ascidian embryos demonstrated that zygotic transcripts first appear in the nuclei, then transcripts are distributed in the entire cytoplasm (e.g. Yasuo and Satoh, 1993). The *pem* hybridization signals were not detected in the nucleus throughout embryogenesis. This also suggests that *pem* transcript is exclusively maternal. Despite the localization of *pem* mRNA, however, the *pem* cDNA sequence contains no localization signal sequence motif in the 3′ untranslated region reported in some other localized messages (reviewed by Ding and Lipshitz, 1993).

The localized domain of *pem* mRNA during the period of the ooplasmic segregation overlaps with the myoplasm, which is partitioned into larval muscle cells and contains determinants for muscle cell differentiation (e.g. Nishida, 1992). The myoplasm is a unique cytoskeletal domain (reviewed by Jeffery and Swalla, 1990), which consists of several cytoskeletal proteins (Nishikata et al., 1987; Swalla et al., 1991; Marikawa, 1995). However, the localization domain of *pem* mRNA becomes narrower as development proceeds to the posteriormost region and no such cytoskeletal components are known to be concentrated in the posteriormost region of the embryo. Therefore, it will be intriguing to examine the molecular and cellular mechanisms underlying the localization of the maternal messenger RNA in future studies.
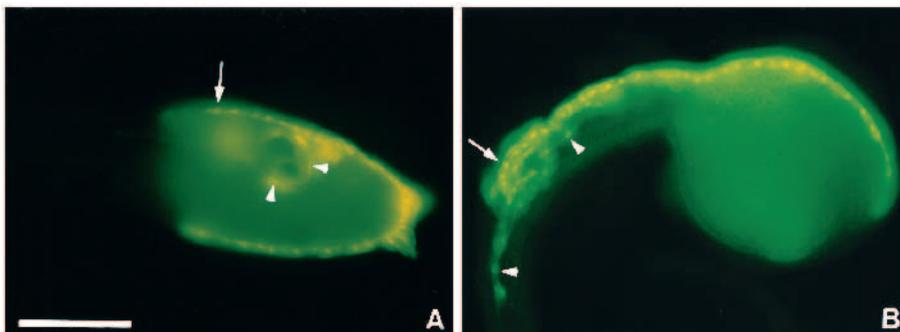

Fig. 5. Effects of *pem* overexpression on the distribution of a4.2 progenitor cells. (A) A control larva showing that a4.2 progenitor cells form structures of the anterior and middle portion of the trunk, including the adhesive organ, epidermis (arrow) and the brain vesicle with otolith and ocellus (arrowheads). The a4.2 progenitor cells do not contribute to the posterior portion of the central nervous system (visceral ganglion and spinal cord) that extends into the tail region. (B) An experimental larva derived from an egg injected with *pem* mRNA showing ectopic distribution of a4.2 progenitor cells. a4.2-derived epidermal cells (arrow) and neuronal cells (arrowheads) were evident in the anterior portion of the tail. Scale bar represents 100 μm.

Overexpression of *pem* affects development of anterior and dorsal structures of the larva

Although we have not yet examined PEM protein distribution in the ascidian embryo, the characteristic distribution of *pem* mRNA implies a significant role of the gene in embryogenesis. To explore its putative function, we prepared synthetic capped *pem* mRNA to examine the effects of overexpression of the gene. As controls, we injected 125 pg of synthesized lacZ-RN3 RNA or 75 pg of synthesized *pem* mRNA with a frame-shift mutation into single fertilized eggs. Eggs from both controls developed into morphologically normal tadpole larvae (Fig. 3A,A′) except for a very few larvae with abnormalities (Fig. 4). In control larvae, differentiation of muscle and endoderm cells was evident by histochemical detection of acetylcholinesterase and alkaline phosphatase, respectively (data not shown). However, microinjection of 50~125 pg *pem* mRNA into fertilized eggs resulted in disturbance of development of the anterior and dorsal structures of the larva, as described below.

Injection of ~125 pg *pem* mRNA into single fertilized eggs did not alter the cleavage pattern. Gastrulation took place normally, followed by neurulation and formation of tailbud embryos. Tadpole-type larvae hatched normally and the tail region of experimental larvae looked normal (Fig. 3B,C). The tail extended straight forward and notochord cells were seen to run straight through the center of the tail. Development of muscle and endoderm cells was demonstrated by expression of acetylcholinesterase and alkaline phosphatase, respectively (data not shown). However, deficiency in development of the anterior and dorsal trunk region was evident. The most marked effect of *pem* mRNA microinjection was lack of the adhesive organ (Fig. 3B,C). At the anterior end of ascidian larvae, there is an adhesive organ that consists of three adhesive papillae arranged in a triangular field (Fig. 3A). As the papillae secrete adhesives that are used to effect settlement at the onset of metamorphosis, this organ may be homologous to the sucker or cement gland of amphibian tadpoles. As summarized in Fig. 4, when single eggs were injected with 50 pg of *pem* mRNA, 64% of the resultant larvae failed to form the adhesive organ (Fig. 3B,C) and 23% showed incomplete development of the organ. In addition, almost all of the larvae (55 of 56; 98%) developed from eggs injected with 125 pg *pem* mRNA failed to form the adhesive organ (Fig. 4). Therefore, the anterior surface of the larva was smooth (Fig. 3B,C).

Another marked deficiency induced by *pem* mRNA injection was evident in the sensory pigment cells of the brain vesicle (the anterior portion of the central nervous system of ascidian larvae). The brain of *Ciona* larvae contains two sensory organs; the otolith and ocellus (reviewed by Satoh, 1994). The otolith or statocyte is an unusual cell which contains a large melanin granule (Fig. 3A′), and the ocellus of the *Ciona* tadpole is composed of a single cup-shaped pigment cell, some 15-20 sensory cells and 3 lens cells (Eakin and Kuda, 1971). The pigment cell forms part of the wall of the sensory vesicle and contains many discrete membrane-bound melanin granules. The otolith pigment cells are distinguishable from ocellus pigment cells (Fig. 3A′). Microinjection of 50 pg of synthesized *pem* mRNA resulted in deficiency of pigment cell formation (Fig. 3B,B′); about 55% of the resultant larvae failed to develop pigment cells (Fig. 4). This effect was more marked when single eggs were injected with 125 pg of *pem* mRNA;

no pigment cells were formed in 96% (54 of 56) of the experimental larvae (Figs 3C,C′, 4), and all of the larvae without the sensory pigment cells failed to develop the adhesive organ. However, in no cases did the experimental larvae develop the adhesive organ but not the sensory pigment cells; i.e. damage was more evident in the more anterior structures. In addition, sections of deficient larvae revealed a lack of the brain vesicle (data not shown). The trunk region of the deficient larva was occupied by endoderm cells.

As described above, injection of *pem* mRNA into fertilized eggs affected formation of the anterior and dorsal structures of the larvae, whereas injection of mutated *pem* mRNA did not. Therefore, it is highly likely that this maternal message produces a functional protein although the predicted amino acid sequence of the *pem* product showed no significant matches to known proteins. The genetic cascade required for *pem* function is an important subject for further studies.

Distribution of a4.2 descendants in *pem*-overexpressed embryos

As described above, the effect of overexpression of the *pem* gene was restricted to the anterior and dorsal structures of the larva; the ventral trunk as well as the tail looked normal in larvae overexpressing *pem*. Disappearance of the anterior and dorsal structures suggests that the presumptive cells of these structures change their developmental fates to other cell types or alternatively the presumptive cells remain undifferentiated somewhere in the embryo. Most of the affected structures are derived from a pair of a4.2 (anterior and animal blastomeres) of the 8-cell embryo (Nishida, 1987). We examined these possibilities by lineage tracing analysis of the progenitor cells. Injection of lineage tracer into b4.2 (posterior and animal blastomere), A4.1 (anterior and vegetal) or B4.1 (posterior and vegetal) showed that overexpression of *pem* had almost no effect on the distributions of the b4.2, A4.1 or B4.1 descendant cells in the larvae (data not shown). This is consistent with the above result that formation of endoderm, mesenchyme, muscle and notochord appeared to be unaffected by overexpression of the gene. However, as shown in Fig. 5, injection of lineage tracer into either right <u>a4.2</u> or left a4.2 demonstrated marked effects of *pem* overexpression on distribution of the a4.2 descendants. In control larvae, the anterior and middle portions of the trunk, including the adhesive organ, epidermis and brain vesicle with two sensory organs, were labeled (Fig. 5A), confirming that these structures are formed by a4.2-derived cells. The labeled region did not cross the boundary that divides the middle and posterior portions of the larval trunk (Fig. 5A). The labeled cells, therefore, do not contribute to the posterior portion of the central nervous system (visceral ganglion and spinal cord) that extends into the tail region. In experimental larvae overexpressing *pem*, labeled epidermal cells and neuronal cells were evident in the anterior portion of the tail (Fig. 5B). This suggests that *pem* overexpression does not affect the differentiation of a4.2-derived neuronal cells, but may change the patterning of the nervous system causing translocation of a4.2 descendants posteriorly to the region that is usually occupied by visceral ganglion and spinal cord derived from b4.2 and A4.1. Together with the posterior localization of *pem* transcript, these observations suggest that the *pem* gene may play an important role in patterning of the embryo.

By fusion of certain cytoplasmic regions of the ascidian egg, Nishida (1994b) showed that the posterior-vegetal cytoplasm contains maternal factors responsible for establishment of the anteroposterior axis of the embryo. In this case, deletion of the posterior-vegetal cytoplasm resulted in alteration of the cleavage pattern from bilateral to radial symmetry. Moreover, transplantation of the posterior-vegetal cytoplasm to an anterior-vegetal position of posterior-vegetal cytoplasm-deficient eggs resulted in reversal of the anterior-posterior axis. These results suggest that the posterior-vegetal cytoplasm is responsible for the establishment of the anterior-posterior axis by generating the cleavage pattern. It is possible that *pem* plays a role in determining this posterior-vegetal cytoplasmic identity, because the initial distribution of the transcript matches the posterior-vegetal cytoplasm and overexpression of this gene affected formation of the anterior and dorsal structures of the larvae. However, overexpression of *pem* mRNA did not disturb the cleavage pattern, major morphogenesis or formation of tadpole larva itself. To elucidate the role of this gene product further, loss-of-function experiments using antisense *pem* RNA or DNA should be carried out in future.

Recent studies of molecular phylogeny and of molecular developmental biology support the hypothesis that the ascidian tadpole is a prototype for the ancestral chordate (Satoh and Jeffery, 1995; Yasuo et al., 1995). For example, ascidian larval actins closely resemble vertebrate muscle-type actins but not those of invertebrates (Kusakabe et al., 1992). An ascidian homologue of the mouse *Brachyury* gene is expressed exclusively in the presumptive notochord cells (Yasuo and Satoh, 1993). These results imply a conserved function of *pem* homologues in vertebrate embryogenesis, which should be investigated further.

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