

# Identification and characterization of maternally expressed genes with mRNAs that are segregated with the endoplasm of early ascidian embryos

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**ABSTRACT** Endoderm cells of the ascidian embryo are specified autonomously dependent on maternal cytoplasmic information or determinants that are localized in the endoplasm. In the present study, we identified three maternally expressed genes (*CsEndo-1*, *CsEndo-2* and *CsEndo-3*) by screening a cDNA library of *Ciona savignyi* fertilized egg mRNAs subtracted with gastrula mRNAs. *CsEndo-1* encoded a protein with nuclear localization signals, *CsEndo-3* predicted a protein containing both a potential transmembrane domain and the PDZ domain, and *CsEndo-2* suggested a protein with no similarity to known proteins. The maternal transcripts of all of these genes were not concentrated during early stages of embryogenesis up to the 8-cell stage, but were concentrated at the endoplasmic region by the 16-cell stage and then segregated later with the endoplasm. At the 110-cell stage, the maternal transcript of *CsEndo-1* was evident only in the primordial endoderm cells, while those of *CsEndo-2* and *CsEndo-3* were found in the primordial endoderm cells as well as the primordial notochord cells. All of the transcripts became barely detectable during gastrulation and neurulation. Later, zygotic expression of the three genes became evident again in the endoderm and notochord cells, suggesting developmental roles in the formation of these types of cell. Although we were not able to deduce their functions, this is the first report of maternal genes with mRNAs that are segregated with the endoplasm of ascidian embryos.

**KEY WORDS:** *ascidians, localized maternal mRNAs, endoplasm, endoderm, notochord*

## Introduction

The endodermal tissue of an ascidian tadpole larva is comprised of endoderm in the trunk region and an endodermal strand in the tail region (reviewed by Satoh, 1994). The lineage of the endodermal tissue is almost completely documented (Conklin, 1905; Nishida, 1987). Most of the endoderm cells, except for a small number of cells of the posterior region of the tail, are derived from the vegetal A4.1 and B4.1 blastomeres of the bilaterally symmetrical 8-cell stage embryo. The developmental fate to form endoderm is segregated into the A5.1, A5.2 and B5.1 of the 16-cell stage embryo, and then into the A6.1, A6.3 and B6.1 of the 32-cell stage embryo. As early as the 32-cell stage, the A6.1 becomes restricted to generate endoderm only. At the 64-cell stage, not only A7.1 and A7.2 (daughter cells of A6.1) but also the A7.5, B7.1, and B7.2 become endoderm-restricted. These primordial cells divide five or six times to form the endodermal tissue of about 500 cells.

Reflecting such an early fate restriction, presumptive endoderm blastomeres show a high potential of autonomous differentiation

when they are isolated from early embryos (Reverberi and Minganti, 1946; Whittaker, 1990; Nishida, 1992). This autonomy is dependent on maternal factors or determinants that are prelocalized in the endoplasm of eggs and early embryos (Nishida, 1993). In addition, it was shown that the endoderm cells induce the differentiation of notochord cells at the 32-cell stage (Nakatani and Nishida, 1994). Although recent studies identified developmental genes that are expressed zygotically in the endodermal tissues, including the LIM class homeobox gene *HrLim* (Wada *et al.*, 1995), *fork head/HNF-3 $\beta$*  (Corbo *et al.*, 1997; Olsen and Jeffery, 1997; Shimauchi *et al.*, 1997) and alkaline phosphatase gene (Kumano and Nishida, 1998), there are no reports on maternally expressed genes with mRNAs that are segregated with the endoplasm. Therefore, the identification of maternal messages in the endoplasm is a current research subject of ascidian embryogenesis.

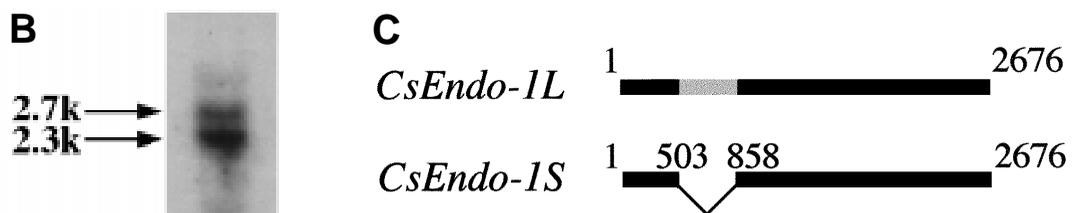
We have attempted to identify maternally expressed genes with localized mRNAs to deduce possible function of the genes. Our strategy to isolate such genes was as follows (Satou and Satoh, 1997). We first constructed a cDNA library of fertilized egg mRNAs

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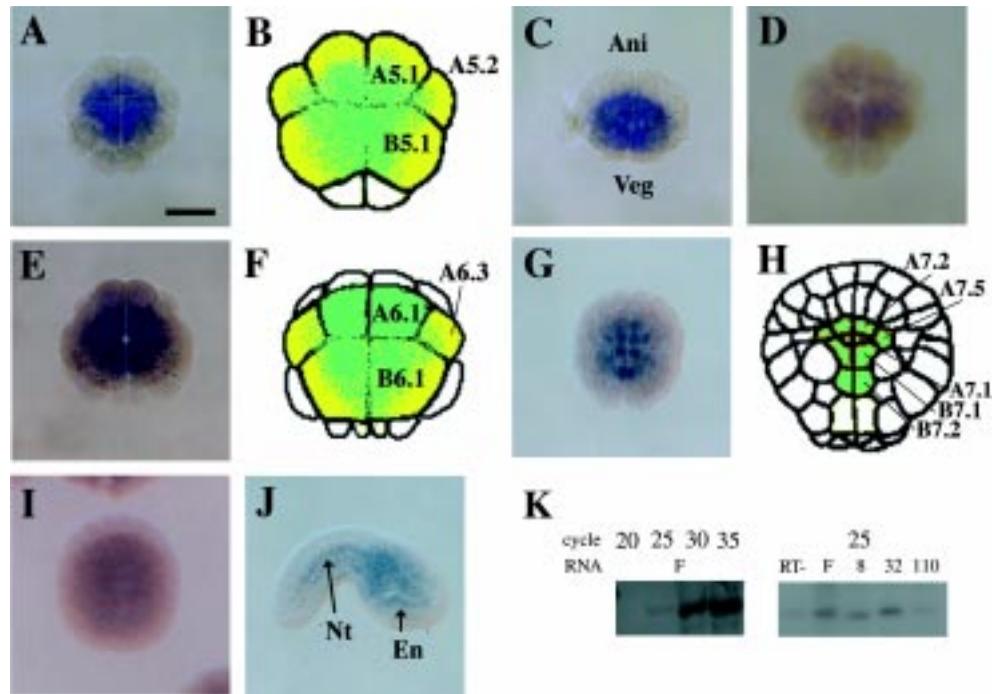
TTTCTATTGAATAAGCTAGGGTTAGTATAACATTAGGGGTATGGAGAACCTACTGGATCTCATATATCTTACCGAAAGTAAAAGAAAAT 90
          M E N L L D L T Y L T E S E R K W
GGATCCAAGAAGTCTTAGACAGGGACGAGACGATACAAAAAGCAGAGGATGCAAGAATAGAGAGGCTGAAGAGCAAAGCGATGCCACAA 170
I Q E V L D R D E T I Q K A E D A R I E R L K S K A M R T N
ACAACCCGACGATTAGGAAGAAGATTAAGATGAGGACGGGAGAATGGTTTCTCGATCTCGCCGATAAAGATCAGACTTTAAAGAAACGTG 270
N P T I R K K I K M R T G E W F L D L A D K D Q T L K K R G
GAGTCGACTCTGTACGGGCTTCCATTCCGGAGAAAAGGAAAAGATCGGCCAAAACCGATTTCACAACGAACCACTCAGATCACAGACCTTG 360
V D S V R A S I R R K G K D R P K P I S Q R T T Q I T D L V
TAGAGAGGTTGACATAACTCCAGTTCCTGATCCTGAACCTATTGTACCAGAAGAGTCGTATTTCTCGCGAACACACAGCAACTATGGAG 450
E Q V D I T P V P D P E P I V P E E S Y F S R T H S N Y G D
AAAATTCCTATGACGAATCCTCTGAAATTGATGACGAATCTAAAAATCAAGAAACCCAGAGTCACTGAACCGAAGATCAGTTGCAGATG 540
N S Y D E S S E I D D E S K N Q E P R V T E P E D Q V A D E
AATCGGTTTTATTGTGATGTCACAAAACATCGGATCGCTGACGAAAACGACCCAGAGAAACCTGTCTCACCTACGTTTGAATCCCCGCTCA 630
S V Y C D V T N N R I A D E N D P E K P V S P T F E S P L T
CTTTGGAAAATGTCACCTCTCATATAGACCCGTCGCCCCAGTTCAGAAGGAGCCTCTCCAACCCCTCTAGAGGATTCACCTTCCCTCCC 720
L E N V H S H I D P S P P V Q K E P L Q P P L E D S T S S Q
AATTACTCCACAACATCCGGAGGATGAGGATGAGGATCAATCTCCGAGGAGGATCAGGAGGATGGGAAGCTCGAGGAGGAAGAGAGAGT 810
L T P Q H P E D E D E D Q A S P R I R R M G S S R R K R E F
TCAAACCGATCCACGATGAACCGGTGGAGCAAATGAAGAAAGAGAAARAGTTCAAGAAATTCAGCGCCATTTTACAAAATA 900
K P I H D E S E T V Q N E E R E K V E E L S S A M T F L Q T I
TAGATAAAGGGAAAACAGCAGAGCGCATGGACGCCATCTTCCATTCTAACTGCACCAAGGAAATCCTGGATCTTATCGGTCGCTAC 990
D K G K T A E R M D A H L P I L N C T K E I L D L I G S L Q
AAGACAGGGCTGCACCTTGTACAGGTAGAGACACTCTTTTAGAAATCGAGAAAGCGTCGAGTCAGGAGCGAGTCAGAAAAGTCGTTG 1080
D Q G C T C T C T G R D T L L E I E K A S S Q E R V I E K V V E
AAATGCTGAGTCAACCCAAAACCGTGTGAGTTGCCGCTTTACTACGAGTTGGCAGATCACGAAATGTCCTTAGTCACCGTGAACAAAATCG 1170
M L S Q P K N A E L P L Y Y E L A D H E M S L V T V K Q I D
ACAAATCAGTGACTCAGGATCACCATGACGAAGATATCTATCTGGGGAACCCCTCAAACACTGCGGCAAATGTCGATGCACCTGATGG 1260
K S V T Q D H H D E D I Y L G E P P Q N L R Q I V D A L M V
TATCGGATGAATCTCAAGACGCTGAAATGCTAGTTTGTACACCAAGTGCCCTTCTGGAACCTTAATTTAGTTATATTCTTTCTTTTC 1350
S D E S Q D A G N C *
AAAGGTTTTAACGACTGTTTCGTTTTGTCCTTTTTCTGTTTCTGTGCGGTGATTGTTTTATTTTCCGTTATTCAATACTGGGGCAAAT 1440
ATGTATAAACTAAACCAGGCATGGGCAACATACTGCACGTAAAGGATTTTGACCAGCCCGCGGACTGTATCTAACACACCCCTGTAATT 1530
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TCTGGGCTAAACTAACACCTTGCCACACTACATTTACTTGTAGATGCATCCCTTTATAAACCTACCCACACCCCTTCCCTTTT 1710
TAATCTGTGACCGCAAATACCACATACATTTCTCATATCATTAAATTTAAATTTCCAAATCFACATTTTGTAGAGTGAAAATATGT 1800
ACCTTCGTGTCATCTGCTACCACATGCCCAAAGTCTAGTTTCCATATTGCCATTTGCTTATAAAGTGTGCTTAAAATTTCCACTGAAAA 1890
TTTTTCAAACAAAAAATCTATAATCAATCCATTAAGTGAATTAATTTGTTTTTAAATGTTGCATTTTTAAATTTGGTTACAGCCAAT 1980
CTTGTATCTTGTGTGTTTTCAATATACATTTCCATACAGTATGCTATGAGCTAAAGAGATTTGTTGATTTTTTGTGCTTTAAGAGAT 2070
TTTFAAGAATCGCCAAATGCTGTGCAATAGATTTACACAATATCTGAATGTTGCGTCACAGTTTGTACCCAATATGTGGGTTATTTTG 2160
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ACCTTCAATAAAAAACCTTTTTTTTTCTGAAATGCAAAATTTTTTTTTTAAATTTGTCAATTTTTTTTTTAAATTTAGTCTAATGTAAC 2340
CTAAGCATAATAGTTTATAAAATCAATAGTTAAATACCCAACATACTTTTTTTCGAAAGTCGGGACCAATTTTAAACGCTACAAAGTCCTA 2430
TTCATCGCACCCCTGAAAACAAATTTTGTGCGCATGACCCACCCTGACTCTAAAAACTAATCTGTGATCTTGTATATGAGAAATAA 2520
CTAGATTTACAACCTATAATCTACATTTCTGTGTTATGCAACGTTTTCGTATTGCTTTTTTGTGTTTTTTTTGCGTTTTTTTACCAGAT 2610
ATTGGATTGCTGCAATAAAATAAGAGATTTCTTCAGAGAAAAAATAAAAAAAAAAAAAAAAAAAAAA 2676
    
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**Fig. 1.** Characterization of cDNA for *CsEndo-1* gene. **(A)** Nucleotide and deduced amino acid sequence of *CsEndo-1L* cDNA. The 2,676bp insert includes a single open reading frame that encodes a polypeptide of 417 amino acids. The asterisk indicates the termination, and potential signal sequences for polyadenylation are dotted-underlined. Two possible nuclear localization signals (amino acid positions 74-90 and 252-255) are underlined. The nucleotide sequences that *CsEndo-1S* cDNA lacks are enclosed by a box. **(B)** A Northern blot of poly(A)<sup>+</sup> RNA of fertilized eggs shows two distinct transcripts of *CsEndo-1*. **(C)** Structural comparison of *CsEndo-1L* and *CsEndo-1S* cDNAs. The shorter transcript (*CsEndo-1S*) lacks the nucleotide sequences between 503 and 858 (gray box) of the longer transcript (*CsEndo-1L*).

subtracted with gastrula mRNAs of the ascidian *Ciona savignyi*. The clones were then randomly picked up from the subtracted library, and they were partially sequenced from the 3'-end to avoid analyzing the same clones any further. After partial sequencing, the localization of corresponding mRNA was examined for each clone by whole-mount *in situ* hybridization. cDNA clones exhibiting the localization of corresponding mRNA were selected for further analyses. Taking advantage of this strategy, we were able to isolate maternal genes with mRNAs that are localized in the posterior end of developing embryos (*pem-2*, *pem-3*, *pem-4*, *pem-5*, and *pem-6*; Satou and Satoh, 1997). In the present study, we

**Fig. 2. Expression of the *CsEndo-1* gene in ascidian embryos.** Localization of *CsEndo-1* transcript at (A) the 16-cell, (E) 32-cell and (G) 110-cell stages, vegetal pole view. (C) The 16-cell stage embryo, posterior view, showing the maternal transcript was restricted to the vegetal side of the embryo. (D) Hybridization of the 16-cell stage embryo with sense probe, showing no signals above background. At these stages, distinct signals were found in the presumptive endoderm cells. (B,F,H) Schematic representations of (A), (E), and (G). Yellow shows blastomeres with the developmental fate of endoderm, and green shows the *CsEndo-1*-expressing cells. The blastomeres with the developmental fate of endoderm are indicated based on Conklin's nomenclature (1905). Only the blastomeres of the right half of the embryo are shown, because the ascidian embryo is bilaterally symmetrical. (I) A gastrula, vegetal view, showing that signals were hardly detected. (J) A tailbud embryo, lateral view, showing distinct zygotic expression of the gene in endoderm (En) and notochord (Nt).



(K) RT-PCR analysis of *CsEndo-1* maternal transcript. (Left) Twenty-five cycles showed an appropriate level of amplification of the signal. (Right) Assay of RNA extracted from fertilized eggs (F), 8- (8), 32- (32) and 110-cell embryos (110). RT- is a negative control.

extended this approach to isolate cDNA clones, the corresponding mRNAs of which exhibit localization and segregation with the endoplasm. We report here the identification and characterization of three maternally expressed genes designated *CsEndo-1*, *CsEndo-2*, and *CsEndo-3*. During early cleavages, these maternal messages gradually concentrated in the endoplasm, and at the 16- to 110-cell stage, *CsEndo-1* mRNA was detected only in the presumptive endoderm cells, while *CsEndo-2* and *CsEndo-3* mRNA was detected in the endoderm cells as well as A-line notochord cells.

## Results

### Isolation of cDNA clones for maternally expressed genes with localized mRNAs

We constructed a cDNA library of fertilized-egg mRNAs subtracted with gastrula mRNAs of *C. savignyi*. In a previous study, we isolated five cDNA clones for maternally expressed genes with mRNAs that were localized to the posterior end of developing embryos (Satou and Satoh, 1997). The genes were named *pem-2*, *pem-3*, *pem-4*, *pem-5*, and *pem-6*. We extended the screening to obtain maternal mRNAs that are segregated with the endoplasm or ectoplasm. In the present study, we isolated three maternally expressed genes, the corresponding mRNAs of which were segregated with the endoplasm. We named these genes *CsEndo-1*, *CsEndo-2* and *CsEndo-3*.

### The *CsEndo-1* gene

#### Characterization of the *CsEndo-1* cDNA clone

A Northern blot analysis identified two distinct *CsEndo-1* transcripts of about 2.3 kb (*CsEndo-1S*) and 2.7 kb (*CsEndo-1L*) in

mRNA of *C. savignyi* fertilized eggs (Fig. 1B). The band intensity of *CsEndo-1S* was stronger than that of *CsEndo-1L* (Fig. 1B). The cDNA clone we initially isolated was about 2.0 kb, which did not contain the entire coding sequence. Two successive 5'-RACE yielded a cDNA clone with the entire coding sequence. As shown in Figure 1, the cDNA sequence of *CsEndo-1L* was 2,676bp long and had a single open reading frame that encoded a polypeptide of 417 amino acids. We found that the cDNA for *CsEndo-1S* had the same sequence as *CsEndo-1L*, except that *CsEndo-1S* lacked the sequence between nucleotide positions 503 and 858 of *CsEndo-1L* (Fig. 1B,C). These transcripts are likely to be products of the alternative splicing of a single gene. The predicted *CsEndo-1L* and *CsEndo-1S* proteins contained nuclear localization signals, suggesting that they were nuclear proteins. However, we were not able to find any consensus motifs of transcription factors in the sequence.

#### The localization and segregation of *CsEndo-1* mRNA

Most of the endodermal cells of the ascidian embryo are derived from the vegetal blastomeres of the 8-cell embryo (A4.1 and B4.1 lines). The developmental fate to form most of the endoderm is further segregated into the A5.1, A5.2 and B5.1 of the 16-cell embryo (Fig. 2B), and then the A6.1, A6.3 and B6.1 of the 32-cell embryo (Fig. 2F). At the 64- and 110-cell stage, the developmental fates of A7.1, A7.2, A7.5, B7.1 and B7.2 blastomeres are restricted to the generation of only endoderm (Fig. 2H).

Although *CsEndo-1* is a maternally expressed gene, whole-mount *in situ* hybridization failed to detect distinct signals until the 16-cell stage (data not shown). At the 16-cell stage, *CsEndo-1* mRNA was detected in the endoplasm of the vegetal hemisphere (Fig. 2A,B,C). No hybridization signals were detected in blas-

TATAGATGATGTTTTGTATATACAAGTGTGGCTGAATAGATAGCCTGAGATAAATCATATTGACACGTAAGTATGGAGAAAGGCGAAATA 90  
 AAGGAAACCAAATATGGAGAGGATCGTTGCTGCTAAATTTTCGGCGACGGGGTTGGAATAAGCTTGGAAAAGCTTGGCAGAGAATACGAT 180  
 K E T K I W R G S L L L N F G D G V G I S L E K L G R E Y D 6  
 GAAATGATAAAGACATTTGAAGAGCAGAAACAAGATTAAGAGAGTTCGTACCACAAGAGAGGCAATAAAGAAGAGCAGTGCAGCAGT 270  
 E M I K T F E E Q K Q R L R E F V P Q E E A I K E E A C S S 36  
 GGCACGTCAAGAAACAATAGTCCAACTCCAGCAATACTAGCTCTAAGAAGATGAAAAGAAGATCATCAACATCAACCTTTTCAATCTTCA 360  
 G T S R N N S P T P S N T S S K K M K R R S S T S T F Q S S 96  
 CTTAGAAGTGCACAAGCAAGTCTGCGGAAGAACTGACCAACAAGTCCCATCTTCAAAGTCCCACCTTGTGATAGGAAACCCACAGGT 450  
 L R S A Q A S L R K K L T N K S H L O K S P L V D R K P T G 126  
 TCAAGCAGAAAAGCAAATTTGATTTGACACTTGTAGTTTGGACAGTGTGTTTCGTTCATTGAAAGACGCAATACAAGTGAAGATACG 540  
 S S T E S K L D S D T C S L D S D V F V I E R R N T S E D T 156  
 AATCATAAATAATCAACGGAACATATATGATGAAAGAACAAGAAAACGACGCTGAATGTACCTGGTAGCGACTCCAGACCACCCATATCA 630  
 N H N N S T E L Y D E R T K K T T C L N V P G S D S R P P I S 186  
 CCAAACCGTATAGATCAGCAACACTATCACAGACCCATGTGCTCCACCAGTGTAGTTGATGGTGAAGTTCGAAAAPACTTTTCAG 720  
 P N R D R S A T L S Q T H V L S P P V L V D G E V R K D F Q 216  
 TATCTGTCACCTTCTATACAGCAAGGTTTAAACACTATGGACAGCGACCAAAGAAATAAAGACTTTTTTCGTTCTGGTAGCTTTGCAAAA 810  
 Y L S P S F T A R L F N T M D Q R N K R L F R S G S F A K 246  
 CTAATCGGAAAACAGACTGTGCGGAATTACCAAAAACAATAAAAGTGAAGATTCACCATTAAAAGCCCTATACAGAGCAACTGTC 900  
 L I G K T D C A G I T K K H N K S E D S P L K A L Y R A T V 276  
 CAAAACAGCGAAAATCTTAACAATAAATAAATACCAAAACAAGGACATAAATATGCAATGTTGAAATCTTCTTAATATTTTCTTCC 990  
 Q N T A E N L N N N N Y Q T K D I I M H G \* 297  
 AGACTAAAGATAAGTGTGTTCTTATTTATGCGGTACGTATGTTATATATGTTATCCTATTTGTTCCCGATAATTTTGTAAACAACAA 1080  
 CATGCCCTCTAAGTTCAAAACAAAATTGTTTGCAGAAGATGTCACAAAATGACTTATTTCAGTTGTTCTAATGTAAGTTAA 1170  
 CAAATACAATCAATAAAGCGGTGAAAATGGTCTAAAAATATTTGTGCCGACCAATTTAAATAGTTTACAGCATTCTCTTAG 1260  
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 AAGATATCAGATTTTTTAAACATTTCCGCACTGTGTAATAGTAAACAATAAATAAAGTGTAGATTTCAAAAAAAAAAAAAAAAAA 1440

Fig. 3. Nucleotide and deduced amino acid sequence of cDNA for the *CsEndo-2* gene. The 1440bp insert includes a single open reading frame that encodes a polypeptide of 297 amino acids. The termination codon is shown by an asterisk, and potential signal sequences for polyadenylation are dotted-underlined.

tomeres of the animal hemisphere (Fig. 2C). The 32-cell stage embryo showed a similar and more distinct localization pattern of the transcript (Fig. 2E,F). At the 64-cell and 110-cell stages (Fig. 2G,H), *CsEndo-1* mRNA was evident in all of the primordial endoderm cells.

It has been noted that, in ascidians, whole-mount *in situ* hybridization usually detects signals of the zygotic transcripts first in nuclei of blastomeres of early embryos (e.g., Yasuo and Satoh, 1993; Satou et al., 1995). Because we were not able to detect the *CsEndo-1* mRNA in nuclei of early embryos until the 110-cell stage, the signal in early embryos was thought to represent the maternal message. This was confirmed by the quantitative RT-PCR analysis. Because twenty-five RT-PCR cycles resulted in an appropriate level of amplification immediately below the level of saturation, we examined changes in the quantity of *CsEndo-1* maternal transcripts by this number of amplification (Fig. 2K, left). As shown in Figure 2K (right), signal that corresponded to the *CsEndo-1* maternal transcript was evident and constant from the stage of fertilized eggs to the 32-cell stage, although the signal became less intense at the 110-cell stage. This suggested that the zygotic expression of *CsEndo-1* did not occur before the gastrulation.

As development proceeded, *CsEndo-1* mRNA became barely detectable during gastrulation (Fig. 2I) and neurulation. However, zygotic transcript was evident in the endoderm cells and notochord cells of the tailbud-stage embryo (Fig. 2J).

### The *CsEndo-2* gene

#### Characterization of the *CsEndo-2* cDNA clone

The cDNA sequence of *CsEndo-2* is shown in Figure 3. It was 1,440 nucleotides in length. We concluded that this cDNA contained the entire coding sequence of *CsEndo-2*, because the 5' end of the cDNA contained stop codons in all three reading frames in front of the initiator methionine at nucleotide position 73. The decoded *CsEndo-2* protein contained 297 amino acid residues. We were not able to find any similarities to other reported proteins.

#### The localization and the segregation of *CsEndo-2* mRNA

As in the case of *CsEndo-1*, although *CsEndo-2* is also a maternally expressed gene, whole-mount *in situ* hybridization failed to detect a distinct pattern of signal localization until the 16-cell stage (data not shown). *CsEndo-2* mRNA was detected in the endoplasmic region of the vegetal hemisphere at the 16-cell (Fig. 4A,B,C) and 32-cell stages (Fig. 4D,E). No hybridization signals were detected in blastomeres of the animal hemisphere (Fig. 4C). At the 64-cell and 110-cell stages, the signal was evident in the primordial endoderm cells; namely, A7.1, A7.2, A7.5, B7.1 and B7.2 blastomeres (Fig. 4F,G). In addition, *CsEndo-2* mRNA was detected in the A-line notochord cells (A8.5, A8.6, A8.13 and A8.14 blastomeres of the 110-cell embryo; Fig. 4F,G). The quantitative RT-PCR analysis demonstrated the presence of *CsEndo-2* maternal transcript in fertilized eggs and early embryos (Fig. 4J).

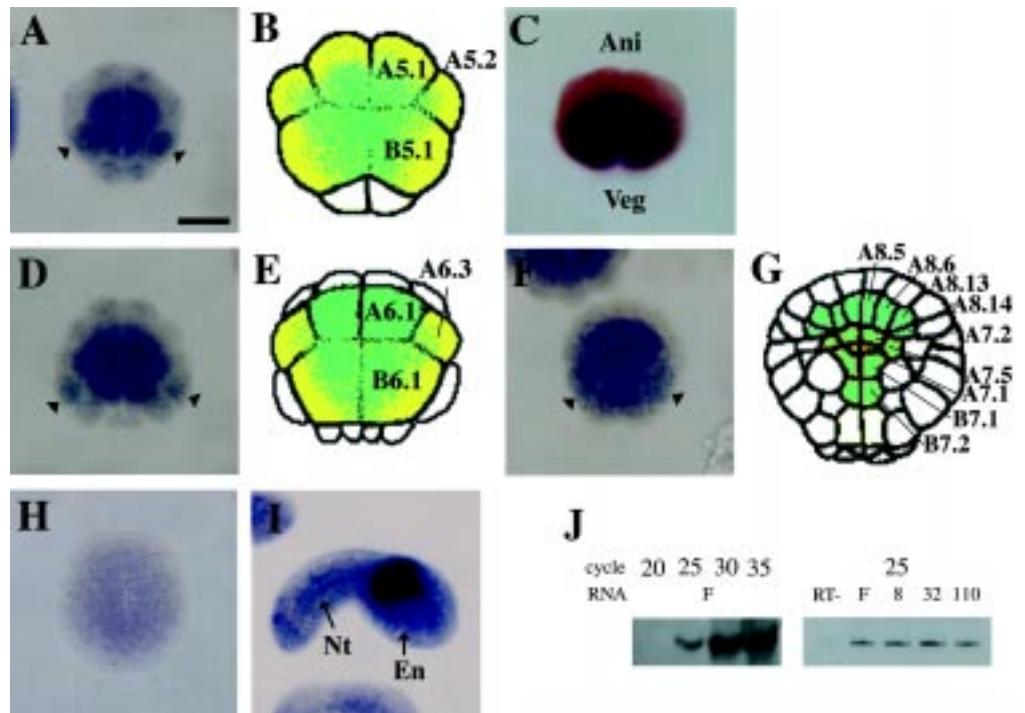
Granules within the myoplasm were sometimes lightly stained (arrowheads in Fig. 4A, D, F). However, this staining was labile and seemed to be non-specific due to longer time period of the staining reaction. A longer reaction without any probes also showed the signals (data not shown).

As in the case of *CsEndo-1*, *CsEndo-2* mRNA was barely detectable in the gastrulae (Fig. 4H), and zygotic transcripts became detectable again in endoderm cells and notochord cells of the tailbud-stage embryos (Fig. 4I).

### The *CsEndo-3* gene

#### Characterization of the *CsEndo-3* cDNA clone

The cDNA sequence of *CsEndo-3* is shown in Figure 5A, and was 881 nucleotides in length. We concluded that this cDNA clone contained the entire coding sequence of *CsEndo-3*, because the 5' end of the cDNA contained stop codons in all three reading frames in front of the initiator methionine at nucleotide position 59. The cDNA encoded a polypeptide of 141 amino acid residues. The *CsEndo-3* protein had a hydrophobic region at the carboxyl terminus, and therefore seemed to be a single-pass transmembrane protein. The *CsEndo-3* protein also contained a PDZ domain (Fig. 5B). The PDZ



**Fig. 4. Expression of the *CsEndo-2* gene in ascidian embryos.** Localization of *CsEndo-2* transcript at (A) the 16-cell, (D) 32-cell and (F) 110-cell stages, vegetal pole view. (C) The 16-cell stage embryo, posterior view, showing the maternal transcript was restricted to the vegetal side of the embryo. At these stages, distinct signals were found in the presumptive endoderm and the notochord cells. Weak background staining was detected in granules of the myoplasm (arrowheads). (B, E, G) Schematic representations of (A), (D), and (F). Yellow shows blastomeres with the developmental fate of endoderm or A-line notochord cells, and green shows the *CsEndo-2*-expressing cells. (H) A gastrula, vegetal view, showing no hybridization signals. (I) A tailbud embryo, lateral view indicating distinct zygotic expression in endoderm (En) and notochord (Nt). (J) RT-PCR analysis of *CsEndo-2* maternal transcript. (Left) Twenty-five cycles showed distinct signal. (Right) Assay of RNA extracted from fertilized eggs (F), 8- (8), 32- (32) and 110-cell embryos (110). RT- is a negative control.

domains are found in diverse membrane-associated proteins including the MAGUK family of guanylate kinase homologs, several protein phosphatases and kinases, neuronal nitric oxide synthase and several dystrophin-associated proteins (reviewed by Ponting *et al.*, 1997; Craven and Bredt, 1998). The PDZ domains of these proteins are thought to bind with other proteins. Generally, it is known that the PDZ-domain proteins of animals are cytoplasmically localized. However, the *CsEndo-3* is a protein that contained both a potential transmembrane domain and a PDZ domain. This is probably the first report of a PDZ domain-containing membrane protein.

*The localization and the segregation of CsEndo-3 mRNA*

As in the cases of *CsEndo-1* and *CsEndo-2*, any distinct localization of the *CsEndo-3* message was not detected until the 16-cell stage (data not shown), although *CsEndo-3* is also a maternally expressed gene. *CsEndo-3* mRNA was evident in the endoplasmic region of embryos at the 16-cell (Fig. 6A,B,C) and 32-cell stages (Fig. 6D,E). The primordial endoderm cells of the 64-cell and 110-cell stage embryos (A7.1, A7.2, A7.5, B7.1 and B7.2) showed distinct signals (Fig. 6F,G). As in the case of *CsEndo-2*, *CsEndo-3* mRNA was also detected in the A-line notochord cells (A8.5, A8.6, A8.13 and A8.14 blastomeres of the 110-cell stage; Fig. 6F, G). The presence of *CsEndo-3* maternal mRNA in fertilized eggs and early embryos was revealed by the RT-PCR analysis (Fig. 6J). Although granules of the myoplasm were weakly stained, the staining was thought to be non-specific, as described above.

*CsEndo-3* mRNA also became barely detectable in the gastrulae (Fig. 6H). In addition, the zygotic expression of *CsEndo-3* became evident in endoderm cells and notochord cells of the tailbud-stage embryos (Fig. 6I).

**Discussion**

In the present study, we isolated and characterized three maternal genes, *CsEndo-1*, *CsEndo-2* and *CsEndo-3*, the mRNAs of which are segregated with the endoplasm. The genes are also expressed zygotically in the endoderm and notochord cells of the tailbud embryo. During ascidian embryogenesis, the specification and subsequent differentiation of endoderm cells take place highly autonomously (reviewed by Satoh, 1994). This autonomy is due to endoderm determinants localized in the endoplasm (Nishida, 1993). In addition, Marikawa and Satoh (1996) suggested that maternal mRNA(s) is a component of the endoderm determinants. It is expected therefore that maternal mRNAs that are segregated together with endoplasm are associated with endoderm determinants. As far as we know, the present study is the first to identify maternal genes, the mRNAs of which are segregated with the endoplasm.

To deduce the possible functions of *CsEndo-1*, *CsEndo-2* and *CsEndo-3*, we microinjected synthetic mRNA of each of these three genes into fertilized eggs to examine effects of the ectopic expression or overexpression of the genes. As a control, we injected *pem* mRNA into fertilized eggs, which induced a deficiency in the formation of the anterior and dorsal larval structures (Yoshida *et al.*, 1996). However, the overexpression experiment failed to deduce the function of these genes, because injected embryos developed normally. Experiments with antisense-oligos to deduce the function of ascidian maternal genes have not yet succeeded. The exploration of their functions is an important subject of further research. In addition, the determination of localization of maternal proteins encoded by these genes will lead to a better understanding of their functions.

## A

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CGGCACGAGGCCCTAACTGGCGATAATTAAATCGACTGTAATATCAAAAACTTAGAAATGGAAGCCCAGCCACGCCATCACCCGTTAC 90
                                     M E A R P R P S P V T 11
CATAAACTTATCTCGGGGATCCAGCGGTCTTGGATTAAATATCAGAGGAGGTGGACCAGCCCCATCTACCAAATGACACTGGGATTTT 180
  I N L S R G S S G L G F N I R G G V D Q P H L P N D T G I F 41
TGTGACCAAGATCAGGAAAAACGAGCAGCTGATAAAGATGGGAGACTAAAGGAAGGAGATAAGCTTTTAGAGATCAATGGAACGAGTT 270
  V T K I R E N A A A D K D G R L K E G D K L L E I N G N E L 71
GCTGGATATAAAGCACAGTGAAGCAGTTGATCACTTCTCTCAGCAGGAGAACATGTTACTTTAAAAGTTGGCACGGCGCTGAAAAGTT 360
  L D I K H S E A V D E F L S A G E H V T L K V W E G A E K F 101
CCTGGTGGATGAATATAGCGAAATGAACAAACCGAAGGGCTGGGGCTCCTTAAAAAATCATGCTCATTTGTAAGCATAGCAACAGCCAT 450
  L V D E Y S E M N K P K G W G S L K K L M L I V S I A T A I 131
AGGCTTCGCAGTATTATGTAAGAGGTCCTAATACGCCCTTATTACCTCTTGAATCTATCTTAATTTAAGCTATTATTCTACCTT 540
  G F A V Y Y V K R S * 141
TTGGAAACCTAATAAATTTTTCAGTTCCATTATTAACAGCAACGGATTTTTCTTCAACTGCAAGTGTTCATTTCAATTACATGTTTACATA 630
GAATTTGAAACTAATTGTAACGCACCGCTTTAAACGTTTTGTTGTTGGAATAATCTTATTACATGTTTAAAGCGAATTTAAACTTTACATC 720
CGGTGCTTTTTCTTAATCATTTTTTGGAATATTGTTATTTTACAAAAAGCATAACGTTCTCTGGATTTTGTGAATATTGATATTATTGT 810
TAGACTTTTTGATAATTAATGCCGATTAATATAAATATGATGTAAAACATAAAAAAAAAAAAAAAAAAAAA 881

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## B

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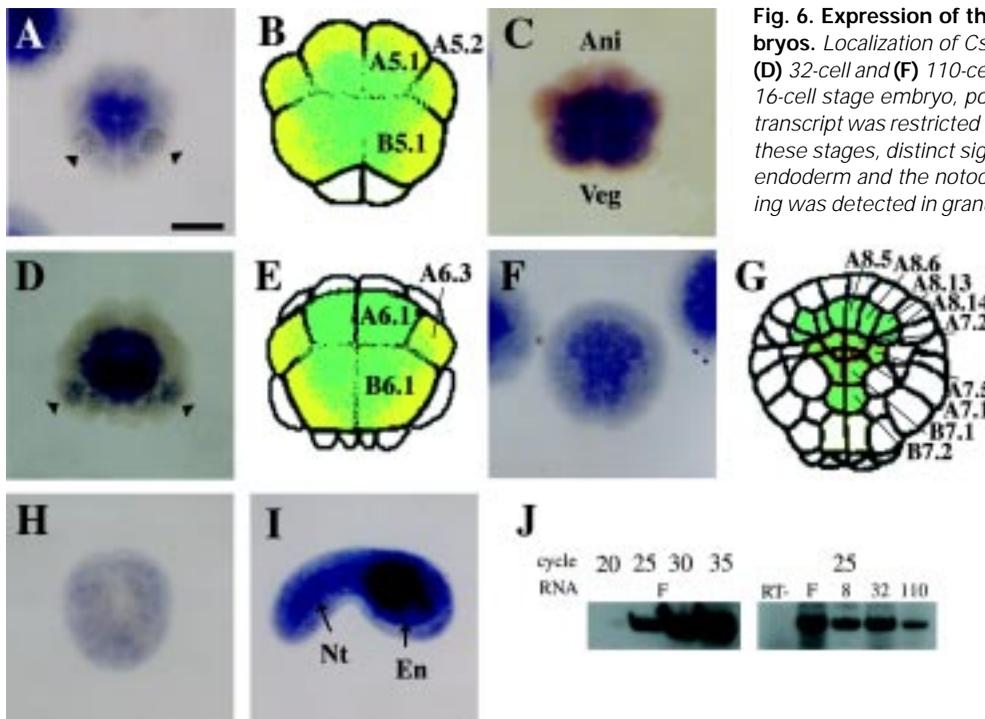
CsEndo3      VTINLSRGSSGLGFNIRGGVDQPHLPNDTGIFVTKIRENAAADKDRLEKEDKLEI--NGNE--LLDIKHSEAVDHFLSAGEH---VTLKVVH
Dsh          VSINMEAV-NFLGISIVGQSNR---GGDGGIYVGSIMKGGAVALDGRIEPGDMILQV--NDVN--FENMTNDEAVRVLREVVQKPGPIKLVVAK
xdsh        VTLNMEKY-NFLGISIVGQSNR---RGDGGIYIGSIMKGGAVAADGRIEPPGDMILQV--NDIN--FENMSNDDAVRVLRDIVHKPGPIVLTVAK
ZO-1        MKLVKFRKGDVGLRLAGND-----VGIFVAGVLEDSPPAAKEG-LEEGDQILRV--NNVD--FTNIIREEAVLFLDLPLKGE-EVTILAQK
PSD95 <1>    EEITLERGNSGLGFSIAGGTDNPHIGDDPSIFITKIIIPGAAAQDGRRLRVNDSILFV--NEVD--VREVTHSAAVEALKEAGSI---VRLYVMR
PSD95 <2>    MEIKLIKGPKGLGFSIAGGVGNQHIPGDNSIYVTKIIEGGAHDKGRLQIGDKILAV--NSVG--LEDVMHEDAVAALKNTYDV---VYLKVAK
PSD95 <3>    RRIVIRHGSTGLGFNIVGGED-----GE-GIFISFILAGPADLSGELRKGQDILSV--NGVD--LRNASHEQAIALKNAGQT---VTIIAQY
DLG <1>      EDIQLERGNSGLGFSIAGGTDNPHIGTDTSIYITKILISGAAAADGRLSINDIIVSV--NDVS--VVDVPHASAVDALKKAGNV---VKLHVKR
DLG <2>      IEIDLKGGKGLGFSIAGGIGNQHIPGDNGIYVTKLTDGGRQAQVDRGLSIGDKLIAVRTNGSEKNLENVTHELAVATLKSITDK---VTLIIGK
consensus   *   b   G*G* * G           G*** * G *   * GD   NG   *   b   ** *b   * * *

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**Fig. 5. (A) Nucleotide and deduced amino acid sequence of *CsEndo-3* cDNA clone and (B) the PDZ domain sequences of *CsEndo-3*, *Dsh* (Theisen et al., 1994), *xdsh* (Sokol et al., 1995), *ZO-1* (Willott et al., 1993), *PSD95* (Cho et al., 1992) and *DLG* (Woods and Bryant, 1991). The consensus residues (Ponting et al., 1997) are indicated below; the asterisks represent hydrophobic residues [ACFILMVWY], and 'b' represents basic residues. Residues that accord with the consensus are shown by bold letters.**

Alkaline phosphatase (AP) activity has been used to monitor the differentiation of endoderm cells of the ascidian embryo (Minganti, 1954; Whittaker, 1990). The AP gene was recently isolated from *H. roretzi* (Kumano and Nishida, 1998). The AP gene is expressed both maternally and zygotically, and zygotic expression initiates around neurulation. In addition, an ascidian member of NK-2 gene family, *Citiff1*, was isolated from *Ciona intestinalis* (Dr. F. Ristorator, personal communication). The *Citiff1* was expressed in all endodermal precursor cells at around the 110-cell stage. A microinjection of *Citiff1* mRNA into fertilized eggs resulted in the development of tadpole larvae with abnormalities in head-trunk development consequent to the formation of excess endoderm, suggesting that this gene plays an important role in normal endoderm differentiation (Dr. F. Ristorator, personal communication). In addition, the LIM class homeobox gene *HrLim* (Wada et al., 1995) and an ascidian homolog of the *fork head/HNF-3 $\beta$*  gene are also expressed in the presumptive endoderm and notochord cells (Corbo et al., 1997; Olsen and Jeffery, 1997; Shimauchi et al., 1997). Future studies should therefore explore the genetic cascade between *CsEndo-1-3* and the developmental genes mentioned above.

Several recent lines of evidence suggest that the A-line precursors of endoderm and notochord have similar characteristics and that some common mechanisms are at work in the specification of the endoderm and notochord. First, lineages of endoderm and notochord cells are common up to the 16-cell stage; namely, A5.1 and A5.2 at the 16-cell stage have the ability to generate both endoderm and notochord, and at the 32-cell stage, A6.1 and A6.3 become endoderm-lineage, while A6.2 and A6.4 are of notochord lineage. Second, the *As-T* gene or an ascidian *Brachyury* is expressed exclusively in notochord cells (Yasuo and Satoh, 1993, 1994), and the ectopic expression of *As-T* in presumptive endoderm cells altered the fate of endoderm cells to notochord cells (Yasuo and Satoh, 1998). Third, the fate of A-line notochord cells was changed to that of endoderm cells by the treatment of early embryos with lithium (Yoshida et al., 1998). The present results regarding the *CsEndo-1*, *CsEndo-2* and *CsEndo-3* genes provide further support for the notion mentioned above. That is, maternal transcripts of *CsEndo-2* and *CsEndo-3* were seen not only in the endoderm cells but also in notochord cells of early cleavage-stage embryos. In addition, the zygotic expression of all three genes was evident in both endoderm and notochord cells of the tailbud



**Fig. 6. Expression of the *CsEndo-3* gene in ascidian embryos.** Localization of *CsEndo-3* transcript at (A) the 16-cell, (D) 32-cell and (F) 110-cell stages, vegetal pole view. (C) The 16-cell stage embryo, posterior view, showing the maternal transcript was restricted to the vegetal side of the embryo. At these stages, distinct signals were found in the presumptive endoderm and the notochord cells. Weak background staining was detected in granules of the myoplasm (arrowheads).

(B,E,G) Schematic representations of (A), (D), and (F). Yellow shows blastomeres with the developmental fate of endoderm or A-line notochord cells, and green shows the *CsEndo-3*-expressing cells. (H) A gastrula, vegetal view showing no hybridization signals. (I) A tailbud embryo, lateral view, indicating distinct zygotic expression in endoderm (En) and notochord (Nt). (J) RT-PCR analysis of *CsEndo-3* maternal transcript. (Left) Twenty-five cycles showed the signal. (Right) Assay of RNA extracted from fertilized eggs (F), 8- (8), 32- (32) and 110-cell embryos (110). RT- is a negative control.

embryos. The effect of lithium was mimicked by the overexpression of  $\beta$ -catenin, suggesting that this mechanism is associated with the Wnt signaling cascade (Yoshida *et al.*, 1998). It would be of interest to address the mechanism underlying the specification of the endoderm in relation to the Wnt signaling cascade and to investigate how *CsEndo-1*, *CsEndo-2* and *CsEndo-3* are involved in this cascade.

*CsEndo-1*, *CsEndo-2* and *CsEndo-3* showed a similar patterns of segregation of the maternal transcript. Namely, although the presence of the maternal transcripts was revealed by the RT-PCR analysis, we did not detect any distinct localization or segregation of the maternal transcripts during ooplasmic segregation and early cleavages up to the 8-cell stage. This was partially due to a large amount of yolk in the endoplasmic region, which sometimes prevents successful whole-mount *in situ* hybridization. However, from the 16-cell stage onwards, the segregation of the maternal transcript with the endoplasm was evident. Several maternal genes have recently been isolated from ascidians; they showed localization of maternal transcripts. Interestingly, most of the maternal genes including *pem* (Yoshida *et al.*, 1996), *pem-2*, *pem-3*, *pem-4*, *pem-5* and *pem-6* (Satou and Satoh, 1997) from *C. savignyi*, and *HrWnt-5* (Sasakura *et al.*, 1998a) and a gene for serine-threonin kinase (Sasakura *et al.*, 1998b) from *H. roretzi* showed similar patterns of localization. Namely, the maternal transcripts of these genes are segregated first with the so called myoplasm up to the 8-cell stage. Then, after the 16-cell stage, the maternal transcripts are not segregated with the myoplasm but rather are localized to the posterior end of developing embryos. There may be an anchoring mechanism for the maternal transcripts at the posterior end of early embryos. In contrast to these *pem*-like maternal genes, *CsEndo-1*, *CsEndo-2* and *CsEndo-3* are concentrated and segregated with the endoplasm. This suggests

that the machinery for the segregation of these maternal genes is different from that for *pem*-like maternal genes. Therefore, the segregation mechanism is also an important subject of further studies.

## Materials and Methods

### Ascidian eggs and embryos

*Ciona 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  $\mu$ g/ml streptomycin sulfate.

### Isolation of cDNA clones and sequencing

The construction of a cDNA library of *C. savignyi* fertilized egg mRNAs subtracted with gastrula mRNAs was described in a previous report (Satou and Satoh, 1997). cDNA clones for the maternal genes of *CsEndo-1*, *CsEndo-2* and *CsEndo-3* cDNA were isolated by screening the cDNA library. Briefly, clones were randomly picked up from the subtracted library and then partially sequenced from the 3'-end to avoid analyzing the same clones any further. After partial sequencing, the localization of corresponding mRNA was examined for each clone by whole-mount *in situ* hybridization. cDNA clones exhibiting the localization of mRNA corresponding to the endoplasm were selected for further analyses.

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

### Northern analysis

Total RNA was isolated from the fertilized eggs by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Poly(A)<sup>+</sup> RNA was purified using Oligotex beads (Roche Japan, Tokyo). Poly(A)<sup>+</sup> RNA was fractionated by agarose gel electrophoresis,

and transferred to a Hybond-N+ membrane (Amersham, Buckinghamshire, UK). Blots were hybridized with a [<sup>32</sup>P]-random-labeled DNA probe in 6xSSPE, 0.5% SDS, 5xDenhardt's solution, 100 µg/ml salmon sperm DNA, and 50% formamide. The filter was washed twice in 2xSSC/0.1% SDS, and exposed to X-ray film.

#### RT-PCR analysis

Quantitative RT-PCR was performed basically according to the method described by Wilson and Melton (1994) with minor modifications. Total RNA was extracted by the AGPC method (Chomczynski and Sacchi, 1987). Twenty fertilized eggs or embryos were lysed in 200 µl GTC solution and stored at -80°C. Following the phenol-chloroform extraction and iso-propanol precipitation, RNA was treated with 1U of RNase-free DNase I (GIBCO BRL) and then with 0.2 mg/ml proteinase K (Sigma). After annealing with 10 pmol oligo(dT), the total RNA was incubated with 200U of Superscript II Reverse Transcriptase (GIBCO BRL) for 50 min at 42°C in a total reaction volume of 20 µl. The reaction mixture contained 1xFirst Strand Buffer, 10 nM DTT, 1.5 nM of each dNTP and 40U of RNase inhibitor (TOYOBO). One-tenth of the RT mixture was used as templates for PCR. Each 100 µl reaction mixture contained 0.2 mM dNTPs, 1.5 mM MgCl<sub>2</sub>, 0.1 µl α-[<sup>32</sup>P]-dCTP, 100 pmol of each primer, 1xTaq Buffer and 2.5U of Taq DNA polymerase (TOYOBO). Reaction proceeded through 20 to 35 amplification cycles (30 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C). Twelve µl of the PCR mixture was resolved on 8% non-denaturing polyacrylamide gels and subjected to autoradiography. The primers used in this study were as follows: *CsEndo1-5'*, ATCGCCAAATGCTGTGCA; *CsEndo1-3'*, ATTGAAGGTGTCAGGGGT; *CsEndo2-5'*, TGCCAAAACACAGCCGA; *CsEndo2-3'*, CTTAGGAGGCGCATGTT; *CsEndo3-5'*, GCACAGTGAAG-CAGTTGA; and *CsEndo3-3'*, AAGCCTATGGCTGTTGCT.

#### 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).

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