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Ascidian Homologs of Mammalian Thyroid Transcription Factor-1 Gene Are Expressed in the Endostyle

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ABSTRACT—The endostyle is a special organ in the pharynx of urochordates, cephalochordates and cyclostomes. During evolution of the primitive chordates, the endostyle was organized in their common ancestor(s) with a shift to internal feeding for extracting suspended food from the water. In addition, the endostyle has an iodine-concentrating activity and is therefore thought to be functionally homologous to the vertebrate thyroid gland. Human *TITF1* and mouse *titf1* are members of the *Nkx-2.1/TTF-1* gene subfamily, which encode an NK-2 type homeodomain transcription factor. The genes are expressed in the thyroid gland and are essential for thyroid-specific structural gene expression. In the present study, we isolated cDNA clones for ascidian homologs of *titf1* from *Halocynthia roretzi* and *Ciona intestinalis*, and examined whether the genes are expressed in the ascidian endostyle. Results clearly indicated that both the *H. roretzi* homolog *Hrtitf1* and the *C. intestinalis* homolog *Cititf1* are expressed specifically in the endostyle. The present finding therefore provide molecular evidence for the functional relationship between the ascidian endostyle and vertebrate thyroid gland. However, the genes are expressed in the supporting element regions but not in the putative iodine-concentrating regions of the endostyle.

INTRODUCTION

We are interested in molecular developmental mechanisms underlying the evolution of chordate body plan. The phylum Chordata consists of the subphyla Urochordata (tunicates), Cephalochordata (amphioxus) and Vertebrata. Chordates are categorized as deuterostomes, together with two other non-chordate invertebrate groups, echinoderms and hemichordates, as supported by molecular phylogenic studies (Wada and Satoh, 1994; Turbeville *et al*., 1994) as well as cladistic analysis (Schaeffer, 1987; Peterson, 1995). Chordates share several characteristic features including a notochord, a dorsal hollow nerve cord, and pharyngeal gill slits (e.g., Brusca and Brusca, 1990; Willmer, 1990; Nielsen, 1995; Gee, 1996). In addition, lower chordates including tunicates, amphioxus and larval lampreys share an endostyle. These are hallmarks of the chordate body plan. Therefore, investigations of molecular developmental mechanisms involved in the organization of these structures are of salient importance in attempts to understand the evolution of chordate body plan.

We have emphasized that these characteristic features of chordates seem to have evolved with the emergence of tadpole larva-like creatures (Satoh and Jeffery, 1995; Satoh, 1995). Coincidently with this change in the mode of larval locomotion, most of the primitive chordates or chordate ancestors shifted their feeding system to the use of the pharyngeal gill slits for extracting suspended food from the water and the endostyle for secreting mucus to catch the food particles. The ascidian endostyle forms a trough-shaped structure in the ventral wall of the pharynx which extends from the fore-part of pharynx to the esophagus (see Fig. 4C; Ogasawara *et al*., 1996 and references therein). The cells of this organ are differentiated into eight or nine strips or zones that run parallel to one another in longitudinal orientation. The cells of each zone are highly specialized in morphology and function (Barrington, 1957, 1958; Fujita and Nanba, 1971; Thorpe *et al*., 1972; Dunn, 1974, 1980). Because the cells of zones 7, 8 and 9 have an iodine-concentrating activity, as do the thyroid cells of higher vertebrates, the endostyle of lower chordates is commonly considered a homolog and primitive antecedent of the vertebrate thyroid gland (e.g., Barrington, 1957; Thorpe *et al*., 1972; Dunn, 1974, 1980).

In previous studies, to obtain insights into the molecular mechanisms responsible for the formation and function of the endostyle, we isolated cDNA clones for the endostyle-specific genes *HrEnds1* and *HrEnds2* from the ascidian *Halocynthia roretzi* (Ogasawara *et al*., 1996) and *CiEnds1*, *CiEnds2*, and *CiEnds3* from the ascidian *Ciona intestinalis* (Ogasawara and Satoh, 1998). All of these genes encode

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secreted proteins. Interestingly, *HrEnds2*, *CiEnds1* and *CiEnds2* are expressed in zone 6 and encode similar secreted proteins, suggesting a molecular marker commonly used to monitor the ascidian endostyle differentiation. Because these genes encode structural proteins, we next focus on ascidian homologs of transcription factor genes that are expressed in the thyroid cells of higher vertebrates. The *Nkx-2.1*/*TTF-1* genes *TITF1* (human) and *titf1* (mouse) belong to the family of NK-2 type homeobox containing genes (Harvey, 1996). They are involved in the thyroid-specific gene expression (Civitareale *et al.*, 1993) and organogenesis of the thyroid (Kimura *et al.*, 1996). In the present study, we investigated whether ascidian homologs of the thyroid-specific transcriptional factor gene *Nkx-2.1*/*TTF-1* are expressed in the endostyle. Our results clearly indicated that the ascidian homologs of *Nkx-2.1* are expressed specifically in the endostyle.

MATERIALS AND METHODS

Biological materials

Adults and juveniles of *H. roretzi* and *C. intestinalis* were collected near the Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate, and the Marine BioSource Education Center of Tohoku University, Onagawa, Miyagi, Japan. After the dissection of adult specimens, tissues and organs were fixed for *in situ* hybridization or quickly frozen in liquid nitrogen and kept at -80°C until use.

Isolation of RNAs

Total RNA was extracted from various organs including the endostyle, pharyngeal gill, body wall muscle, intestine, gonad, and digestive gland of *H. roretzi* and *C. intestinalis* by the AGPC method (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was purified with oligotex dT30 beads (Roche Japan, Tokyo).

RT-PCR amplification

Reverse-transcription (RT) was carried out using *H. roretzi* endostyle Poly(A)$^+$ RNA with hexanucleotide mix (Boehringer Mannheim, Heidelberg, Germany). After purification of the cDNA, degenerated polymerase chain reaction (PCR) was performed using two degenerated primers. In order to amplify the DNA fragment of NK-2 type homeodomain, the first-round PCR was done using primers NKX-F1 (5'-TTYAG-YCARGCNCARGTNTAYGARYT-3') and NKX-R (5'-KTTYTGRAA-CCADATYTTNACYTG-3') (shown by IUPAC code). After the purification of DNA of the expected size from agarose gel, the DNA was used for the second-round PCR with the same primers. The PCR conditions were 30 cycles of 94°C for 1 min, 50°C for 2 min, and 72°C for 30 sec for both rounds.

Isolation and sequencing of cDNA clones for an ascidian *titf1* gene

The DNA fragment isolated by RT-PCR was subcloned into the EcoRV site of the vector pBluescript II SK(-). cDNA clones for *H. roretzi titf* (*Hrtitf1*) were obtained by screening the *H. roretzi* endostyle cDNA library with this DNA fragment as the probe. Plaques which showed positive hybridization were selected and isolated by two rounds of screening. The specificity of the clones positive for the endostyle was confirmed by a Northern blot analysis. The clones were prepared for sequencing by controlled nested deletion from either the T3 or T7 side and sequenced using the ABI PRISM 377 DNA Sequencer (Perkin Elmer, Norwalk, CT, USA). The isolation and characterization of cDNA clones for *C. intestinalis titf1* (*Cititf1*) were reported elsewhere (Ristoratore *et al.*, submitted).

Northern blot analysis

The Northern blot hybridization was carried out by the standard procedure (Sambrook *et al.*, 1989), and the filters were washed under high-stringency conditions. DNA probes for blot hybridizations were labeled with [^{32}P]-dCTP using a random primed labeling kit (Boehringer Mannheim).

In situ hybridization

Tissues of *H. roretzi* and *C. intestinalis* were fixed in 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS buffer at 4°C for 12 hr. For the young adult *H. roretzi*, the tunic was stripped off with a razor-knife prior to fixation as above. In the case of young adult *C. intestinalis*, the specimens were treated with L-menthol seawater to induce relaxation of the body-wall muscle, and then the tunic was stripped off with tungsten needles in the fixation buffer. Probes were synthesized by following the instructions from the kit supplier (DIG RNA Labeling kit; Boehringer Mannheim). The *in situ* hybridization of whole-mount specimens was carried out basically as described previously (Ogasawara *et al.*, 1996). For the *in situ* hybridization of sectioned specimens, samples were dehydrated with a graded series of alcohol, embedded in polyester wax (BDH) and sectioned at 10 μm.

RESULTS

Isolation and characterization of cDNA for *Hrtitf1* of *Halocynthia roretzi*

With the aid of the conserved NK-2 type homeodomain sequence of Nkx-2.1/TTF-1, we amplified a target fragment from the adult endostyle poly(A)$^+$ RNA by RT-PCR. After confirming that the fragment contained the predicted sequence of the NK-2 type homeodomain, we screened the *H. roretzi* endostyle cDNA library (Ogasawara *et al.*, 1996) with the fragment as a probe, and obtained candidate cDNA clones.

Fig. 1 shows the nucleotide and predicted amino acid sequences of cDNA for the *H. roretzi Hrtitf1* gene. The nucleotide sequence will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under Accession No. AB017704. The sequence of the cDNA encompassed 2,820 bp including 19 adenylyl residues at the 3' end. The ATG at the position 144-146 represented the putative start codon of the *Hrtitf1*-encoding protein. The cDNA contained a single open reading frame, which predicted the HrTTF-1 protein of 557 amino acids. The molecular mass of HrTTF-1 was calculated to be 60,038. A data base search indicated that the polypeptide contained the TN domain at amino acid position 15-25, the homeodomain at 276-335, and the NK-2 domain at 415-432, respectively (Fig. 1).

Characterization of HrTTF-1

The NK type homeobox genes were first cloned from *Drosophila* (Kim and Nirenberg, 1989), and four *Drosophila* genes so far isolated include *NK1* (Dohrmann *et al.*, 1990), *NK2* (Jiminez *et al.*, 1995), *NK3* (Azpiazu and Frasch, 1993), and *NK4* (Bodmer, 1993). The products of these genes were later classified into the two homeodomain protein classes, NK-1 (containing NK1) and NK-2 (containing NK2 to NK4) (Burglin, 1993). NK-2 homeobox genes were isolated from various organisms (Harvey, 1996). At least five members of this family were isolated from mouse, including *TTF-1*/*Nkx-2.1* (Oguchi

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Fig. 1. Nucleotide and predicted amino acid sequences of cDNA for *Hrtitf1* of *Halocynthia roretzi*. The sequence of the cDNA encompasses 2,820 bp including 19 adenylyl residues at the 3' end. The ATG at the position 144-146 represents the putative start codon of the *Hrtitf1*-encoding protein. An asterisk indicates the termination codon. The TN domain at amino acid portion 15-25 is double-underlined, the homeodomain at 276-335 is boxed, and the NK-2 domain at 415-432 is underlined. The nucleotide sequence will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under Accession No. AB017704.

et al., 1995), *Nkx-2.2* (Price *et al*., 1992), *Nkx-2.3* (Price *et al*., 1992), and *Nkx-2.5* (Lints *et al*., 1993). To identify subfamily to which HrTTF-1 belongs, we compared amino acid sequences of the HrTTF-1 TN domain (Fig. 2A) and homeodomain (Fig. 2B) with those of other Nkx-2 family members. First, it was noted that NK-2 type homeodomains (those of mouse Nkx-2.1, Nkx-2.2, Nkx-2.3 and Nkx-2.5, and *Drosophila* NK2, NK3 and NK4) share a tyrosine residue at position 54 within this domain. As was evident in Fig. 2B, the amino acid residue at position 54 of the HrTTF-1 homeodomain is tyrosine (arrow). This strongly suggests that HrTTF-1 is a member of the NK-2 family.

The amino acid sequence of the TN domain of HrTTF-1 was identical to that of CiTTF-1 (TTF-1 of *C. intestinalis*, see below; Fig. 2A; the Accession No. AJ009607). The grade of identity of this domain was about 90% between the ascidian (HrTTF-1 and CiTTF-1) and mammalian TTF-1/Nkx-2.1. However, the grade was relatively low between the ascidians and mammalian Nkx-2.2, Nkx-2.3 and Nkx-2.5. This suggests that HrTTF-1 is related to the Nkx-2.1 family.

A similar relationship was evident when we compared the amino acid sequences of the homeodomain (Fig. 2B). Only two amino acid residues were different between HrTTF-1 and CiTTF-1, the identity being 97%. In addition, the degree of identity of the homeodomain was 90% between the ascidian HrTTF-1 and mammalian TTF-1/Nkx-2.1 (Fig. 2B). However, the identity decreased when compared between the ascidian HrTTF-1 and mouse Nkx-2.2 (83%), Nkx-2.3 (82%) or Nkx-2.5 (78%). Furthermore, the degree of identity of this domain was about 82% between the ascidian HrTTF-1 and *Drosophila* NK2, but the degree was relatively low when compared with NK3 (65%), NK4 (65%), and NK1 (50%). This strongly suggests that HrTTF-1 and CiTTF-1 are members of the Nkx-2.1 family.

To test the above-mentioned results, we performed a molecular phylogenetic analysis. Using 60 confidently aligned sites of the homeodomain amino-acid residues, a molecular phylogenetic tree was constructed by the neighbor-joining

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A TN domain

```
HrTTF-1          TPFSVTDILSP
CiTTF-1          ...........      (100.0)
Human TTF-1      .....S.....      ( 90.9)
Mouse TTF-1      .....S.....      ( 90.9)
Mouse Nkx-2.2    .G...K...DL      ( 63.6)
Mouse Nkx-2.3    .....K...NL      ( 72.7)
Mouse Nkx-2.5    .....K...NL      ( 72.7)
```

B Homeodomain

```
HrTTF-1          RRKRRVLFSQAQVFELERRFKQQKYLSAPEREHLAQMIRLTPTQVKIWFQNHRYKNKRAL
CiTTF-1          ..................................H.................S.    ( 96.7)
Human TTF-1(Nkx-2.1)  ............Y.....................S..H.............M..QA  ( 90.0)
Mouse TTF-1(Nkx-2.1)  ............Y.....................S..H.............M..QA  ( 90.0)
Mouse Nkx-2.2    K........K..TY......R..R...........SL.................M...R  ( 83.3)
Mouse Nkx-2.3    ...P..................R...........SSLK..S.........R...C..QR  ( 81.7)
Mouse Nkx-2.5    ............Y.........R.......DQ..SVLK..S.........R...C..QR  ( 78.3)
Drosophila NK2   K.......TK..TY......R..R...........SL.................T...Q  ( 81.7)
Drosophila NK3   KKRS.AA..H.........A..R...G...SEM.KSL...E.........R...T..KQ  ( 65.0)
Drosophila NK4   K..P.........L...C..RLK...TGA...II..KLN.SA.........R...S..GD  ( 65.0)
Drosophila NK1   ..RA.TA.TYE.LVS..NK..TTR...VC..LN..LSLS..E.........R.T.W.KQN  ( 50.0)
```

Fig. 2. (**A**) Comparison of amino acid sequences of the HrTTF-1 TN domain with those of CiTTF-1 and five mammalian NK-2-like gene products. The percentage of identity is shown at the right side. The dots represent identical amino acids. (**B**) Comparison of amino acid sequences of the HrTTF-1 homeodomain with those of CiTTF-1, five other mammalian NK-2-like gene products and *Drosophila NK1* to *NK4* gene products. The tyrosine at position 54 is a feature of NK-2 homeodomain proteins (arrow). Sources: CiTTF-1 (Ristoratore *et al.*, submitted), human TTF-1 (Saiardi *et al.*, 1995), mouse TTF-1 (Oguchi *et al.*, 1995), mouse Nkx-2.2 (Price *et al.*, 1992), mouse Nkx-2.3 (Price *et al.*, 1992), and mouse Nkx-2.5 (Lints *et al.*, 1993), *Drosophila* NK2 (Jiminez *et al.*, 1995), *Drosophila* NK3 (Azpiazu and Frasch, 1993), *Drosophila* NK4 (Bodmer, 1993) and *Drosophila* NK1 (Dohrmann *et al.*, 1990).

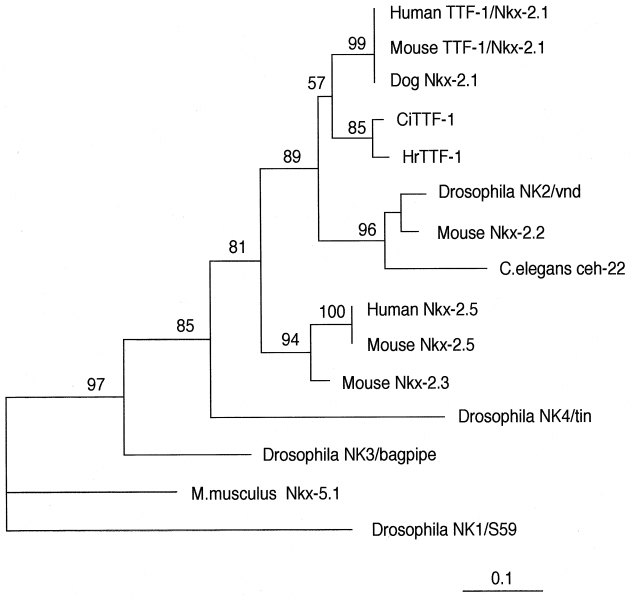

Fig. 3. Evolutionary relationships of HrTTF-1 with other NK-2 proteins. Using 60 confidently aligned sites of the homeodomain amino-acid residues, a molecular phylogenetic tree was constructed by the neighbor-joining method. The branch length is proportional to the number of amino acid substitutions; 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.

method (Saitou and Nei, 1987). As shown in Fig. 3, the ascidian HrTTF-1 and CiTTF-1 form a discrete group with vertebrate Nkx-2.1 (mouse TTF-1/Nkx-2.1, human TTF-1/Nkx-2.1 and dog Nkx-2.1). This grouping was supported by the bootstrap value of 57%. In addition, this group was included in a larger clade with *Drosophila* NK2, mouse Nkx-2.2 and *C. elegans* CEH-22. This larger clade was supported by the bootstrap value of 89%. From this data together with the results mentioned above, we concluded that HrTTF-1 and CiTTF-1 are members of the Nkx-2.1 family.

Hrtitf1 is expressed in the endostyle

The ascidian endostyle forms a trough-shaped structure in the ventral wall of the pharynx and extends from the forepart of the pharynx to the esophagus (Fig. 4C). The *in situ* hybridization of whole-mount specimens demonstrated that the *Hrtitf1* signal was specific to the endostyle (Fig. 4A, B). No signal was detected in organs and tissues other than the endostyle. This was confirmed by Northern blot analysis. As shown in Fig. 4F, a distinct band of 2.8 kb was found in the endostyle. Although a very weak band was seen in the digestive gland (hepatopancreas), the signal was undetectable in the gonad, intestine, body-wall muscle and pharyngeal gill (Fig. 4F).

The endostyle cells are differentiated into eight or nine strips or zones and the cells of each zone are highly specialized in morphology and function (Fig. 4C; Barrington, 1957; Thorpe *et al.*, 1972; Dunn, 1974, 1980). The cells of zones 7, 8 and 9 have an iodine-concentrating activity, as do the thy-

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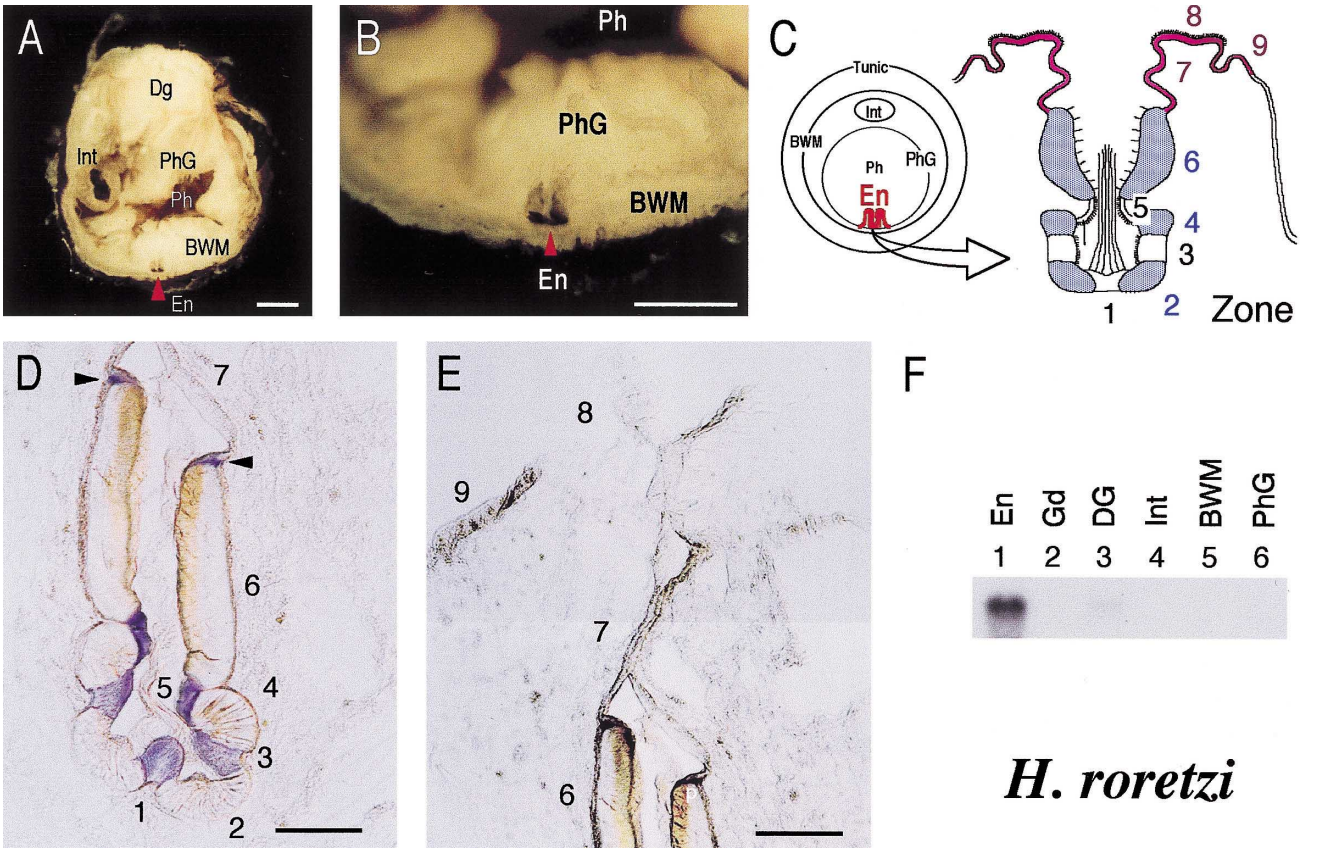

Fig. 4. Expression of *Hrtitf1* in the endostyle. (**A, B**) A whole-mount specimen of a 1-month-old young adult *H. roretzi* showing that the hybridization signal is restricted to the endostyle (En, red arrowhead). BWM, body-wall muscle; Dg, digestive gland; Int, intestine; PhG, pharyngeal gill; Ph, pharynx. Scale bar is 1 mm. (**C**) Diagram of the ascidian endostyle, showing compositional elements or zones of the endostyle. Zones 1, 3 and 5 are supporting elements, zones 2, 4 and 6 are protein-secreting glandular elements, and zones 7, 8 and 9 are iodine-concentrating elements, equivalent to the thyroid gland of vertebrates. [Based on descriptions of Barrington (1957), Thorpe *et al.* (1972), Fujita and Nanba (1971), and Dunn (1974)]. (**D, E**) Cross-sections of the endostyle showing the gene expression in the entire zones of 1, 3 and 5 of the endostyle and in the basement region of the zone 6 (**D**, arrowheads). Scale bar is 100 μm. (**F**) Northern blots of poly(A)$^+$ RNA prepared from the endostyle (En), gonad (Gd), digestive gland (DG), intestine (Int), body-wall muscle (BWM), and pharyngeal gill (PhG) were hybridized with random-primed [^{32}P]-labeled DNA probe, and the membrane was washed under high-stringency conditions. The *Hrtitf1* transcript of about 2.8 kb in length was mainly detected in the endostyle. Each lane was loaded with 10 μg of poly(A)$^+$ RNA.

roid cells of higher vertebrates. The cells of zones 2, 4 and 6 have numerous secretory granules. These cells are believed to secrete the proteins or mucoprotein related to the digestion of food. The cells of zones 1, 3 and 5 are considered supporting elements and also as elements that might play a role in catching and transporting food. The *in situ* hybridization of sectioned specimens demonstrated that the signal was not distributed over the entire area of the endostyle rather restricted to several zones (Fig. 4D, E). The transcript was evident in the entire region of zones 1, 3 and 5 (Fig. 4D), and in basement region of the zone 6 (Fig. 4D, E). No signal was detected in zones 2, 4, 7, 8, and 9 (Fig. 4D, E).

Cititf1 is also expressed in the endostyle

The isolation and characterization of a cDNA clone for *Cititf1* of *C. intestinalis* will be described in detail together with their expression pattern during *Ciona* embryogenesis, and experiments to deduce its function (Ristoratore *et al.*, submitted). The present analysis of CiTTF-1 (Figs. 2 and 3) clearly indicated that CiTTF-1 is also a member of the Nkx-2.1 family.

The *in situ* hybridization of whole-mount specimens of a 1-month-old young adult *C. intestinalis* demonstrated that the signal was evident in the endostyle (Fig. 5A). No signal was detected in organs and tissues other than the endostyle. The Northern blot analysis supported this result. As shown in Fig. 5D, a distinct band of 2.7 kb was found in the endostyle. The signal was undetectable in the pharyngeal gill, body-wall muscle, intestine, and gonad.

The *in situ* hybridization of sectioned specimens demonstrated that the signal was restricted to several zones of the endostyle (Fig. 5B, C). The *Cititf1* transcript was found in the entire region of zones 3 and 5 (Fig. 5B), and in part of the regions of zones 1, 2 and 6 (Fig. 5B, C). As was the case for *Hrtitf1*, *Cititf1* transcript was not found in the zones 7, 8, and 9 (Fig. 5B, C) with iodine-concentrating activity.

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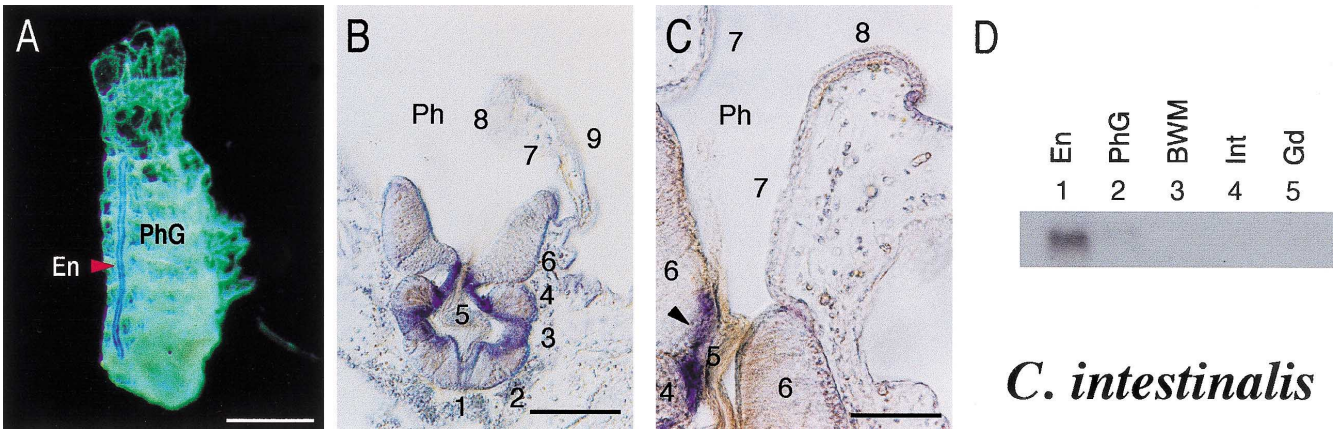

Fig. 5. Expression of *Cititf1* in the endostyle. (**A**) A whole-mount specimen of a 1-month-old young adult *C. intestinalis* showing that the hybridization signal is restricted to the endostyle (En, red arrowhead). PhG, pharyngeal gill. Scale bar is 1 mm. (**B, C**) Cross-sections of a young adult (scale bar is 100 μm) showing the *Cititf1* gene expression in the entire zones of 3 and 5, and in part of the zones 1, 2 and 6 (arrowhead in **C**) of the endostyle. No signal is evident in the zones 7, 8 and 9, iodine-concentrating elements equivalent to the vertebrate thyroid gland. (**D**) Distribution of *Cititf1* transcript in tissues and organs of the adult. Northern blots of poly(A)$^+$ RNA prepared from the endostyle (En), pharyngeal gill (PhG), body-wall muscle (BWM), intestine (Int), and gonad (Gd) were hybridized with the random-primed [^{32}P]-labeled DNA probes, and the membrane was washed under high-stringency conditions. The *Cititf1* transcript of about 2.7 kb in length was detected in the endostyle. Each lane was loaded with 8 μg of poly(A)$^+$ RNA.

DISCUSSION

The present study investigated ascidian homologs of the transcription factor gene *TTF-1*/*Nkx-2.1*, which is expressed in the thyroid gland of higher vertebrates (human, Saiardi *et al.*, 1995; mouse, Oguchi *et al.*, 1995; rat, Oguchi and Kimura, 1998; dog, Van Renterghem *et al.*, 1995; and chick, Pera and Kessel, 1998). We isolated cDNA clones for the *H. roretzi* gene *Hrtitf1* and the *C. intestinalis* gene *Cititf1*. Both genes have a typical NK-2 type homeodomain (Fig. 2B) and are classified as members of the TTF-1/Nkx-2.1 family. The Northern blotting of adult tissues (Figs. 4F and 5D) and whole-mount *in situ* hybridization (Figs. 4A, 4B and 5A) clearly showed that the transcripts are present only in the endostyle. These results provide molecular evidence that the ascidian endostyle is homologous to the vertebrate thyroid gland.

The *in situ* hybridization of sectioned specimens showed that transcripts of *Hrtitf1* are detected in the entire regions of zones 1, 3 and 5, and in part of the zone 6. The *Cititf1* transcript is also expressed in the entire regions of zones 3 and 5, and in part of the zones 1, 2 and 6 of the endostyle. As was mentioned above, zones 7, 8 and 9 are thought to be homologous to the thyroid cells of higher vertebrates, because the zones have an iodine concentration activity (Thorpe *et al.*, 1972; Dunn, 1974) and thyroperoxidase activity (Fujita and Sawano, 1979). Interestingly, both *Hrtitf1* and *Cititf1* were not expressed in these zones with iodine-concentrating activity. Instead, *Hrtitf1* was expressed primarily in the supporting zones and mucus secretion zones.

During the evolution of chordates, their ancestor(s) may have obtained an internal feeding system, using the pharyngeal gill slits and endostyle for extracting suspended food from the water. The endostyle of ascidians and amphioxus consists of two different regions, a mucus-secretory region and an iodine-concentrating region. The endostyle of larvae of cyclostomate lamprey has several cell types including protein secretory cells and iodine-concentrating cells (Fujita and Honma, 1968). During metamorphosis, the endostyle loses the protein secretory cells and transforms into the thyroid gland (Wright and Youson, 1976). The endostyle is therefore a key structure for clarifying not only the origin of chordates, but also the evolution to the thyroid gland of higher vertebrates. As shown in this study, *Hrtitf1* and *Cititf1* were not expressed in the region with iodine-concentrating activity but rather were expressed primarily in the supporting region and mucus secretion region as well. Therefore, further examinations of the pattern of *titf1* expression (e.g., during the transformation of the endostyle of larval lamprey) are required to understand the relationships between the *titf1* expression and formation of the thyroid-like organs during chordate evolution.

In previous studies, we isolated genes specific to the endostyle (Ogasawara *et al.*, 1996; Ogasawara and Satoh, 1998). The *HrEnds1*, *HrEnds2*, *CiEnds1* and *CiEnds2* genes are expressed in the whole region of zone 6, *CiEnds3* in zone 2, and *CiEnds4* or a cytoplasmic actin gene in zones 3, 5 and 7. Although the expression of the ascidian *titf1* genes only partially overlap with that of these structural genes, it is an intriguing research subject to determine whether *HrTTF-1* or *CiTTF-1* regulates the expression of these structural genes.

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