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Original Article

Isolation and characterization of cDNA clones for β-tubulin genes as a molecular marker for neural cell differentiation in the ascidian embryo

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ABSTRACT The central nervous system (CNS) of an ascidian tadpole larva is composed of about 340 cells, the lineages of which are well documented. To elucidate the mechanisms underlying the neural induction of ascidians, appropriate molecular markers are required. In this study, to obtain an early differentiation marker of the neural cells, we isolated and characterized cDNA clones for two β-tubulin genes (*HrTBB1* and *HrTBB2*) of the ascidian *Halocynthia roretzi*. We found that the HrTBB1 and HrTBB2 amino acid sequences are highly conserved, with 91-98% identities to other invertebrate and vertebrate β-tubulins. The expression of *HrTBB1* was found to be maternal, while *HrTBB2* is expressed both maternally and zygotically. We observed that the zygotic expression of *HrTBB2* commences at the neural plate stage and is specific to cells of the differentiating CNS. In the larvae, *HrTBB2* expression was restricted to cells of the CNS, some cells of the papilla and cells of the peripheral nervous system. These results indicate that *HrTBB2* will be a useful early molecular marker for neural cell differentiation in the ascidian embryo.

KEY WORDS: *neural cell differentiation, β-tubulin genes, ascidians, molecular marker*

Introduction

Ascidian embryos have served as experimental systems with which to explore the genetic circuitry required for cell specification and morphogenesis (reviewed by Satoh, 1994; Satoh *et al.*, 1996). The fertilized egg develops quickly into a tadpole larva, which consists of a small number of tissues including an epidermis, a dorsal central nervous system (CNS) with two sensory organs (otolith and ocellus), endoderm, mesenchyme, notochord and muscle. The lineage of embryonic cells is completely described up to the early gastrula stage (Conklin, 1905; Nishida, 1987; Nicol and Meinertzhagen, 1988). Since the work of Chabry (1887), which described the first blastomere destruction experiment in the history of embryology, many descriptive and experimental studies have been performed to elucidate cellular and molecular mechanisms underlying the autonomous and conditional specification of ascidian embryonic cells (reviewed by Satoh, 1994). The neurogenesis of the ascidian embryo is one of these intensive research subjects, because of the compositional simplicity and functional complexity of the ascidian CNS (reviewed by Okamura *et al.*, 1993).

As in the case of vertebrates, the neurulation of ascidian embryos is accomplished by the folding of the presumptive neural cells (Satoh, 1978). A serial section study of *Ciona intestinalis* tadpole larvae revealed that the CNS is comprised of only about 340 cells, about 80 neurons and sensory cells and the 260 ependymal cells (Nicol and Meinertzhagen, 1988, 1991). The three cup-shaped adhesive organs (papillae) are located at the rostral end of the head. The bipolar sensory neurons in the papillae send axons to the nerve cells in the neck (K. Takamura, personal communication) and are presumed to play a role in the settlement of the tadpole and the initiation of metamorphosis. Motor axons originate from the head nerve cells and run through the nerve cord, surrounded by lateral ependymal cells. Motor nerves form cholinergic synpase directly on the striated muscle cells while they run down the tail. In addition, the caudal epidermis in the tail contains several pairs of putative primary sensory neurons along the tail (Okamura *et al.*, 1993). Although the physiological function of these neurons in swimming behavior is unknown, they extend a cilium into the tunic fin, suggesting that they are mechanosensory neurons.

The differentiation of ascidian neuronal cells is also accomplished by so-called "neural induction" (Rose, 1939; Reverberi *et al.*, 1960; Nishida and Satoh, 1989; Nishida, 1991), and thus the ascidian embryo is regarded as a prototype of vertebrate neurogenesis (Okamura *et al.*, 1993). In addition, a very attractive, simple neural induction system has been developed by Okado and Takahashi (1988). When a single a4.2 blastomere was isolated from the 8-cell embryo and its division was arrested with cytochalasin B, it differentiated into epidermis judging from

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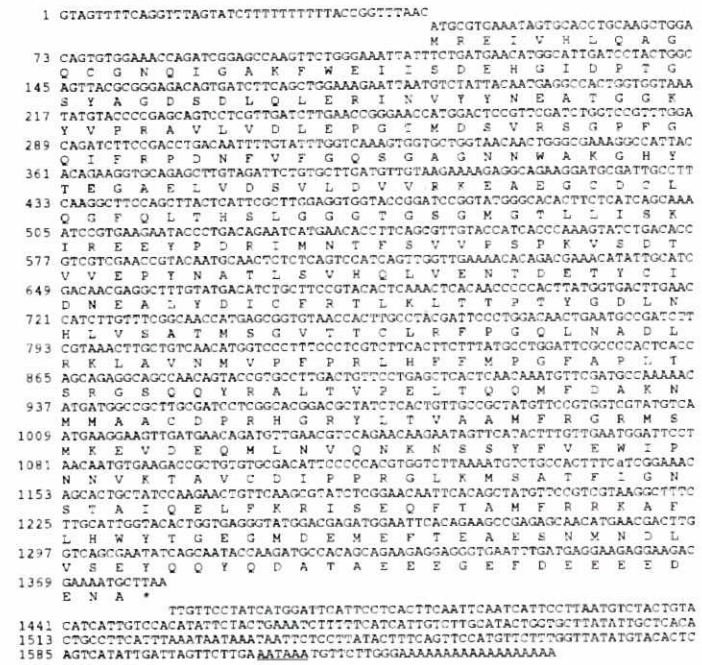

Fig. 1. Nucleotide and predicted amino acid sequences of a cDNA clone for *HrTBB1*, a β-tubulin gene of the ascidian *Halocynthia roretzi*. *The cDNA encompasses 1,640 nucleotides including 18 adenylyl residues at the 3' end. The ATG at the position 43-45 represents the putative start codon, and the asterisk indicates the termination codon. The predicted HrTBB1 protein consists of 445 amino acids. A potential signal sequence for polyadenylation is underlined. The nucleotide sequence will appear in the DDBJ, EMBL, and Genbank Nucleotide Sequence Databases under Accession No. D89793.*

the membrane excitability and immunoreactivity. However, when the same blastomere was cultured in contact with a single A4.1 blastomere, which includes the presumptive notochord and nerve cord, it displayed Na spikes and showed no expression of the epidermal markers, suggesting that neural induction resulted in a single cell during the interaction with a single neighboring cell.

Under such research circumstances, the isolation of molecular markers for ascidian neural differentiation has been a key goal. Okamura *et al.* (1994) isolated a cDNA clone for the Na-channel α subunit gene *TuNa1* of the *Halocynthia roretzi*. The *TuNa1* gene expression is specific to nerve cells, but *in situ* hybridization was able to detect its first signal at the early tailbud stage, suggesting that this probe is useful as a marker for the later process of neural cell differentiation. In the present study, we attempted to isolate cDNA clones for early differentiation markers of the ascidian CNS. In vertebrates, β-tubulin genes are expressed specifically in cells of the CNS, and the gene expression is used to monitor the neural cell differentiation (Denoulet *et al.*, 1986; Good *et al.*, 1989; Oschwald *et al.*, 1991). We therefore attempted to isolate and characterize cDNA clones for ascidian β-tubulin genes as an early molecular marker of neural cell differentiation.

Results

Isolation and characterization of ascidian β-tubulin genes

To isolate cDNA clones for ascidian β-tubulin genes, we designed two degenerate oligonucleotide primers that correspond to the conserved sequences of the β-tubulins (Fig. 3). Using these primers, we generated PCR fragments from a cDNA library of *H. roretzi* early tailbud-stage embryos. Sequence analysis revealed two independent PCR fragments with sequence similarity to β-tubulin. The corresponding genes were designated *HrTBB1* (*Halocynthia roretzi* tubulin beta-1) and *HrTBB2*. Using the *HrTBB1* and *HrTBB2* PCR fragments as probes, we screened the cDNA library several times and obtained cDNA clones that contained the entire coding sequences.

The structure of the *HrTBB1* cDNA clone is shown in Figure 1. The insert of the clone consisted of 1,640 nucleotides. A northern blot showing a transcript of about 1.7 kb (Fig. 5A) suggested that the clone is close to full-length. The clone contained a single open reading frame of 1,335 nucleotides that encode a polypeptide of 445 amino acids. The calculated relative molecular mass (Mr) of the predicted polypeptide was 50×10^3.

The structure of the *HrTBB2* cDNA clone is shown in Figure 2. The insert of the clone consisted of 1,578 nucleotides. Because the northern blot showed a transcript of about 1.7 kb (Fig. 5B), the clone is also close to full-length. The clone contained a single open reading frame of 1,338 nucleotides that encode a polypeptide of 446 amino acids. The calculated relative Mr of the predicted polypeptide was 50×10^3.

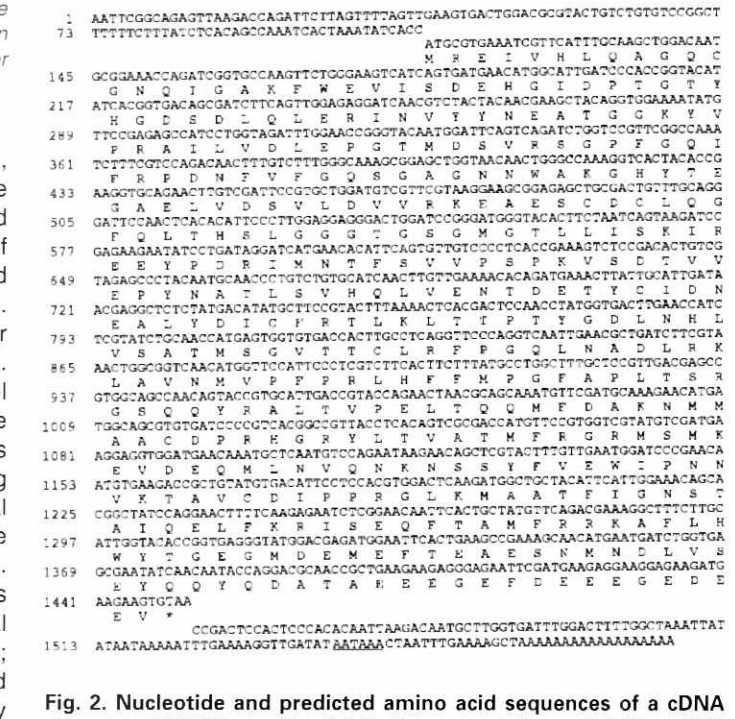

Fig. 2. Nucleotide and predicted amino acid sequences of a cDNA clone for *HrTBB2*, another β-tubulin gene of *H. roretzi*. *The cDNA encompasses 1,578 nucleotides including 18 adenylyl residues at the 3' end. The ATG at the position 111-113 represents the putative start codon, and the asterisk indicates the termination codon. The predicted HrTBB2 protein consists of 446 amino acids. A potential signal sequence for polyadenylation is underlined. The nucleotide sequence will appear in the DDBJ, EMBL, and Genbank Nucleotide Sequence Databases under Accession No. D89794.*

```
                                                                >>F1>>>
HrTBB1:         MREIVHLQAGQCGNQIGAKFWEIISDEHGIDPTGSYAGDSDLQLERINVYYNEATGGKYV
HrTBB2:         .....V..........T.H.....................................
Sea urchin TBB: .....M.....V..........T.H.....................................
Xenopus TBB4:   .....V..........A.H.....................................
Xenopus TBB2:   .....V..........H.....................N.F.
Chick TBB2:     .....I.....V..........H.....................N...

HrTBB1:         PRAVLVDLEPGTMDSVRSGPFGQIFRPDNFVFGQSGAGNNWAKGHYTEGAELVDSVLDVV
HrTBB2:         ...I.....
Sea urchin TBB: .....
Xenopus TBB4:   .....
Xenopus TBB2:   ...I.....
Chick TBB2:     ...I.....

HrTBB1:         RKEAEGCDCLQGFQLTHSLGGGTGSGMGTLLISKIREEYPDRIMNTFSVVPSPKVSDTVV
HrTBB2:         .....S.....
Sea urchin TBB: .....S.....
Xenopus TBB4:   .....S.....
Xenopus TBB2:   ...S.S.....M.....
Chick TBB2:     ...S.S.....M.....

HrTBB1:         EPYNATLSVHQLVENTDETYCIDNEALYDICFRTLKLTTPTYGDLNHLVSATMSGVTTCL
HrTBB2:         .....
Sea urchin TBB: .....
Xenopus TBB4:   .....
Xenopus TBB2:   .....
Chick TBB2:     .....

HrTBB1:         RFPGQLNADLRKLAVNMVPFPRLHFFMPGFAPLTSRGSQQYRALTVPELTQQMFDAKNMM
HrTBB2:         .....
Sea Urchin TBB: .....
Xenopus TBB4:   .....
Xenopus TBB2:   .....S....
Chick TBB2:     .....S....

                                        <<<R2<<
HrTBB1:         AACDPRHGRYLTVAAMFRGRMSMKEVDEQMLNVQNKNSSYFVEWIPNNVKTAVCDIPPRG
HrTBB2:         .....T.....
Sea urchin TBB: .....I.....
Xenopus TBB4:   .....I.....
Xenopus TBB2:   .....I.....S.....
Chick TBB2:     .....I.....

HrTBB1:         LKMSATFIGNSTAIQELFKRISEQFTAMFRRKAFLHWYTGEGMDEMEFTEAESNMNDLVS
HrTBB2:         ...A.....
Sea urchin TBB: .....
Xenopus TBB4:   .....
Xenopus TBB2:   .....
Chick TBB2:     .....

HrTBB1:         EYQQYQDATAEEEGEFDEEE-EDENA-
HrTBB2:         .....G...EV-
Sea urchin TBB: .....EG..E.A
Xenopus TBB4:   .....E.G.-.E...-
Xenopus TBB2:   .....D.Q...E..-—...A—
Chick TBB2:     .....D.Q...E..GE...A--
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Fig. 3. Comparison of the amino-acid sequence of the HrTBB1 and HrTBB2 with those of other β-tubulins. *The sequences are compared with those of sea urchin β-tubulin (Di Bernardo et al., 1989), Xenopus β4 tubulin (Bieker and Yazdani-Buicky, 1992), Xenopus β2 tubulin (Good et al., 1989), and chick β2 tubulin (Valenzuela et al., 1981; Sullivan et al., 1985). Dots indicate identities to HrTBB1. For maximal similarity, gaps (shown by dashes) were introduced. Primer-designed regions used for amplification of HrTBB target fragments are shown.*

The nucleotide and predicted amino acid sequences were found to be highly conserved between *HrTBB1* and *HrTBB2*; they are 98% identical at the amino acid level, and 80% identical at the level of nucleotide of the coding regions.

Figure 3 shows the comparison of the amino acid sequence of the HrTBB1 and HrTBB2 with those of sea urchin β-tubulin (Di Bernardo *et al.*, 1989), *Xenopus* tubulin β4 (Bieker and Yazdani-Buicky, 1992), *Xenopus* tubulin β2 (Good *et al.*, 1989), and chick tubulin β2 (Valenzuela *et al.*, 1981; Sullivan *et al.*, 1985). HrTBB1 and HrTBB2 showed the highest grade of amino acid identity with sea urchin β-tubulin (98%) and *Xenopus* tubulin β4 (98%). The amino acid sequences of HrTBB1 and HrTBB2 also resembled vertebrate β-tubulins at the level of 91-97% identity.

Genomic Southern analysis

We determined the number of different sequences that correspond to *HrTBB1* and *HrTBB2* in the ascidian genome by genomic Southern hybridization. Because the coding sequences of *HrTBB1* and *HrTBB2* highly resemble each other, we examined this issue with specific probes of the 5' flanking regions. As shown in Figure 4A, regarding *HrTBB1*, only one band was detected in the lanes of *Eco*RI (about 1.7 kb), *Hin*dIII (about 5.2 kb) and *Pst*I (about 5.2 kb). This result suggests that *HrTBB1* is present as a single copy per haploid genome of *H. roretzi*. As for *HrTBB2* (Fig. 4A), only one band was detected in the lanes of *Eco*RI (about 1.7 kb) and *Pst*I (about 7.4 kb), while in the lane of *Hin*dIII, a major (about 4.4 kb) and a minor band (about 9.5 kb) were detected. This result suggests that *HrTBB2* is also present as a single copy per haploid genome of *H. roretzi*. The sizes of *Eco*RI bands for both genes were about 1.7 kb, suggesting that both genes contain no introns or a very short intron.

However, when the blot hybridization was performed with a probe from the coding region and the membrane was washed under low-stringency conditions, we obtained many bands in the lanes (Fig. 4B). These results suggest that the *H. roretzi* genome contains several tubulin genes other than the two β-tubulin genes.

Expression of HrTBB1 is maternal

As shown in Figure 5A, the northern blots revealed that the expression of *HrTBB1* is exclusively maternal. An *HrTBB1* transcript of about 1.7 kb appeared to be abundant in unfertilized eggs. However, the hybridization signals for *HrTBB1* maternal transcripts became weaker in the 64-cell embryos and early gastrulae,

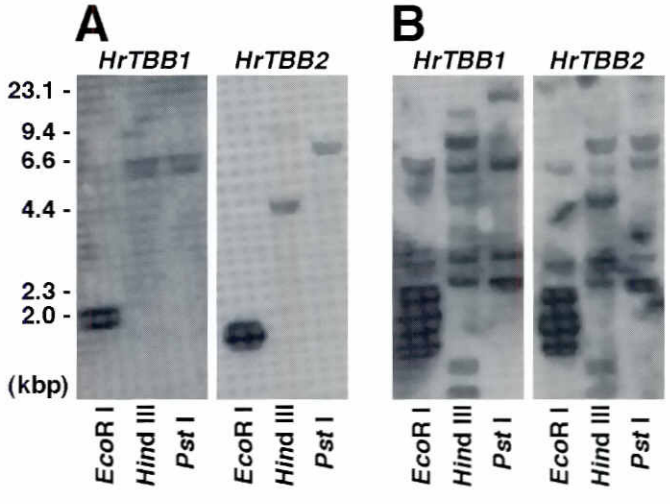

Fig. 4. Genomic Southern blot analysis of the *HrTBB1* and *HrTBB2* genes. (A) *Genomic DNA was isolated from a single adult H. roretzi, and aliquots were digested separately with EcoRI (lane 1), HindIII (lane 2), and PstI (lane 3). The blots were hybridized with random-primed [^{32}P]-labeled DNA probes including specific 5' untranslated regions, and the membrane filter was washed under high-stringency conditions. Five micrograms of digested genomic DNA were loaded per lane. The numbers indicate the sizes (in kb) of marker fragments.* **(B)** *The blots (the same filters as used for A) were hybridized with random-primed [^{32}P]-laabeled DNA probes for the coding region, and the filter was washed under low-stringency conditions. Five micrograms of digested genomic DNA were loaded per lane.*

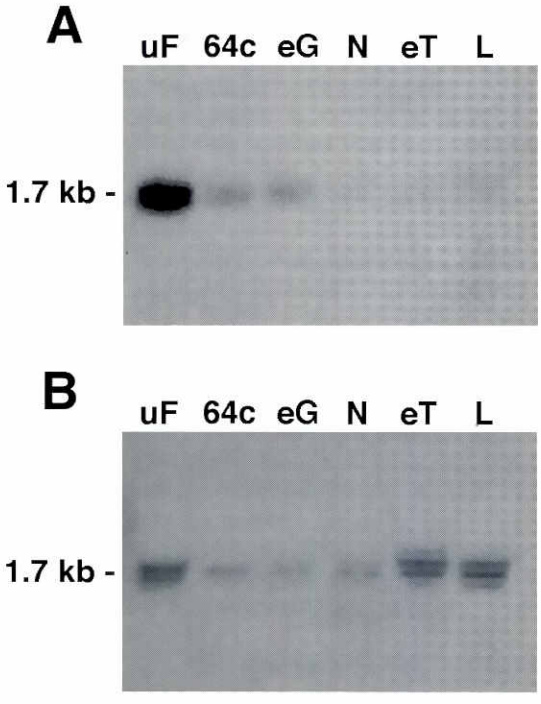

Fig. 5. Temporal expression of (A) *HrTBB1* and (B) *HrTBB2*. *Northern blots of total RNA prepared from unfertilized eggs (uF), 64-cell stage embryos (64c), early gastrulae (eG), neurula (N), early tailbud embryos (eT), and larvae (L) were hybridized with [^{32}P]-labeled DNA probes, and the membrane was washed under high-stringency conditions. Each lane was loaded with 5 μg total RNA.*

and were barely detectable in neurulae and later stages. However, we could isolate the cDNA clone from the tailbud embryo cDNA library, suggesting the presence of the transcript at that stage.

The characterization of the spatial distribution of *HrTBB1* transcripts by whole-mount *in situ* hybridization demonstrated that the transcript was distributed over the entire cytoplasm of fertilized eggs without showing any specific localization pattern (Fig. 6A). A similar distribution pattern was found in the 8-cell embryo, although the signals were scarce in the vegetal cytoplasm with heavy yolk (Fig. 6B). Hybridization signals for maternal *HrTBB1* transcripts were undetectable in early tailbud embryos (Fig. 6C).

Expression of HrTBB2 is both maternal and zygotic, and the zygotic expression is specific to cells of the nervous system

The northern blot analysis revealed that the expression of *HrTBB2* is both maternal and zygotic (Fig. 5B). An *HrTBB2* transcript of about 1.7 kb was evident in unfertilized eggs. The hybridization signals became weaker as development proceeded, suggesting a gradual diminution of the *HrTBB2* maternal transcripts. The whole-mount *in situ* hybridization demonstrated that the maternal transcript was distributed over the entire cytoplasm of fertilized eggs (data not shown) and the 8-cell embryo (Fig. 7A), although the signals were weak in the vegetal cytoplasm with heavy yolk . No localization was evident in the hybridization signals of the maternal *HrTBB2* transcripts (data not shown).

We then characterized the spatial distribution of the zygotic *HrTBB2* transcripts by whole-mount *in situ* hybridization (Fig. 7). Control embryos hybridized with sense probe did not show signals above the level of background (data not shown). The hybridization signals of zygotic *HrTBB2* transcripts were undetectable in early embryos up to the gastrula stages (data not shown).

We first obtained clear signals at the neural plate stage. At this stage, the zygotic *HrTBB2* transcript was detected in almost all of the cells constituting the neural plate (Fig. 7C). During neurulation, signals were evident in the presumptive cells of the CNS (Fig. 7D). In addition, signals were evident in cells of the anterior-most region, which give rise to papilla or the adhesive organ of the larva (Fig. 7D).

The early tailbud embryo also exhibited a characteristic signal distribution (Fig. 7E). When the embryo was viewed from the dorsal side (Fig. 7E), signals were found in cells of the CNS along the midline of the entire dorsal trunk region. The signals of this region extended to the posterior region of the tail, although there were several intermissions (Fig. 7E). Hybridization signals were also evident in cells of the anterior part of the CNS in the dorsal trunk region of the embryo (Fig. 7E). In the tadpole larvae, signals were evident in cells of the CNS and adhesive organ (Fig. 7F, G). In addition, signals were found in cells of the tail region (Fig. 7G); they may be of peripheral sensory neurons.

Discussion

In this study, to obtain an early differentiation marker of neural cells of ascidian embryos, we isolated and characterized cDNA clones for two β-tubulin genes *(HrTBB1* and *HrTBB2)* of *H. roretzi*. The predicted amino acid sequences of HrTBB1 and HrTBB2 show a very high degree of identity to other invertebrate and vertebrate β-tubulins. The expression of *HrTBB1* was found to be maternal,

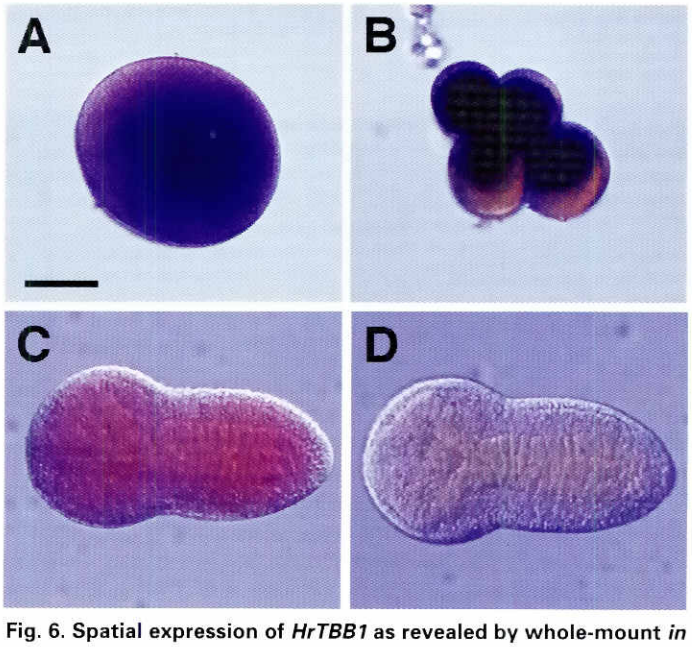

Fig. 6. Spatial expression of *HrTBB1* as revealed by whole-mount *in situ* hybridization with a digoxigenin-labeled antisense probe. (A) *A fertilized egg and* **(B)** *an 8-cell embryo showing hybridization signals in the entire region of the cytoplasm.* **(C,D)** *The early tailbud embryos hybridized with* **(C)** *antisense and* **(D)** *sense probe. Bar, 100 μm for all panels.*

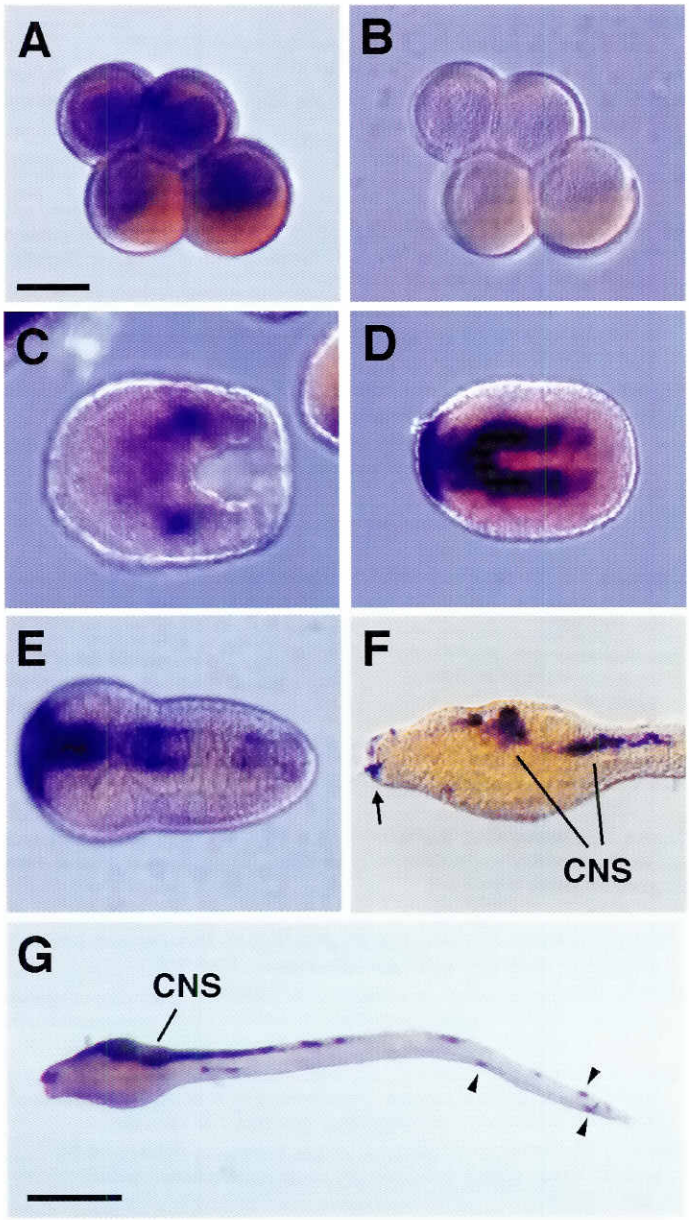

Fig. 7. Spatial expression of *HrTBB2* as revealed by whole-mount *in situ* hybridization with a digoxigenin-labeled antisense probe. (A,B) *The 8-cell stage embryos hybridized with* **(A)** *antisense and* **(B)** *sense probes. Hybridization signals of maternal transcripts are seen in the entire region of the embryo. Because of dense yolk granules, the vegetal region of the embryo does not show the hybridization signals. Bar, 100 μm in A-F.* **(C)** *Neural-plate stage embryo, viewed from the dorsal side. Signals were seen in cells of the neural plate.* **(D)** *A neurula viewed from the dorsal side. Signals were seen in cells of the neural plate and the anterior-most region of the embryo.* **(E)** *Early tailbud embryo viewed from the dorsal side. Signals are seen in the CNS.* **(F)** *Head-trunk region of a tadpole larva showing signals in the CNS and in the papilla (arrow).* **(G)** *Lateral view of tadpole larva. Signals are found in the papilla, CNS, and peripheral sensory neurons (arrowheads) in the tail. Bar, 200 μm.*

and its transcript is distributed in the egg cytoplasm without localization. *HrTBB2* is expressed both maternally and zygotically, the zygotic expression of *HrTBB2* commencing at the neural plate stage. The zygotic *HrTBB2* expression is specific to cells of the differentiating CNS, and in the larvae, *HrTBB2* expression is found in cells of the CNS, some cells of the papilla and cells of the peripheral nervous system.

Whole-mount *in situ* hybridization revealed the first signals as early as the neural plate stage. These results therefore indicate that *HrTBB2* can be used as an early molecular marker for neural cell differentiation in the ascidian embryo. In practice, gene expression has been used as a marker of neural cell differentiation in some experiments. For example, an ascidian homolog of vertebrate BMP2/4 or *HrBMPb* plays a role in the inhibition of neural cell differentiation (Miya *et al.*, 1997). This was demonstrated by an experiment in which synthetic mRNA of *HrBMPb* was injected into fertilized eggs. Zygotic *HrTBB2* expression was used to monitoring the neural cell differentiation.

The neural induction of the ascidian embryos has also been examined by experiments using blastomere isolation and combinations. Reverberi *et al.* (1960) suggested that the induction signals emanate from the presumptive notochord cells of *Ascidiella aspersa* and *Phallusia mammillata*. In contrast, Nishida (1991) showed that in *H. roretzi*, nerve cord cells are indispensable for the neural induction. In these experiments, however, the differentiation of neuronal cells was monitored by the occurrence of pigment cells or sensory receptor cells of the otolith and ocellus. This morphological marker does not always provide an appropriate marker of neural cell differentiation. Therefore, the neural induction of ascidian embryos should be explored again with a specific marker such as *HrTBB2*.

In vertebrates, several β-tubulin genes are expressed in neural tissues. In particular, differential expression between β-tubulin isotypes during vertebrate brain development is reported (Bond *et al.*, 1984; Ginzburg *et al.*, 1985; Denoulet *et al.*, 1986). Ascidians are phylogenetically close to vertebrates, and the CNS of the ascidian embryo is considered a prototype of vertebrate neurogenesis (Okamura *et al.*, 1994). In this study, we isolated two ascidian β-tubulin genes, *HrTBB1* and *HrTBB2*. Of them, only *HrTBB2* is neural-specific. It is unknown whether ascidians have other neural-specific β-tubulin genes. However, the results of genomic Southern analysis (Fig. 4) showed that the ascidian genome contains many β-tubulin genes other than *HrTBB1* and *HrTBB2*. Thus it is possible that there are other neural-specific β-tubulin genes in the ascidian genome. If so, the elucidation of the regulation of β-tubulin gene expression during ascidian brain development may provide further insight into the neurogenesis of the vertebrate CNS.

Materials and Methods

Animals and embryos

Halocynthia roretzi was purchased during the spawning season from fishermen near the Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, Japan. *H. roretzi* is hermaphroditic and self-sterile. Naturally spawned eggs were fertilized with a suspension of non-self sperm. When fertilized eggs were cultured at about 12°C, they developed into gastrulae and early tailbud embryos about 12 and 24 h after fertilization, respectively. Tadpole larvae hatched after about 40 h of development.

Eggs and embryos at appropriate stages were packed by low-speed centrifugation and frozen with chilled ethanol for Northern blotting or fixed for *in situ* hybridization.

PCR amplification of β-tubulin related fragments

The amino acid sequences of β-tubulins are highly conserved among various animal groups (cf., Fig. 3). We synthesized a sense-strand oligonucleotide, F1 [5'AA(TC)GTNTA(TC)TA(TC)AA(TC)GA(AG)AC-3'] and an antisense oligonucleotide, R2 [5'-GGNGC(AG)AANCCNGGCAT(AG)AA-3'] by means of an automated DNA synthesizer (Applied Biosystems Inc., Foster City, CA, USA). Using these as primers, we amplified by polymerase chain reaction (PCR) the target fragments from a cDNA library of the early tailbud-stage embryos which was constructed with the λZapII cloning vector (Stratagene, La Jolla, CA, USA). The reaction mixture contained $1x10^5$ pfu of cDNA library, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each of dATP, dCTP, dGTP and dTTP, 1 μM of each primer and 1 unit of Taq DNA polymerase (Toyobo, Osaka, Japan) in a total volume of 50 μl. Amplification was performed for 40 cycles of 94°C (1 min), 55°C (2 min) and 72°C (2 min). The PCR products were purified by gel electrophoresis and cloned into pBluescript II SK(+) (Stratagene). The cDNA clone was sequenced on both strands by means of dideoxy chain-termination (Sanger *et al.*, 1977) by an automated DNA sequencer (ABI PRISM 377, Perkin Elmer, Norwalk, CT, USA).

Isolation of cDNA clones for ascidian β-tubulin genes and nucleotide sequencing

Probing with a cDNA fragment labeled with [^{32}P]-dCTP, we screened a cDNA library of early tailbud-stage embryos. Several candidate cDNA clones were obtained. The longest clones were subcloned into the plasmid vector pBluescript SK(-) by *in vivo* excision. The clones were used as a template for sequencing and *in situ* hybridization. The cDNA clones were sequenced on both strands by means of dideoxy chain-termination (Sanger *et al.*, 1977) by the automated DNA sequencer mentioned above.

Genomic Southern analysis

High-molecular weight genomic DNA was extracted from the gonad of a single adult by the standard procedure (Sambrook *et al.*, 1989). After exhaustive digestion with *Eco*RI, *Hin*dIII and *Pst*I and 0.7% agarose gel electrophoresis, the DNA fragments were blotted onto a Hybond-N$^+$ nylon membrane (Amersham, Buckinghamshire, UK). The blots were hybridized with random-primed [^{32}P]-labeled DNA probes from the 5' flanking region of each gene at 42°C in 50% formamide, 5xSSPE, 0.5% SDS, 5xDenhardt's solution, and 100 μg/ml denatured salmon sperm DNA for 16 h and washed under high-stringency conditions (2xSSC, 0.1% SDS, 65°C for 20 minx2; 0.1xSSC, 0.1% SDS, 65°C for 20 min). The blots were also hybridized with random-primed [^{32}P]-labeled DNA probes from the coding region of the genes at 37°C in 30% formamide, 5xSSPE, 0.5% SDS, 5xDenhardt's solution, and 100 μg/ml denatured salmon sperm DNA for 16 h and washed under low-stringency conditions (2xSSC, 0.1% SDS, 37°C for 20 minx2; 1xSSC, 0.1% SDS, 37°C for 20 min). Both probes did not contain *Eco*RI, *Hin*dIII and *Pst*I sites.

Isolation of RNA and northern blotting

Total RNA was extracted using acid guanidinium-thiocyanate-phenol-chloroform (Chomczynski and Sacchi, 1987). Northern blot hybridization was performed by standard procedures (Sambrook *et al.*, 1989) with random-primed [^{32}P]-labeled DNA probe from the 5' flanking region of each gene, and the membranes were washed under the high-stringency conditions described above.

In situ hybridization

Whole-mount specimens were hybridized *in situ* at 42°C using digoxigenin-labeled antisense probes, essentially as described by Miya *et al.* (1996). Probes were synthesized following the instructions supplied with the kit (DIG RNA Labeling Kit; Boehringer Mannheim, Mannheim, Germany), and used at a concentration of 1 μg/ml in the hybridization buffer. Hybridization was visualized using alkaline phosphatase.

Embryos were dehydrated in a graded series of ethanol and rendered transparent with xylene.

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