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Original Article

Characterization of an ascidian maternal T-box gene, *As-mT*

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ABSTRACT The T-box genes encode a novel family of transcriptional factors that seem to play crucial roles in various processes of animal development, in particular, mesoderm formation of chordate embryos. The ascidian egg has been regarded as a typical mosaic egg, in which several types of embryonic cells are specified autonomously dependent on prelocalized maternal factors or determinants. In the present study, we investigated a possible role of a maternal T-box gene *(As-mT)* of the ascidian *Halocynthia roretzi*. A cDNA clone we obtained predicted As-mT protein of 891 amino acids with a distinct T-domain, which was divergent from those of other T-box genes. Expression of *As-mT* was exclusively maternal. Although the transcript became barely detectable by the gastrula stage, no zygotic expression was evident during embryogenesis. The maternal transcript was distributed rather evenly within eggs and early embryos without any special localization. Injection of synthetic *As-mT* mRNA into fertilized eggs induced retardation of embryogenesis. Although cleavage occurred normally, the initiation of gastrulation was delayed, and delay in the morphogenesis resulted in dumpy larvae. Expression of a muscle-specific actin gene, a notochord-specific *Brachyury* gene, and an epidermis-specific gene was not detected at the early gastrula stage, all of the three genes being expressed in normal embryos at that stage. However, the expression of these genes as well as a mesenchyme-specific gene and histochemical activity of endoderm-specific alkaline phosphatase were evident by the mid-tailbud stage.

KEY WORDS: *ascidians, maternal T-box gene, spatial expression, gene overexpression embryogenesis retardation*

Introduction

A novel family of transcriptional factors that appears to play critical roles in various aspects of animal development was recently uncovered on the basis of homology to the DNA binding domain of the mouse *Brachyury* or *T* gene product (reviewed by Herrmann and Kispert, 1994: Smith, 1997; Papaioannou and Silver, 1998). The *T* locus was originally identified about 70 years ago as homozygotic mutant mouse embryos died on day 11 of gestation with deficiencies in the posterior mesoderm formation in the primitive streak, an absent notochord and severe reduction of the allantois (Dobrovolskaïa-Zavadskaïa, 1927). In 1990, this gene was cloned (Herrmann *et al.*, 1990). Transient expression of the *Brachyury* gene is detected in nascent and migrating mesoderm generated from the primitive streak, then continuously in the notochord (Wilkinson *et al.*, 1990). The cloning of mouse *T* was followed by isolation and characterization of its homologs in chick (Kispert *et al.*, 1995b), *Xenopus* (Smith *et al.*, 1991), zebrafish (Schulte-Merker *et al.*, 1992), amphioxus (Holland *et al.*, 1996; Terazawa and Satoh, 1997), ascidians (Yasuo and Satoh, 1993; Corbo *et al.*, 1997), hemichordate (Tagawa *et al.*, 1998), sea urchin (Harada *et al.*, 1995), and insects (Kispert *et al.*, 1994).

The Brachyury protein acts as a transcriptional factor, which is achieved through DNA binding activity of the "T-domain" or "T-box", a highly conserved region in the N-terminal half of *T* genes (Kispert *et al.*, 1995a). The T-box itself, however, is not unique to *Brachyury*, but shared with *optomotor-blind (omb)* of *Drosophila* (Pflugfelder *et al.*, 1992). In addition, recent clonings of T-box genes, other than the *Brachyury* subfamily, from the mouse, chick, *Xenopus* and nematodes, suggest a novel gene-family of *T*-related genes (reviewed by Herrmann and Kispert, 1994; Smith, 1997; Papaioannou and Silver, 1998). Furthermore, three or more maternally expressed T-box genes have been isolated from *Xenopus*: *Xombi* is involved in mesoderm patterning and blastopore lip formation (Lustig *et al.*, 1996); *VegT* is localized to the vegetal cortex during oogenesis and is involved in mesoderm patterning (Zhang and King, 1996); *Antipodean* encodes a vegetally localized maternal mRNA and can trigger mesoderm formation

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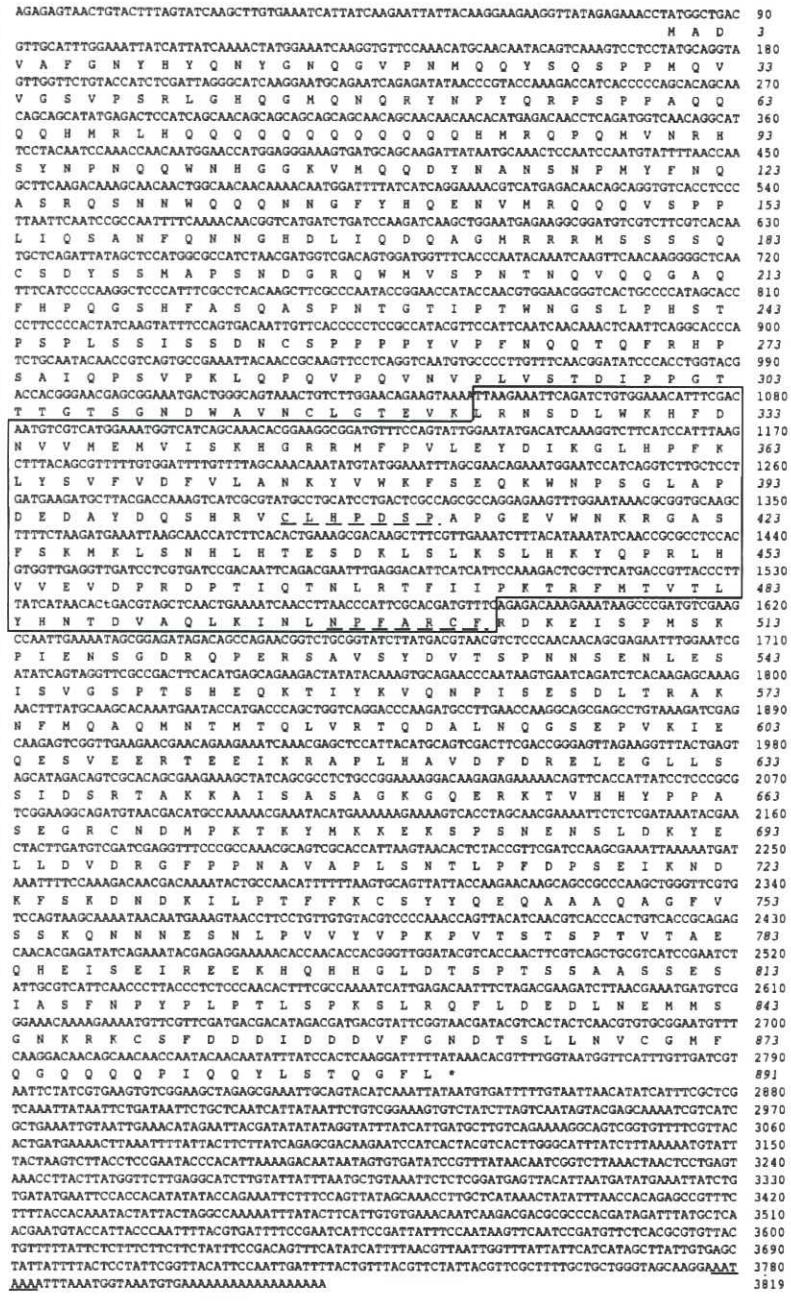

Fig. 1. The nucleotide and predicted amino acid sequences of a cDNA clone for the *As-mT* gene. *The sequence of the cDNA encompasses 3,819bp including 18 adenylyl residues. The ATG at the position 82-84 represents the putative start codon of the As-mT-encoded protein of 891 amino acids. An asterisk indicates the termination codon and the potential signal sequence for polyadenylation is underlined. The T-domain is boxed. Sequences for PCR primers are shown by dotted lines.*

cells are specified autonomously dependent on prelocalized egg cytoplasmic factors or determinants. Recent experimental embryological studies have provided convincing evidence for the localization of the determinants responsible for the differentiation of muscle, epidermis and endoderm, factors for the establishment of the embryonic antero-posterior axis, and those for the initiation of gastrulation (for reviews see Satoh, 1994; Nishida, 1997). The molecular identification of localized maternal factors, the elucidation of the machinery responsible for the localization, and the exploration of the mode of action of the localized factors are therefore key research subjects for the elucidation of the pattern formation of ascidian embryos.

In the present study, we attempt to isolate a maternally expressed T-box gene in ascidian eggs. We show here that expression of the gene named *As-mT* is exclusively maternal, that the *As-mT* transcript is not localized to a particular region of the egg cytoplasm, and that overexpression of the protein by microinjection of synthetic mRNA results in a general retardation of embryogenesis.

Results

Isolation and characterization of cDNA for an ascidian maternal T-box gene (As-mT)

The T-box, a DNA-binding domain of about 190 amino acids usually positioned in the N-terminal half of *T* gene products, is highly conserved among vertebrates and invertebrates (Herrmann and Kispert, 1994; Papaioannou and Silver, 1998). Using degenerate oligonucleotide primers corresponding to the shared sequences (see Materials and Methods; Fig. 1), we amplified target fragments from an *H. roretzi* unfertilized-egg cDNA library by means of PCR. Sequencing the amplified fragments after subcloning them into plasmid vectors, revealed that the library contained cDNA clones for an ascidian maternal T-box gene, named *As-mT*.

Using the amplified fragment as probe, we screened the library again and isolated seven candidate cDNA clones, which were revealed to encode an identical polypeptide. As shown in Figure 1, the longest cDNA sequence was 3,819 base pairs long and had a single open reading frame that predicted a polypeptide of 891 amino acids. Because Northern blot analysis suggested a 3.8-kb *As-mT* transcript (Fig. 3), the cDNA was close to the full length. The T-domain was evident in the middle portion of the predicted protein (boxed in Fig. 1). Besides the T-domain, we could not find any consensus sequences with suggested functions.

(Stennard *et al.*, 1996); and *Xbrat* is also essential for embryonic mesoderm formation (Horb and Thomsen, 1997). All of these results strongly suggest that T-box family transcriptional factors play essential roles in early processes of the mesoderm formation in *Xenopus* embryos.

Since the work of Chabry (1887), the ascidian egg has been regarded as a mosaic egg, in which several types of embryonic

The As-mT encodes a divergent T-box protein

Although the overall degree of amino acid identity was not high, the amino acid sequence of As-mT T-domain was highly conserved when compared with other T-domains. Recent molecular

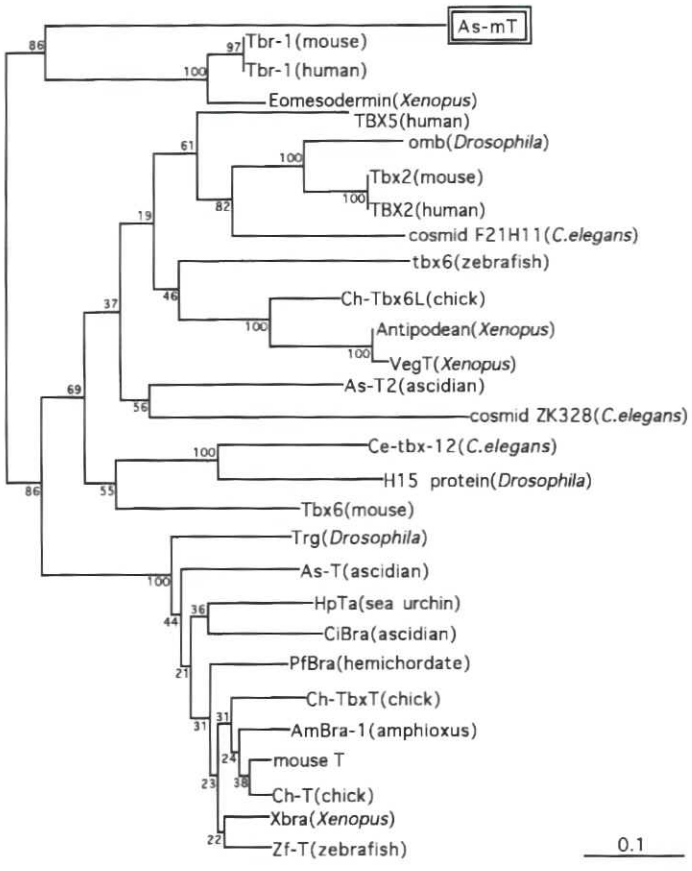

Fig. 2. Evolutionary relationships of As-mT with other T-domain proteins. *Using 135 confidentially aligned sites of the T-domain amino acid residues, a molecular phylogenetic tree was constructed by the neighbor-joining method. Branch length is proportional to the number of amino acid substitutions; the scale bar indicates 0.1 amino acid substitutions per position in the sequence. The numbers at each branch indicate the percentage of times that a node was supported in 100 bootstrap pseudoreplications.*

phylogenetic analyses suggest that products of the T-box gene family members are categorized into several subfamilies, including T (Brachyury), Tbx1, Tbx2 (including *Drosophila* omb), Tbx6 (including *Xenopus* VegT, Xombi and Antipodean, but not mouse Tbx6), and Tbr1 (including *Xenopus* Eomesodermin) (Herrmann and Kispert, 1994; Papaioannou and Silver, 1998). In order to deduce which subfamily the *As-mT* belongs to, we performed a molecular phylogenetic analysis, using confidentially aligned 135 amino acid sites of the T-domains, by the neighbor-joining method (Saitou and Nei, 1987). As shown in Figure 2, in this tree, the grouping of various *Brachyury* homologs was supported by 100% bootstrap value. The tree suggested that *As-mT* encodes a divergent T-box, which was not grouped with any of the subfamilies, although the subfamily with more affinity to *As-mT* was Tbr1.

Temporal expression of As-mT

The result of Northern blot analysis is shown in Figure 3. The *As-mT* maternal transcript was abundant in unfertilized eggs and early embryos up to the 64-cell stage. However, the band intensity reduced quickly as development proceeded. By the gastrula stage, the transcript became barely detectable, although a very faint band was seen at the neurula and tailbud stages. Judging from the band intensity profile, expression of *As-mT* is exclusively maternal, and is unlikely to be expressed zygotically during embryogenesis up to the hatch of larvae.

Spatial distribution of As-mT transcript

Figure 4 shows results of *in situ* hybridization to determine the spatial distribution of *As-mT* maternal transcript. The *As-mT* transcript appeared to be distributed evenly in the middle portion of the cytoplasm of unfertilized eggs (Fig. 4A). No special localization of *As-mT* transcript was observed in unfertilized eggs. In ascidians, fertilization evokes a dynamic rearrangement of the egg cytoplasm called ooplasmic segregation. The first phase of ooplasmic segregation involves rapid movement of the peripheral cytoplasm including the myoplasm (the cytoplasm segregated into muscle lineage) to form a transient cap near the vegetal pole of the egg. After the first phase of ooplasmic segregation, localized maternal transcripts such as *pem* became accumulated near the vegetal pole (Yoshida *et al.*, 1996). 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. However, *As-mT* transcript was not localized in particular regions during ooplasmic segregation (data not shown).

At the 8-cell stage, *As-mT* transcript was distributed in the middle portion of the cytoplasm of the a4.2 and b4.2 animal-blastomeres and B4.1 vegetal-blastomere, although signal was weak in the A4.1 vegetal-blastomere (Fig. 4C). This pattern of *As-mT* transcript distribution was inherited by embryos at later stages; namely, the hybridization signal was detected mainly in animal blastomeres (Fig. 4D). Signals for *As-mT* transcript became weaker in gastrulae (Fig. 4E) and barely detectable in neurulae (Fig. 4F).

Injection of As-mT mRNA induced retardation of embryogenesis

The T-box genes encode transcriptional factors that are impli-

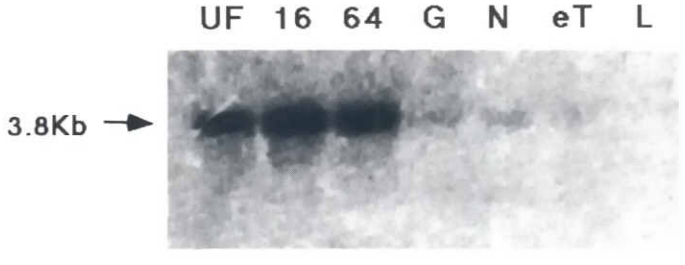

Fig. 3. Northern blot analysis of *As-mT*. *Occurrence of As-mT transcript during embryogenesis of H. roretzi. Northern blots of poly(A)⁺ RNA prepared from unfertilized eggs (UF), 16-cell stage embryos (16), 64-cell stage embryos (64), gastrulae (G), neurulae (N), early tailbud embryos (eT) and swimming larvae (L) were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the membrane was washed under high-stringency conditions. The As-mT transcript was detected in unfertilized eggs, 16- and 64-cell stage embryos, but became barely detectable as development proceeded. Each lane was loaded with 2 μg of poly(A)⁺ RNA.*

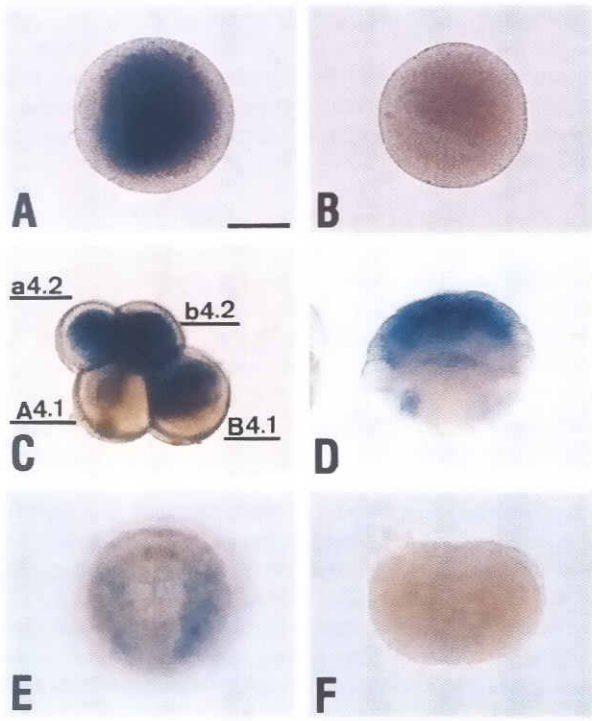

Fig. 4. Spatial distribution of *As-mT* transcripts, as revealed by whole-mount *in situ* hybridization. (A) *Unfertilized egg showing hybridization signals in the middle portion of the egg cytoplasm.* **(B)** *Control unfertilized egg hybridized with sense probe.* **(C)** *An 8-cell stage embryo, side view.* **(D)** *A 64-cell stage embryo, lateral view.* **(E)** *Gastrula, animal pole view. Hybridization signals became faint.* **(F)** *Neurula, lateral side view, showing no signals. Bar, 100 μm.*

cated in various aspects of animal development. In conjunction with maternally expressed T-box genes, *Xenopus Xombi* (Lustig *et al.*, 1996) and *VegT* (Zhang and King, 1996), for example, they are reported to be involved in embryonic mesoderm formation. To infer a putative function of *As-mT*, we performed experiments in which overexpression of *As-mT* was induced by a microinjection of synthesized *As-mT* mRNA into fertilized eggs.

As control, we injected 0.4 μg/μl synthesized lacZ-RN3 RNA into fertilized eggs. Three independent series of total 71 eggs were injected with synthesized lacZ-RN3 RNA and allowed to develop into tailbud embryos. Of these, 69 eggs developed into embryos with normal morphology (data not shown), and 2 into embryos with a slightly abnormal tail. LacZ activity was histochemically detected in almost all of the constituent cells of the embryo (data not shown).

To produce the overexpression of *As-mT*, four independent series of total 81 eggs were injected with 0.4 μg/μl synthesized *As-mT* mRNA and then allowed to develop into tailbud embryos. All of these 81 embryos showed slight morphological abnormality (Fig. 5C,F). Abnormality was evident in both the trunk and tail regions, and resultant tailbud embryos appeared dumpy. When two series of 40 eggs were injected with 0.2 μg/μl *As-mT* mRNA, 36 of them also developed into dumpy embryos, and the remaining 4 developed into embryos with normal morphology. Abnormality was evident again in both the trunk and tail regions. Although their morphology was not normal, cell differentiation including epidermis, muscle and notochord, appeared to take place, judging from the morphology (Fig. 5F). We therefore examined the expression of tissue-specific genes in *As-mT* mRNA-injected embryos. *In situ* hybridization revealed the expression of a muscle-specific actin gene *HrMA4* (Fig. 5C), a notochord-specific *Brachyury* gene *As-T* (data not shown; Fig. 5F of a living specimen shows clearly differentiation of notochord cells), an epidermis-specific gene *HrEpiC* (data not shown), and a mesenchyme-predominant cytoplasmic actin gene *HrCA1* (data not shown). We also confirmed the occurrence of endoderm-specific alkaline phosphatase activity in the ventral trunk region of the embryo (data not shown). All of the differentiation markers appeared in appropriate regions of the dumpy embryo; no ectopic expression of the markers was detected in experimental embryos. Therefore, it might be concluded that, although the overall morphology was slightly abnormal, the tissues differentiated in *As-mT*-overexpressed tailbud embryos.

Careful observation of cleavage pattern and developmental tempo in *As-mT*-overexpressed embryos indicated that the cleavage pattern and tempo were the same as normal embryos. However, the timing of initiation of gastrulation was delayed a few hours in the *As-mT*-overexpressed embryos compared with normal embryos. This delay was followed by retardation of gastrulation, neurulation and tailbud embryo formation. When we examined whether the timing of the gene expression was altered in *As-mT*-overexpressed embryos, it became evident that the tissue-specific gene expression was delayed. *HrMA4* expression was observed in very few experimental embryos (Fig. 5B) at the early gastrula stage, at that time normal gastrulae showing the gene expression in primordial muscle cells (Fig. 5A). No *As-mT*-overexpressed embryos showed *As-T* expression at the early gastrula stage (Fig. 5E). The number of primordial epidermal cells exhibiting *HrEpiC* expression decreased in *As-mT*-overexpressed embryos (Fig. 5H). Therefore, it is likely that the injection of *As-mT* mRNA induced general retardation of embryogenesis.

Discussion

The present study characterized a maternal T-box gene named *As-mT* of the ascidian *H. roretzi*. The expression of *As-mT* was exclusively maternal. In conjunction with maternally expressed T-box genes, up to date, four independent cDNA clones have been isolated from *Xenopus*, *Xombi* (Lustig *et al.*, 1996), *VegT* (Zhang and King, 1996), *Antipodean* (Stennard *et al.*, 1996) and *Xbrat* (Horb and Thomsen, 1997). Molecular phylogenetic analysis shown in Figure 2 suggested a structural affinity of all of the first three *Xenopus* maternal T-box genes with the Tbx6 subfamily based on the amino acid sequence similarity of the T-box region. These genes are expressed not only maternally but also zygotically. Maternal transcripts of *Xombi* (Lustig *et al.*, 1996), *VegT* (Zhang and King, 1996), *Antipodean* (Stennard *et al.*, 1996), and *Xbrat* (Horb and Thomsen, 1997) are localized to the oocyte's vegetal cortex and cytoplasm. Ectopic expression of the genes in the animal blastomeres induced expression of mesodermal genes, suggesting a crucial role of these genes in the mesoderm formation during *Xenopus* embryogenesis (Lustig *et al.*, 1996; Stennard *et al.*, 1996; Zhang and King, 1996; Horb and Thomsen, 1997). Mis-expression of *VegT* in dorsal animal blastomeres fated to contribute to brain also suppressed head formation (Zhang and

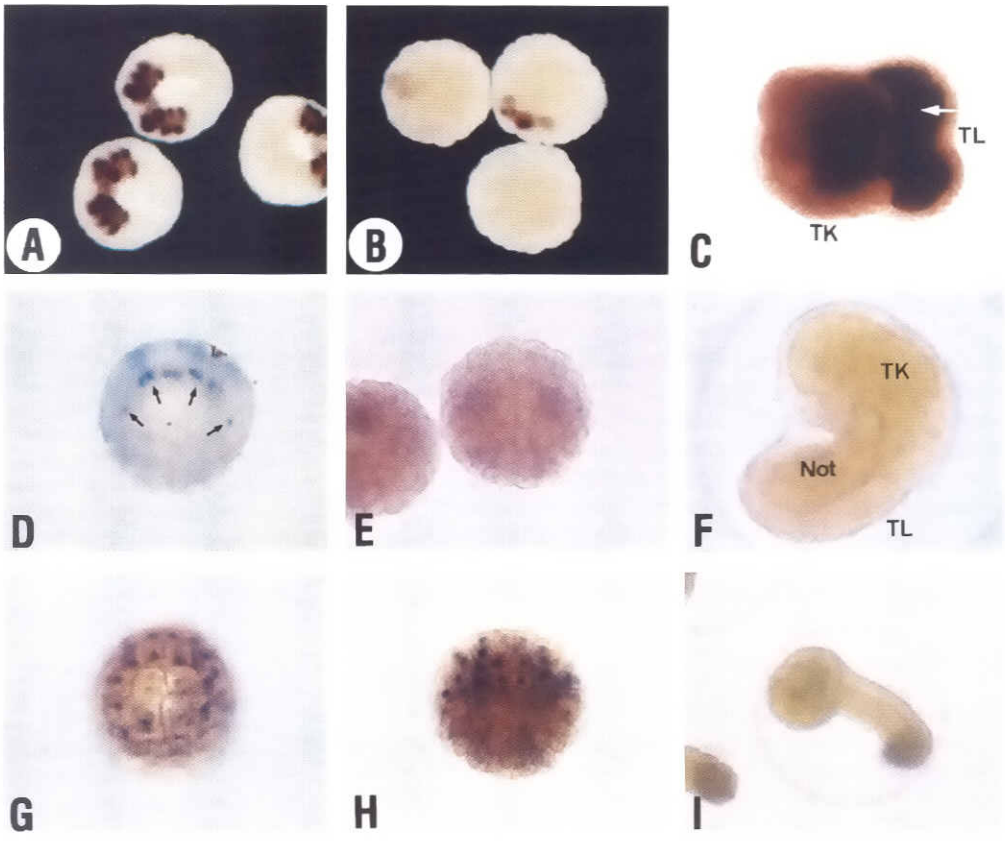

Fig. 5. Effects of injection of *As-mT* mRNA into fertilized eggs upon embryogenesis. (A-C) *Muscle actin gene expression, assessed by whole-mount in situ hybridization, in control gastrulae (A) and experimental (As-mT mRNA injected) gastrulae (B) and tailbud embryo (C). TK, trunk; TL, tail. A white arrow in C indicates the actin gene expression.* **(D,E)** *Brachyury gene expression, assessed by whole-mount in situ hybridization, in control gastrula (D, arrows) and experimental (As-mT mRNA injected) gastrula (E).* **(F)** *Experimental tailbud embryo showing differentiation of notochord (Not).* **(G,H)** *Epidermis-specific gene expression in control gastrula (G) and experimental (As-mT mRNA injected) gastrula (H), assessed by whole-mount in situ hybridization.* **(I)** *Control tailbud embryo at the stage corresponding to C and F.*

King, 1996). In contrast, the molecular phylogeny suggested that *As-mT* encodes a polypeptide with a divergent T-box. The *As-mT* expression is exclusively maternal. The maternal transcript is not localized to a particular egg cytoplasmic region. Overexpression of the gene induced general retardation of the zygotic expression of tissue-specific genes and initiation of morphogenesis, and thus resulting in dumpy tailbud embryos. Therefore, it is not reasonable to correlate the structure and function of maternal *As-mT* directly to that of the *Xenopus* maternal T-box genes.

Recent studies have demonstrated that antisense oligodeoxynucleotides (ODNs) can be applied successfully to deduce possible functions of zygotic products of ascidian developmental genes. Swalla and Jeffery (1996), Olson and Jeffery (1997), and Satou and Satoh (unpublished data) used ODNs to deduce functions of *Manx*, *HNF-3β* and *pem-3*, respectively. However, at present, we are unable to analyze the function of the maternal product of ascidian developmental genes, because techniques such as *C. elegans* RNA interference (e.g., Fire *et al.*, 1998) are not applicable to ascidian embryos.

The fertilized egg of ascidians 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 (reviewed by Satoh, 1994). Lineage of these embryonic cells is almost completely described. Taking advantage of these features of the ascidian embryo, we investigated expression and possible function of ascidian T-box genes. We found that the *H. roretzi Brachyury (As-T)* is expressed exclusively in blastomeres of notochord lineage and that the *As-T* transcripts become detectable immediately after the developmental fate of the blastomeres is restricted to give rise to the notochord (Yasuo and Satoh, 1993,1994). Overexpression of this gene induces notochord differentiation in embryonic cells of non-notochord lineage, suggesting a master control of *As-T* over notochord formation (Yasuo and Satoh, 1998). In addition, we isolated another T-box gene *(As-T2)*, which is transiently expressed in differentiating muscle cells and in cells at the tip of the elongating tail (Yasuo *et al.*, 1996). Overexpression of *As-T2* induces muscle-specific gene expression in ectodermal cells (Mitani *et al.*, unpublished data). The *Halocynthia* genome also contains an ascidian homolog of *omb* of *Drosophila*. Because all of the T-box genes including *As-mT* encode transcriptional factors with different functions, it is a very intriguing research subject to determine and compare targets of these ascidian T-box genes.

The ascidian egg is 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, epidermis and endoderm, factors for the establishment of antero-posterior axis of the embryo, and those for initiation of gastrulation (reviewed by Satoh, 1994; Nishida, 1997). The molecular identification of localized maternal factors, the elucidation of the machinery responsible for the localization, and the exploration of the mode of action of the localized factors are therefore key research subjects for the elucidation of the pattern formation of ascidian embryos. Recently, Yoshida *et al.* (1996) isolated a novel maternal gene, *posterior end mark (pem)*, of which transcript is initially concen-

trated in the posterior-vegetal cytoplasm of the fertilized egg, and later the distribution of the transcript marks the posterior end of developing embryos. Overexpression of PEM results in development of larvae with deficiencies of the anterior-most adhesive organ, dorsal brain and sensory pigment cells, suggesting that *pem* may be involved in pattern formation of the embryo (Yoshida *et al.*, 1996, 1997). In addition, maternal genes with localized mRNA were further isolated by Satou and Satoh (1997). Interestingly, all of the five genes showed a distribution pattern of the maternal mRNA very similar to that of *pem*, and therefore named *posterior end mark 2 (pem-2), pem-3, pem-4, pem-5,* and *pem-6* (Satou and Satoh, 1997). The predicted amino acid sequence, for example, 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), and that *pem-3* encodes a probable RNA-binding protein with two KH domains (Satou and Satoh, 1997). Although *As-mT* transcript is not localized, functional relationships between *As-mT* and *pem*s should be explored in future studies to understand the molecular mechanisms underlying cell specification and pattern formation of the mosaic embryo.

Materials and Methods

Ascidian eggs and embryos

Naturally spawned eggs of the ascidian *Halocynthia roretzi* were fertilized with a suspension of non-self sperm, and then raised in filtered seawater at about 12°C. Embryogenesis proceeded synchronously in various batches of eggs. The first cleavage occurred about 2 h after insemination, and the embryo divided at about hourly intervals. They became gastrulae about 12 h after fertilization and developed into early tailbud embryos about 24 h after fertilization. Tadpole larvae hatched at about 40 h of development.

Eggs and embryos at appropriate stages were collected by low speed centrifugation, and were frozen for Northern blot analysis or fixed for *in situ* hybridization as whole-mount specimens.

Isolation of cDNA clones for As-mT and sequencing

For PCR amplification, we synthesized primers 5'-TG(C/T)(T/C)T(A/C/G/T)CA(C/T)CC(A/C/G/T)GA(C/T)TC(A/C/G/T)CC-3' as the sense-strand oligonucleotide and 5'-(A/G)AA(A/G)GA(A/C/G/T)CT(A/C/G/T)GC(A/G)AA(A/C/G/T)GG(A/G)TT-3' as the antisense oligonucleotide. Target fragment was amplified from an *H. roretzi* unfertilized-egg cDNA library constructed with λZAPII (Stratagene; Fujiwara *et al.*, 1993). Amplification proceeded for 30 cycles of 94°C (1 min), 50°C (1 min), 72°C (1 min). PCR product was purified by gel electrophoresis and cloned into pBluescript II SK(+) (Stratagene). Sequence of the fragment was determined by the ABI PRISM dye primer cycle sequencing kit (Perkin Elmer).

The PCR-derived clone was random-labeled with [^{32}P]dCTP (Amersham). The *H. roretzi* unfertilized-egg cDNA library was screened using the probes under high stringency conditions (hybridization: 6xSSC, 0.5% SDS, 5xDenhardt's solution, 0.1 mg/ml salmon sperm DNA, 50% formamide at 42°C; washing: 2xSSC, 0.1% SDS at 65°C). Several positive clones were obtained. The longest was prepared for sequencing by controlled nested deletion from either the T3 or the T7 side and sequenced using the ABI PRISM dye primer cycle sequencing kit (Perkin Elmer).

Sequence comparisons and molecular phylogenetic analysis

Amino acid sequences of the T-box regions of T-related gene products were aligned on the basis of maximum similarity using a SeqApp 1.9 manual aligner for the Macintosh (Gilbert, 1993). Gaps and insertions were excluded from the analysis. Molecular phylogenetic relationships among the T-related gene products were estimated using the neighbor-joining (Saitou and Nei, 1987) with the PHYLIP ver. 3.5c package (Felsenstein, 1993). The distance matrix was constructed according to the Dayhoff model (Dayhoff *et al.*, 1978). The degree of support for internal branches of the tree was assessed by bootstrapping (Felsenstein, 1985).

RNA isolation and northern blot hybridization

Total RNA was isolated from eggs, embryos and larvae by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified by use of Oligotex-dT30 Latex beads (Roche Japan, Tokyo) according to the manufacturer's protocol. Two μg of poly(A)$^+$ RNAs isolated from each specimen was separated on a 1% agarose gel containing 6% formaldehyde, and bands of RNA were transferred to a Hybond-N+ nylon membrane filter (Amersham). The filter was prehybridized in 5xSSPE (0.75 mol/l NaCl, 50 mmol/l sodium phosphate, pH 7.7, 0.075 M Na-citrate), 5xDenhardt's solution, 50% formamide, and 100 μg/ml salmon sperm DNA for 2 h. The filter was then exposed to random-primed [^{32}P]-labeled DNA probes at a concentration of 2x10^5 cpm/ml overnight at 42°C. Finally, that was washed three times in 2xSSC, 0.1% SDS at 50°C for 15 min.

In situ hybridization

Digoxigenin (DIG)-labeled sense and antisense *As-mT* probes were synthesized following the instructions from the suppliers of the kit (DIG RNA Labeling kit; Boehringer Mannheim). Their final sizes were reduced to approximately 150 nucleotides by alkaline hydrolysis. Whole-mount specimens were hybridized *in situ* using the probes essentially according to the method described by Ishida *et al.* (1996). Briefly, specimens were fixed in 4% paraformaldehyde in 0.1 M MOPS buffer (pH 7.5), 0.5 M NaCl. After being thoroughly washed with PBT (phosphate-buffered saline containing 0.1% Tween 20), the specimens were treated with 2 μg/ml proteinase K (Sigma) in PBT for 30 min at 37°C, then post-fixed with 4% paraformaldehyde in PBT for 1 h at room temperature. After a 1-h period of prehybridization at 42°C, the specimens were allowed to hybridize with the DIG-labeled probes at a concentration of 1 μg/ml for at least 16 h at 42°C. After hybridization, the specimens were washed with 4xSSC, 50% formamide, 0.1% tween-20, 15 min, twice, and 2xSSC, 50% formamide, 0.1% tween-20, 15 min, twice, and replaced by Solution-A (0.5 M NaCl, 10 mM Tris (p 8.0), 5 mM EDTA, 0.1% tween-20). Then the specimens were digested with 20 μg/ml RNase A (Sigma). Thereafter, the samples were incubated for 1 h with 500 μl anti-DIG-alkaline phosphatase conjugate, and color was developed according to the Boehringer protocol.

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

Probes used to examine tissue-specific gene expression in experimental embryos, were for a muscle-actin gene *HrMA4* (Kusakabe *et al.*, 1991), an ascidian homolog of *Brachyury* or *As-T* (Yasuo and Satoh, 1993), an epidermis-specific *HrEpiC* (Ueki *et al.*, 1991), and a mesenchyme-specific cytoplasmic actin *HrCA1* (Araki *et al.*, 1996).

Injection of synthetic capped mRNA

Synthetic capped mRNA for *As-mT* was synthesized from *As-mT* cDNA cloned into pBluescript RN3 vector (a gift from Dr. Patrick Lemaire) using a Megascript kit (Ambion, Austin, TX, USA). To obtain a capped mRNA, the concentration of GTP was lowered to 1.5 mM and the cap analog 7mGpppG was added at a final concentration of 6 mM. As a control, *lacZ*mRNA was synthesized from *lacZ*gene cloned into pBluescript RN3 vector in the same way. The injection of mRNAs into the fertilized eggs was performed as described previously (Yoshida *et al.*, 1996).

Histochemical staining for alkaline phosphatase

The differentiation of endoderm cells was monitored by the histochemical reaction of alkaline phosphatase as described by Marikawa and Satoh (1996).

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The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number (AB001770).

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