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Abstract

Ascidians are primitive chordates, and their critical evolutionary position expected to offer an excellent experimental system to understand the origin and the evolution of vertebrates (Satoh, 1994). Especially, neural tube is one of the characteristic features of chordates. In this study, we focused on the mechanism of the neural tube formation, and described the detailed expression profiles of two neural tube specific genes, *CiNut1* and *CiNut2*. Although the amount of *CiNut2* expression is about 1/1000 compared to that of *CiNut1*, both two genes are expressed in the entire neural plate and neural tube during the course of the development. Both two genes are situated in an adjacent position of the chromosome in the same direction. Comparative analysis of the upstream regulatory sequence of two genes revealed the conserved sequences, which suggested having a role for the neural tube specific expression.

Keywords: ascidians, regulatory sequence, *in situ* hybridization, quantitative RT-PCR, neurulation

INTRODUCTION

The neural tube is one of the characteristic features of chordates (Satoh 1994), and the central nervous system (CNS) arisen from the neural tube is well developed in vertebrates. The ascidian tadpole larva has a CNS consisting of about 330 cells, of which only about 100 cells are neurons and the others are neuroglia (ependyma cells) (Meinertzhagen and Okamura, 2001). Ascidian neural tube has basically the same structure as those of vertebrates, and its morphogenesis, rolling up of a neural plate into a neural tube, is also conserved among the chordates (Nicol and Meinertzhargen, 1988). Moreover, a draft sequence of the ascidian genome was generated in a widely studied species, *Ciona intestinalis* (Dehal *et al.*, 2002). Owing to its relatively simple structure, its conservation among chordates and its abundant molecular resources, the ascidian offers an excellent experimental system for analyzing the molecular mechanisms of neural tube formation. Furthermore, the studies about the ascidian neural tube formation will lead us to understand the origin and evolution of vertebrates.

We have already reported the ascidian neural tube specific gene, which was designated as *CiNut1* (*Ciona intestinalis* neural tube specific gene 1; Etani and Nishikata, 2002). *CiNut1* encoded the G-protein coupled receptor, and expressed specifically in the neural plate and the entire neural tube during neural tube formation. In the *Ciona* genome project databases, we found a similar sequence adjacent to the *CiNut1* gene.

In this study, we describe a novel G-protein coupled receptor gene, which designated as *CiNut2*. According to the genome project databases, it situated in the downstream of the *CiNut1* gene in a tandem array and in the same direction. Then we quantitatively compared the expression profiles of *CiNut1* and *CiNut2*, including spatial and temporal expression pattern, in detail. From the real-time RT-PCR analysis, the amount of *CiNut2* expression is about 1/1000 compared to that of *CiNut1* during the course of the development. During embryogenesis, spatial expression pattern of *CiNut2* was basically the same as that of *CiNut1*. *CiNut2* was expressed specifically in the neural tube. But the *CiNut2* expression in the egg was not detected. And in the swimming tadpole stage, *CiNut2* increased its expression and continued to express during metamorphosis. According to these expression patterns, *CiNut1* and *CiNut2* suggested to have a redundant function and a similar transcriptional control mechanism, at least in the neural tube formation. The comparative analyses of upstream sequence of *CiNut1* and *CiNut2* have revealed the possible important regulatory sequences, which conserved between *CiNut1* and *CiNut2* upstream sequences.

MATERIALS AND METHODS

Animals and embryos

The adults of ascidian *Ciona intestinalis* were collected near the Onagawa Field Science Center (Tohoku University, Onagawa Bay, Japan), the International Maritime Education and Research Center (Kobe University, Osaka Bay, Japan), the Maizuru Fisheries Research Station (Kyoto University, Wakasa Bay, Japan), and the harbor in Murotsu (Hyogo, Japan). Adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. Eggs were inseminated with a diluted sperm suspension from other individuals. Fertilized eggs were reared at 16℃. At this temperature, first cleavage takes place about 60 min after insemination, and tadpole larvae hatch about 20 h after insemination.

Searching for the genome data base

We searched for the *CiNut1* homologous genes on the *C. intestinalis* and *C. savignyi* genomes by comparing the full length cDNA sequence of *CiNut1* with the whole-genome sequences of the Kyoto *C. intestinalis* Genome Project Database (Satou *et al.*, 2002; GHOST; http://ghost.zool.kyoto-u.ac.jp/indexr1.html), the JGI *Ciona* Genome Project Database (Dehal *et al.*, 2002; http://www.jgi.doe.gov/ciona) and *C. savignyi* genome database on Broad Institute (http://www.broad.mit.edu/annotation/ciona/) using the Aniseed V3.0 *Ciona intestinalis* genome browser (http://crfb.univ-mrs.fr/ciona-bin/gbrowse/intestinalis).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was carried out according to the method described previously (Wada *et al.*, 1998) with minor modifications. Total RNAs were extracted from 200 manually counted embryos. PCR programs are as follows: for *CiNut1*; 25 cycles of 94℃ 1 min, 55℃ 1 min, 72℃ 1 min: for *CiNut2*; 40 cycles of 94℃ 1 min, 53℃ 1 min, 72℃ 1 min. The primers for *CiNut1* were a28-OU (5'-TGGATTGTTCTCAGGTTACG-3'), a28-OL (5'-CATCGGTTGTGGTTCTTTAG-3'), and for *CiNut2* were Nut2-up-bgl2 (5'-GGAAGATCTATGGAGCTGGAATTCG-3'), Nut2-low-bgl2 (5'-GGAAGATCTGACGACTCGTTTTGCT-3'). The expression of *tubulin* and *YWHAZ* (*Tyrosine-3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide*: CLSTR00726) was assayed as internal control for RNA recovery and cDNA synthesis. The primers for ascidian *beta-tubulin* were TB2-TU (5'-CCCTCGTCTCCACTTCTTCA-3'), TB2-TL (5'-CTTGGATGGCTGTGCTGTTT-3'), and for ascidian *YWHAZ* were YWHAZ-up (5'-GCAGTTGCAAAGCTTGGAGTT-3'), YWHAZ-low (5'-AGGGTGTGTTGGTGGAAGGTT-3'). These primers for the internal control

genes were designed according to the report by Vandesompele *et al.* (2002).

Quantitative real-time RT-PCR

For the quantification of transcripts, mRNA was extracted from 200 embryos by TRIzol Reagent (Invitrogen), and reverse transcribed with SuperScript III (Invitrogen). The same amount of each cDNA was used for the real time RT-PCR (7300 Real Time PCR System, Applied Biosystems). We estimated the amount of *CiNut1* and *CiNut2* mRNA relative to the *Ciona Glyceraldehyde-3-phosphate dehydrogenase* (CiGAPD) gene (CLSTR00881) mRNA as an inner control. Primers and TaqMan probes are as follows:
CiNut1FP primer (5'-CTATTTGCCGTTCGCCATCT -3'),
CiNut1RP primer (5'-AGAGGCGACGACCATGGA -3'),
CiNut1 TaqMan probe (5'FAM-ATCACCGTCGGACTCCCCCCAC-TAMRA3'),
CiNut2FP primer (5'-TTGCTTACAAATCGGAAGAGGAA -3'),
CiNut2RP primer (5'-TAAATATGGGAGATAACCGAGTACGA -3'),
CiNut2 TaqMan probe (5'FAM-CGGTTGTGATCGCTGCACCAGTTTC -TAMRA3'),
CiGAPD FP primer (5'ACATGGTTTACATGTTCAAGTATGATTCA -3'),
CiGAPD RP primer (5'-AATTTGCCGCCTTCTTCAGA -3'), and
CiGAPD TaqMan probe (5'FAM-ACACGGCGTTTTCAACGGCACAG -TAMRA3').

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization of staged *C. intestinalis* embryos was carried out as described (Etani and Nishikata, 2002). Specimens were fixed in 4% paraformaldehyde in 0.1 M Mops (pH 7.5), 0.5 M NaCl at 4℃ 16 h, prior to storage in 80% ethanol at –20℃. DIG-labeled RNA probes were synthesized using Anti-Digoxigenin-AP Fab fragments (Roshe), 10× transcription buffer, and T3/T7 RNA polymerase (Roshe). Rehydration of specimens was followed by proteinase K treatment (200 μg/ml, 50°C, 30 min), postfixation (4% paraformaldehyde), acetic anhydride treatment (0.27% in 0.1 M triethanolamine), prehybridization (50% formamide, 5× SSC, 50 μg/ml heparin, 100 μg/ml tRNA, 1% SDS), and hybridization (1 μg/ml (*CiNut1*) and 2.5 μg/ml (*CiNut2*) DIG-labeled probe, 50℃, 16 h). Hybridized probe was immuno-detected with alkaline phosphatase-conjugated anti-DIG antibody and the color reaction was carried out with NBT (nitroblue tetrazolium; Wako) and BCIP (5-bromo-4-chloro-3-indolyl phosphate; Wako). Otherwise, the specimens were dehydrated in a graded series of ethanol, and then cleared in a 1:2 mixture of benzyl alcohol:benzyl benzoate (BABB).

The sequence comparison and the Harr-plot analysis

The upstream sequences of *CiNut1* and *CiNut2* genes were obtained from the

whole-genome sequences of above-mentioned databases. About 1.1 kb upstream sequence of *CiNut1* and 4.3 kb upstream sequence of *CiNut2* were analyzed with the Genetics Mac ver. 8. The dot matrix of Harr-plot analysis was also carried out by the soft ware packaged with the Genetics Mac ver. 8. These upstream sequences were the longest sequences, which were available in these databases without gaps.

RESULTS

Search for the *CiNut1* homologues in the *Ciona intestinalis* and *Ciona savignyi* genome

We have already described a novel G-protein coupled receptor, *CiNut1*, which specifically expressed in the entire neural tube. Thanks to the genome project database, we could find a *CiNut1* homologous gene, *CiNut2*, in the *C. intestinalis* genome. The total length of their amino acid sequence was identical, and the over all amino acid sequence identity between CiNut1 and CiNut2 was 62%. Especially, the amino acid sequence from 112 to 159 amino acid residues of CiNut1 and CiNut2, which was around the fourth transmembrane domain, was completely conserved (Fig. 1). This high similarity between *CiNut1* and *CiNut2* strongly suggested that these two genes were homologs. Other similar sequences

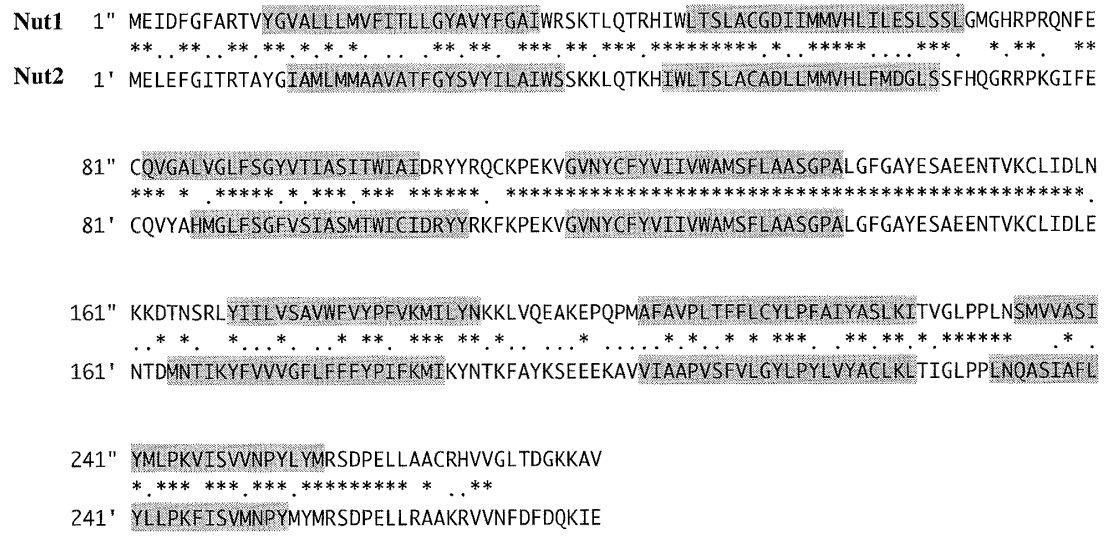


Fig. 1. Amino acid sequence comparison of CiNut1 and CiNut2. Amino acid sequence of CiNut1 (upper line) and CiNut2 (lower line) are aligned. Asterisks (*) denote identical residues and dots (.) denoted conserved residues. Shaded regions are predicted transmembrane domains, which predicted by SOSUI (Hirokawa *et al.*, 1998). CiNut1 and CiNut2 have the same amino acid sequence length.

to *CiNut1* in the *C. intestinalis* genome were rhodopsin family genes (Kusakabe *et al.*, 2002; Nakashima *et al.*, 2003). Compared to the sequence similarity between *CiNut1* and *CiNut2* (E-value = 7E-95), the sequence similarity between CiNuts and *Ciona* rhodopsin family genes seemed not to be significant (for example, *CiNut1* and *Ci-opsin3*; E-value = 8E-35, *CiNut1* and *Ci-opsin1*; E-value = 2E-6). This meant that *CiNut1* and *CiNut2* could form a paralogous group in the *C. intestinalis* genome.

Using the gene models of genome projects (Kyoto grail and JGI gene model version 1 and version 2), the chromosome location of *CiNut1* and *CiNut2* was revealed. They were adjoined on the chromosome 14p in the same direction (Fig. 2, *CiNut1*; ci0100148961, *CiNut2*; ci0100152713). Both *CiNut1* and *CiNut2* consisted of 7 exons. The length of their corresponding exons were almost identical, in other words, the locations of the intron were conserved. Moreover, the length of their corresponding introns was almost identical, except for the fourth and fifth introns.

In the *C. savignyi* genome (Broad Institute), we found one scaffold (scaffold reminder 27704), in which two *CiNut1* or *CiNut2* homologous sequences adjoined in the same direction. Unfortunately, since the database had no gene model and did not contain the sequence around the first exon of *CiNut1*, we could not find

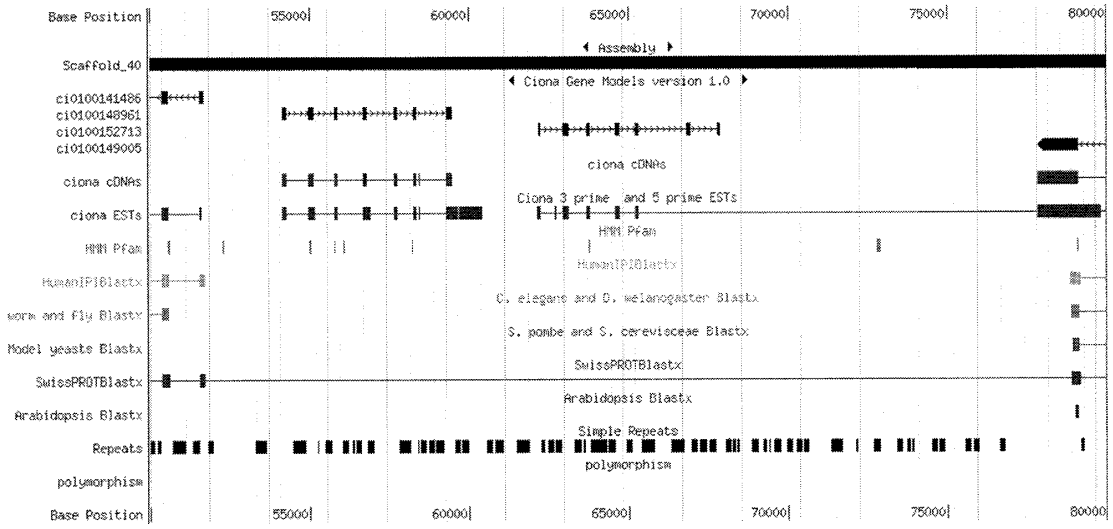


Fig. 2. Screen shot from the JGI genome browser, ver. 1. *CiNut1* and *CiNut2* were found on the scaffold 40 (chr 14 in the gene model ver. 2), and designated as ci0100148961 and ci0100152713, respectively. EST clones were assigned to both *CiNut1* and *CiNut2* (ciona ESTs), but no cDNA clones has been reported as *CiNut 2* homolog (ciona cDNAs). Homologs of *CiNut1* and *CiNut2* could not be found in the genome databases of other organisms (human, nematode, fly, yeast and Arabidopsis).

precise homologous sequences, namely *CsNut1* and *CsNut2*. So we tentatively assembled the partial *CsNut1*-coding sequence and full-length *CsNut2*-coding sequence, according to the sequence similarity to the *C. intestinalis* exon sequences. Using these tentative sequences, we also predicted their amino acid sequences. The total amino acid sequence identities between two species were very high; for example, between CiNut2 and CsNut2 were 80%. Moreover, the nucleotide sequence length between the coding sequences of *CsNut1* and *CsNut2* was about 3 kb, which is similar to that in the *C. intestinalis* genome.

Expression profiles of *CiNut1* and *CiNut2* during early development

According to the semi-quantitative RT-PCR analyses (Fig. 3a), *CiNut1* was

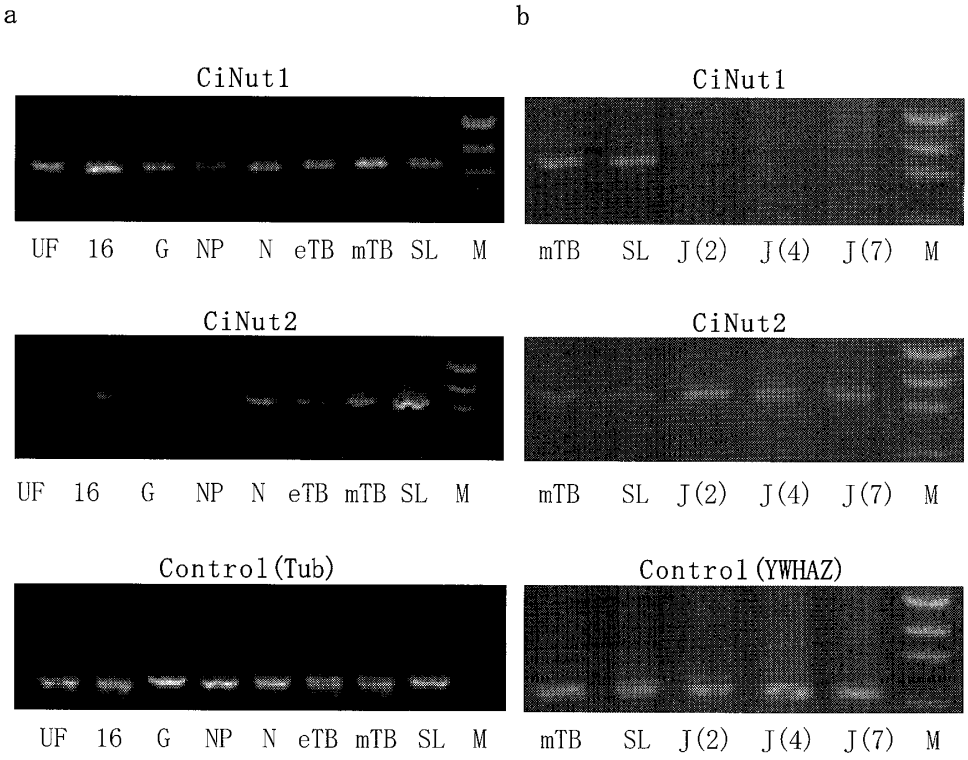


Fig. 3. Temporal expression pattern of *CiNut1* and *CiNut2*. Semi-quantitative RT-PCR was carried out on the following stages: UF, unfertilized egg; 16, 16-cell; G, gastrula; NP, neural plate; N, neurula; eTB, early tail-bud; mTB, middle tail-bud; SL, swimming larva; J, juvenile. Juvenile stages are distinguished by days after metamorphosis, which are shown in parentheses. Analyses during embryogenesis (**a**) and during and after metamorphosis (**b**) were carried out separately, because the amount of *CiNut2* transcript was drastically changed during metamorphosis. In **a**, *CiNut1* amplification was carried out for 25 cycles, while, *CiNut2* was carried out for 40 cycles. In **b**, both amplifications were carried out for 30 cycles.

maternally expressed in the unfertilized egg and constantly expressed from cleavage stages to the swimming larval stage. On the other hand, the expression of the *CiNut2* was not detected in unfertilized egg nor cleavage stage embryos. *CiNut2* expression was detected from gastrula stage and continued to the swimming larval stage, however, it was very faint during the early development.

To analyze the *CiNut1* and *CiNut2* expression quantitatively, we analyzed these transcripts by the real-time RT-PCR. We calculated their amount of transcripts relative to the amount of the inner control gene, *GAPD*. As shown in the Figure 4, the expression of *CiNut1* was slightly increased during cleavage stages and decreased at the gastrula stage. Then at the neurula and tail-bud stage, it was up-regulated specifically in the neural plate cells. This period was corresponding to the neululation. The amount of *CiNut2* transcript was less than 1/1000 of *CiNut1* during the early development. Even in the neurula and initial tail-bud stages, when the *CiNut2* expression was most eminent, *CiNut2* expression was about 1/3000 of *CiNut1*. Though, at the swimming larval stage, *CiNut2* expression was up-regulated to about ten-fold, while *CiNut1* expression was down-regulated to about ten-fold.

Spatial expression patterns of *CiNut1* and *CiNut2* were compared by whole-

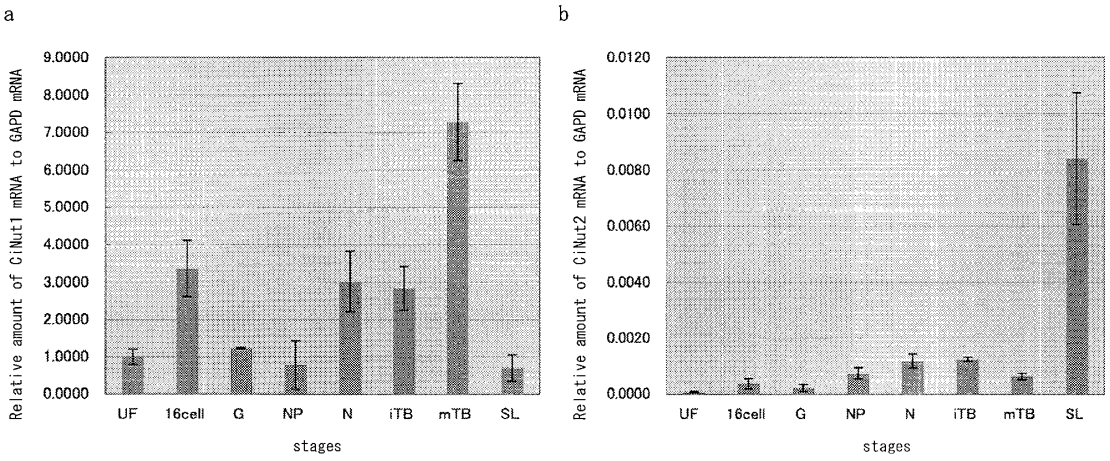


Fig. 4. Quantitative comparison of *CiNut1* and *CiNut2* transcripts. Quantitative real-time RT-PCR was carried out on the following stages: UF, unfertilized egg; 16cell, 16-cell; G, gastrula; NP, neural plate; N, neurula; iTB, initial tail-bud; mTB, middle tail-bud; SL, swimming larva. Vertical axis represents relative amount of *CiNut1* (**a**) and *CiNut2* (**b**) mRNA to the *GAPD* mRNA, which was selected as the inner control. Three independent analyses were done and the standard deviation is shown on each column. These results are in a good accordance with the results of the semi-quantitative analyses and whole-mount *in situ* hybridization.

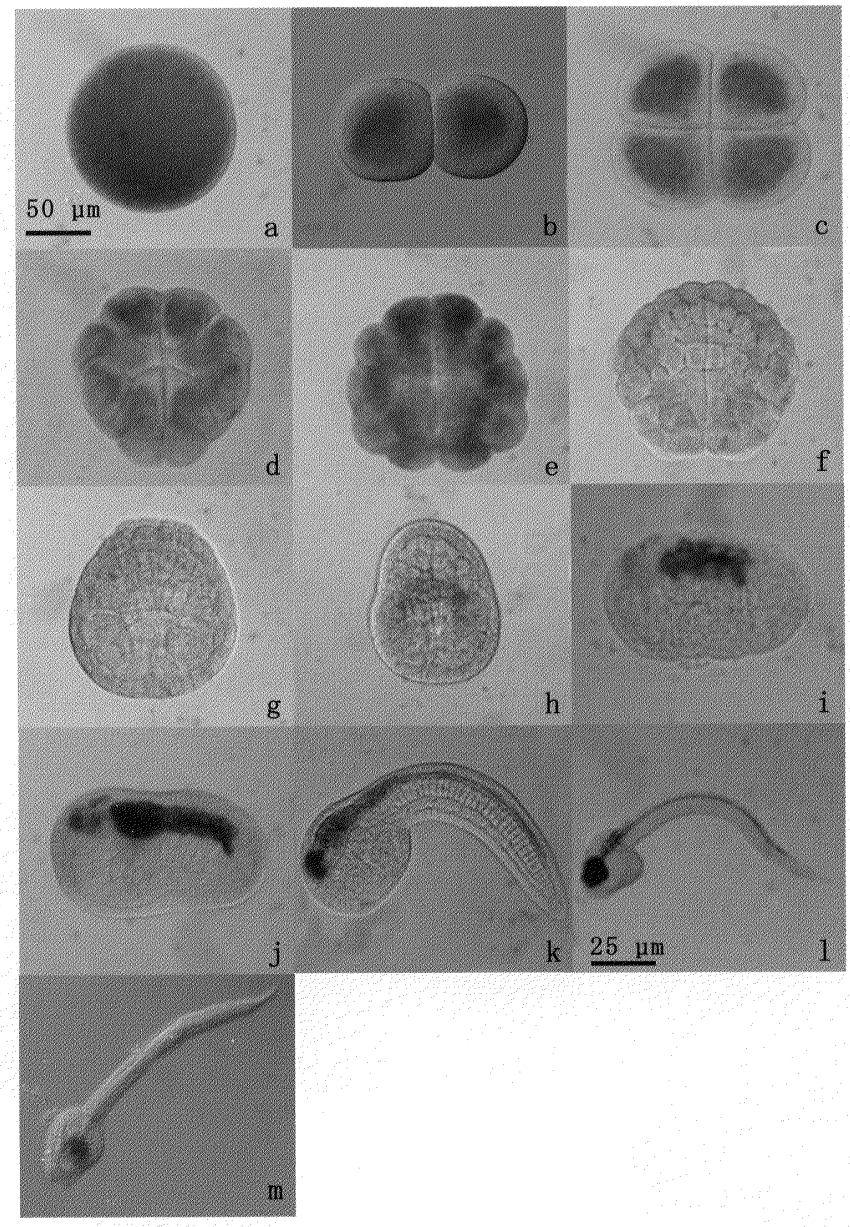


Fig. 5. Spatial expression pattern of *CiNut1* during early embryogenesis. Whole-mount *in situ* hybridization was carried out in the following stages: **a**, unfertilized egg; **b**, 2-cell; **c**, 4-cell; **d**, 16-cell; **e**, 32-cell; **f**, 110-cell; **g**, gastrula; **h**, neural plate; **i**, neurula; **j**, early tail-bud; **k**, middle tail-bud; **l**, late tail-bud; **m**, swimming larva. Maternal *CiNut1* transcripts were evenly distributed to all blastomeres during early cleavage stages (**a-e**). From gastrula stage, neural plate cells started to express weak *CiNut1* signal (**g**). *CiNut1* strong signals were appeared from neural plate stage (**h**). During the tail-bud stages (**j-l**), *CiNut1* expression was restricted to the entire neural tube. Scale bar for **a-k** appears on **a**; 50 μm. Scale bar for **l** and **m** appears on **l**; 25 μm.

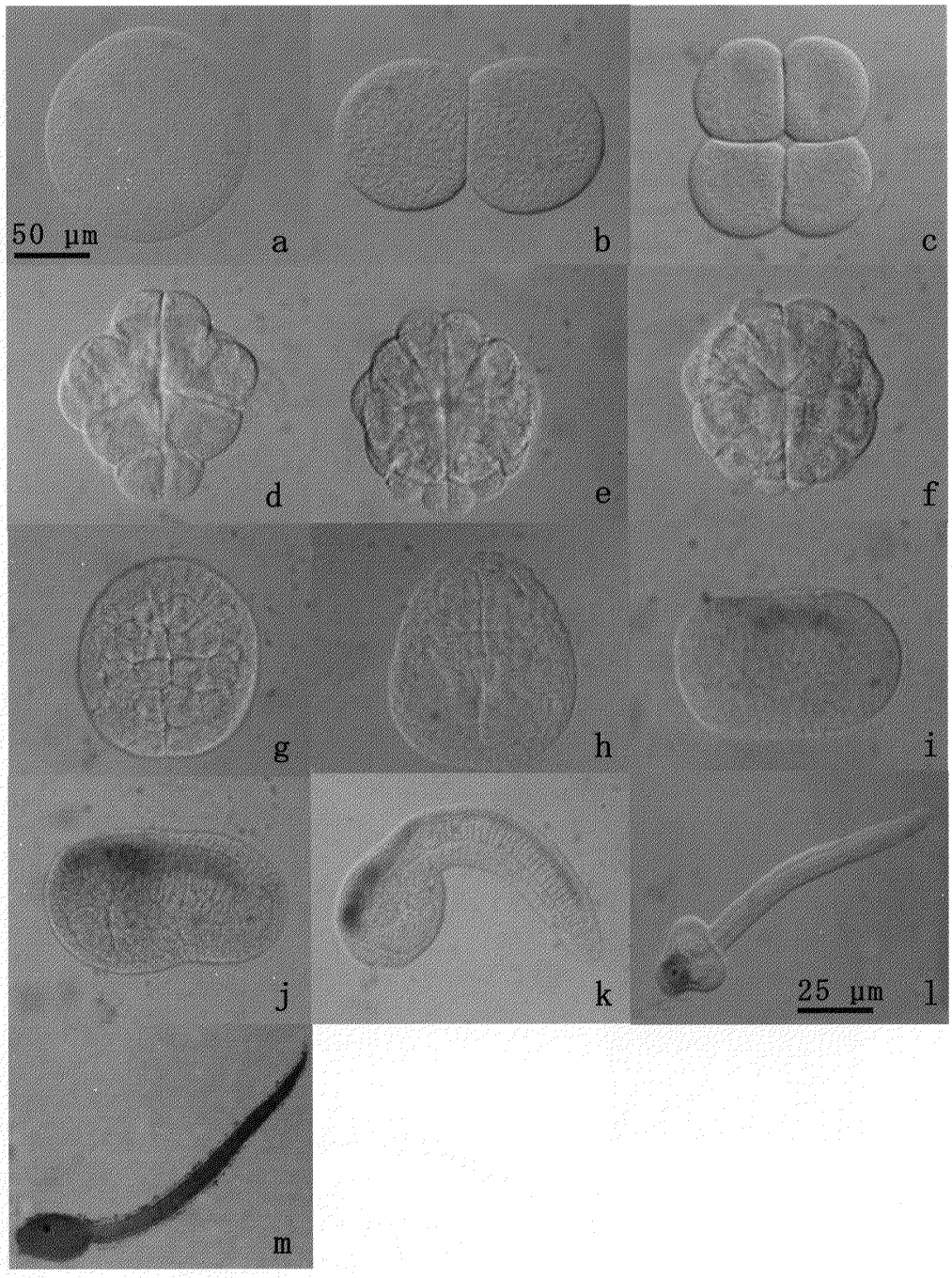


Fig. 6. Spatial expression pattern of *CiNut2* during early embryogenesis. The stages of whole-mount *in situ* hybridization were same as in Fig. 5. Different from the *CiNut1* expression, *CiNut2* was not detected from unfertilized egg to gastrula stage (**a-g**). From the neural plate stage through the tail-bud stages (**h-l**), although the signals were faint, *CiNut2* expressions within the neural plate and neural tube were almost identical to the *CiNut1* expression. In the swimming larval stage (**m**), *CiNut1* started to express in the entire body. Scale bars are also same as in Fig. 5.

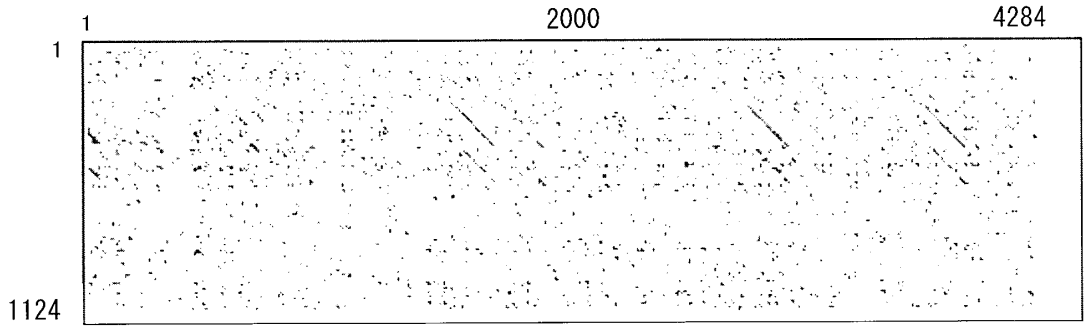
mount *in situ* hybridization (Fig. 5 and 6). The maternally expressed *CiNut1* was distributed evenly in the unfertilized egg and every blastomeres during cleavage stages. In the beginning of gastrula stage, the entire signals became faint, especially in the presumptive endoderm, notochord and nerve cord cells. From the gastrula stage, weak signals were appeared in the neural plate cell. At the neural plate stage, strong signals were appeared in the nerve cord cells. At the neurula stage, presumptive brain cells also started to express strong *CiNut1* signals. During the tail-bud stage, entire neural tube, except for the small portion of the brain, which thought to be sensory pigment cells, expressed *CiNut1*. The neural tube specific expression of *CiNut1* was gradually decreased during the swimming larval stage. On the other hand, *CiNut2* expression was not detected from unfertilized egg to neural plate stage. From neurula to tail-bud stage, although the signals were not so strong, the expression pattern was similar to that of *CiNut1* and restricted to the neural tube. At the swimming larval stage, *CiNut2* was expressed in the whole embryo. This strong expression was in a good accordance with the results of the quantitative analyses.

CiNut2 expression was up-regulated during metamorphosis

As the *CiNut2* expression was very faint during early development and increased during swimming larval stage, *CiNut2* seemed to have a role for the metamorphosis and/or post-metamorphic events. In order to reveal the expression profile of *CiNut2* during and after metamorphosis, we carried out semi-quantitative RT-PCR. As shown in Figure 4b, *CiNut1* was expressed up to swimming larval stage, and ceased to express in juvenile stage at least one week. In case of *CiNut2*, compared to the faint expression during tail-bud and swimming larval stages, it was actively translated in young juvenile for at least one week. This showed the clear difference between the roles of *CiNut1* and *CiNut2*.

Comparison of upstream sequence of _CiNut1_ and _CiNut2_

Although, some gaps were existed around this region, we could compare about 1.1 kb and 4.3 kb of the upstream sequences of *CiNut1* and *CiNut2*, respectively. According to the Harr-plot analysis, the upstream sequences of *CiNut1* and *CiNut2* had conserved sequence, which we designated as Box1 (Fig. 7a). The Box1 sequence was recognized once in *CiNut1* and three-times in *CiNut2*. In comparison of the Box1 sequences of *CiNut1* and *CiNut2*, they had 70 to 80% sequence similarity (Fig. 7b). In comparison of *C. intestinalis* and *C. savignyi* genome sequences, we could not find obvious Box1-like sequence in the upstream of *CsNut1* and *CsNut2*.



b

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Nut1  1"  GTATAGTAGGTTTAGATGGGTCACCTTTACCACAAATAACATCCAAATATCCTGAGCATGTTTTAAACATTTAACAACGGTATATGTGGA
          *** *** **   ** **** ******** ***  * ** ********** *** * ********** ********* * **  ***
Nut2  1'  GTAGAGTGGGGGAAGTTGGGACACCTTTAGCAC--AGAATATCCAAATATCTTGATCGTGTTTTAAACAATTAACAACGTTCTA--TGGG

      91" AGTTGCGAGAATACGGTTTGATAATTCATTAAATATGCTTTGTTTACTACAAAATGGGACGGGAAAATAAAATAAAAAGGTGTCCTATCT
          *** * *** ********* * ***** ** *** * ***********  ***** * ** * ***** *** ********** ** *
      87' AGTCGTGAGGATACGGTTTTACAATTCTTTGAATGTTCTTTGTTTACTAGCAAATGAGGCGAGGAAATAGAATGAAAAGGTGTCCCATTT

     181" TCCCCCATCCTACTATATATA
          ********************
     177' TCCCCCATCCTACTATATATT
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Fig. 7. Comparison of upstream sequences of *CiNut1* and *CiNut2*. Entire upstream sequences, which were available in the genome database at this point, were used. **a**, Harr-plot analysis with the about 1.1 kb *CiNut1* upstream (vertical line) and 4.3 kb *CiNut2* upstream (horizontal line). Considerably conserved sequence, which designated as Box1, was found once in *CiNut1* and three times in *CiNut2*. **b**, Sequence comparison of the Box1 sequences of *CiNut1* (upper) and the most proximal Box1 sequence of *CiNut2* (lower). Asterisks (*) show the conserved nucleotides.

DISCUSSION

In this study, we described two paralogous genes, *CiNut1* and *CiNut2*. These two genes encoded G-protein coupled receptor. As the sequence similarities between rhodopsin family genes and *CiNuts* were not high, we concluded that *CiNut1* and *CiNut2* should not be included in rhodopsin family. The amino acid sequence similarity between *CiNut1* and *CiNut2* were very high and their genome structure, such as intron position and length, were highly conserved, moreover, they are located in an adjoining location. These data were strongly suggested that these two paralogous genes were tandemly duplicated genes. Since, in the *Ciona savignyi* genome, *CsNut1* and *CsNut2* were also located in an adjoining location,

this duplication must be occurred prior to the diversion of *Ciona intestinalis* and *Ciona savignyi*. The result that the sequence similarities between species were higher than those among paralogous group supports this idea.

According to the expression profiles of *CiNut1* and *CiNut2*, their major functions were suggested to be different. While *CiNut1* might be involved in the neural tube formation, *CiNut2* might have roles during and after metamorphosis. During the neural tube formation, although *CiNut2* expressed in a very low level and suggested to have a small contribution, *CiNut2* must be functionally redundant with *CiNut1*. Moreover, during neural tube formation, expression patterns of *CiNut1* and *CiNut2* were almost identical, suggesting a similar transcriptional regulatory mechanism, which restricted their expression to the entire neural tube.

In this situation, we could find Box1 sequences, which conserved in the upstream of both *CiNut1* and *CiNut2*. If we can experimentally analyze the upstream sequences of them, the importance of the Box1 sequence and the regulatory mechanism of neural tube specific expression will be revealed. These molecular mechanisms should provide a clue to understand the key mechanism of the neural tube formation. Moreover, it will lead us to understand the origin and evolutional process of vertebrates.

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