Novel Endostyle-Specific Genes in the Ascidian Ciona intestinalis

Authors: Sasaki, Akane, Miyamoto, Yuki, Satou, Yutaka, Satoh, Nori, and Ogasawara, Michio

Source: Zoological Science, 20(8) : 1025-1030

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.1025

ZOOLOGICAL SCIENCE **20**: 1025–1030 (2003)

Novel Endostyle-Specific Genes in the Ascidian *Ciona intestinalis*

Akane Sasaki[1*], Yuki Miyamoto[2], Yutaka Satou[1], Nori Satoh[1] and Michio Ogasawara[2]

[1]*Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*
[2]*Department of Biology, Faculty of Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263-8522, Japan*

ABSTRACT—The endostyle is a pharyngeal organ of Urochordata, Cephalochordata and larval Cyclostomata. This organ secretes mucus-proteins for internal filter feeding, a feeding system that must have developed in the common ancestor of these subphyla. Therefore, the endostyle is a key structure to understanding the origin and evolution of chordates. A previous study of the overall gene expression in *Ciona intestinalis* young adults yielded several candidates for ascidian endostyle-specific genes. In the present study, we determined in detail the expression profiles of six novel endostyle-specific genes. *Ci-VWFL1* and *Ci-VWFL2* encode related proteins similar to vertebrate von Willebrand factor, and were continuously expressed in zones 4 and 2 of the developing endostyle, respectively. The expression of *Ci-Ends8* was observed in the entire region of zone 6 in young adults; however, the expression of this gene was restricted to the dorsal- and ventral-regions of zone 6 in the adult endostyle. The expression of *Ci-Ends9* and *Ci-Ends10* was observed in zones 6 and 4 in young adults, respectively, and was downregulated in the adult endostyle. *Ci-Ends11* showed an expression pattern similar to that of *Ciona TTF-1,* which encodes a thyroid-related transcription factor. The predicted amino acid sequence of Ci-Ends10 showed similarity to Trip230, and that of Ci-Ends11 resembled Ptp4E. These molecules might be useful for further analysis of the development, function and evolution of the endostyle.

Key words: ascidian, *Ciona intestinalis*, endostyle, VWF, evolution of chordates

INTRODUCTION

Urochordates (ascidians) represent one of the basal chordates in addition to cephalochordates (amphioxus) and cyclostomates (lampreys), and have some primitive features of chordates. One of these features, the endostyle, which is located in the ventral midline of the adult pharynx (Fig. 1A, B), is a characteristic organ for mucoprotein secretion. This organ is generally thought to have arisen in the common ancestor of chordates during the shift to internal feeding. Furthermore, the endostyle has functions that parallel those of the vertebrate follicular thyroid, such as iodine uptake and thyroid peroxidase activity (reviewed by Eales, 1997). Therefore, the endostyle is a key structure for understanding the origin and evolution of chordates. Cells of the ascidian endostyle are differentiated into nine zones that run parallel to one another in longitudinal orientation (e.g., Dunn, 1974). The cells of zones 1, 3 and 5 are considered to be supporting elements involved in catching and transporting food. Cells of zones 2, 4 and 6 are protein-secreting glandular cells used for filter feeding. Cells of zones 7, 8 and 9 have iodine-binding and peroxidase activities, like the follicular thyroid cells of vertebrates (Fig. 1C).

Several cDNA clones for ascidian endostyle-specific genes (*HrEnds1* and *HrEnds2*) have been isolated from *Halocynthia roretzi* (Ogasawara *et al*., 1996) and homologous cDNA clones have been isolated from *Ciona intestinalis* (Ogasawara and Satoh, 1998). The proteins deduced for these genes have no similarity to reported proteins, and therefore no insights into their function or phylogenetic relationships have been obtained by comparison to known proteins. *Ciona* homologs of thyroid-related transcription factor TTF-1 and differentiation marker TPO have been isolated (Ogasawara *et al*., 1999a, 1999b; Ristoratore *et al*., 1999). Exclusive expression of these genes in the endostyle strongly suggests that this organ is homologous to the follicular thyroid. However, the molecular mechanisms of the

* Corresponding author: Tel. +81-75-753-4082;
FAX. +81-75-705-1113.
E-mail: akane@ascidian.zool.kyoto-u.ac.jp

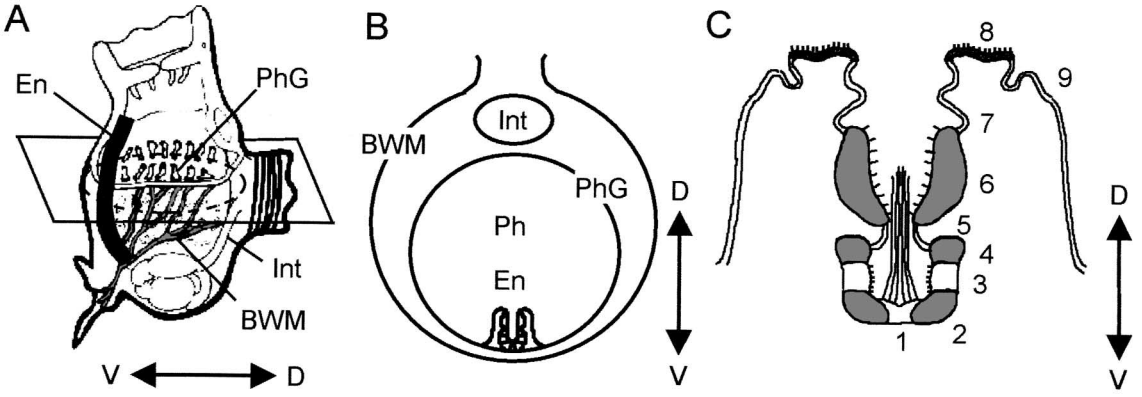

Fig. 1. Diagram of the ascidian endostyle. (A) Global morphology of young adult specimen without tunic. (B) Transverse section of young adult illustrated in A showing compositional elements. (C) Transverse section of the adult endostyle. Numbers are names of each zone. Zones 1, 3 and 5 are supporting elements. Zones 2, 4 and 6 are glandular regions for filter feeding. Zones 7, 8 and 9 are iodine-concentrating elements that constitute a thyroid-equivalent region. Abbreviations: En, endostyle; PhG, pharyngeal-gill; BWM, body-wall muscle; Int, intestine; Ph, pharynx; D, dorsal; V, ventral.

development, function and evolution of the endostyle are still unclear.

Ciona intestinalis has a small genome of about 1.6×10^8 bp/haploid genome containing approximately 15,800 genes (Simmen *et al*., 1998; Dehal *et al*., 2002). Comprehensive analyses of expressed sequence tags (ESTs) and gene expression profiles have been performed for several developmental stages of this animal (Satou *et al*., 2002). We have determined the expression profiles of 976 non-redundant genes in young adults 2-3 weeks after metamorphosis (Ogasawara *et al*., 2002), and this analysis identified several candidates for endostyle-specific genes. In the present study, we characterized cDNAs and the expression profiles of the novel endostyle-specific genes.

MATERIALS AND METHODS

Characterization of endostyle-specific cDNA clones

Candidates for endostyle-specific cDNA clones of *Ciona intestinalis* were identified by previous global analyses of gene expression profiles in *Ciona* young adults (Ogasawara *et al*., 2002). Reconfirmation of candidates for endostyle-specific gene was performed by *in situ* hybridization in young adults and adult endostyles, and by dot blotting in adults, leading to the identification of six candidate clones. Nucleotide sequences were determined for both strand by standard procedures using BigDye terminators and an ABI PRISM 377 sequencer (Applied Biosystems). Public protein databases were searched with each sequence using the BLASTX algorithm at the NCBI server.

In situ hybridization

Young adult specimens 2–3 weeks after metamorphosis and adult specimens of 2–3 months after metamorphosis were prepared as described in Ogasawara *et al*. (2002). DIG-labeled RNA probes were synthesized from PCR-amplified templates which contained a T7 RNA polymerase promoter, and purified using a centrifugal ultrafilter as described in Ogasawara *et al*. (2001). Whole-mount *in situ* hybridization of young adult specimens was performed by the method described by Ogasawara *et al*. (2002). *In situ* hybridization of adult endostyle specimens and sections was carried out essentially as described in Ogasawara and Satoh (1998).

mRNA dot blotting and Northern blotting

Poly(A)$^+$ RNA of adult organs (endostyle, pharyngeal-gill, body-wall muscle, intestine, and gonad) was isolated using Oligotex dT30 beads (Roche). For dot blotting, 100 ng of mRNA of each organ was blotted on a Hybond-N$^+$ nylon membrane (Amersham Biosciences). Northern blotting using 10 μg of mRNA of each organ was carried out using standard procedures (Sambrook *et al*., 1989). Hybridization was carried out using a DIG-labeled RNA probe, followed by washing of the filter under high-stringency conditions (hybridization: 6x SSPE, 0.1% SDS, 1× Denhardt's solution, 50% formamide at 68°C for 16 hr; washing: 2× SSC, 0.1% SDS at 68°C for 30 min, 1× SSC, 0.1% SDS at 68°C for 30 min, 0.5× SSC, 0.1% SDS at 68°C for 30 min).

RESULTS AND DISCUSSION

Twenty-one cDNA candidates for endostyle-specific genes were identified in a previous study of gene expression profiles in *Ciona* young adults (Ogasawara *et al*., 2002). The expression of these genes was prominent in the endostyle; however, several of these genes were also expressed in other organs of young adults (data not shown). Reconfirmation of the expression pattern by means of *in situ* hybridization, mRNA dot blotting analyses, and sequencing of cDNA clones revealed that six of these genes were novel endostyle-specific genes.

von Willebrand factor-like genes were expressed exclusively in the glandular cells of the ascidian endostyle

Whole-mount *in situ* hybridization using *Ciona* young adults showed that genes designated ID03415 and ID05762 were expressed exclusively in the endostyle (Fig. 2A, F). Although Northern blotting using mRNAs of adult organs showed several transcripts of these genes, the expression of these genes was detected only in the endostyle, and this was confirmed by dot blotting (Fig. 2D, E, I, J). The predicted amino acid sequences of these genes indicated that both genes encoded sequences similar to vertebrate von Willebrand factor (VWF) which is expressed in the vascular

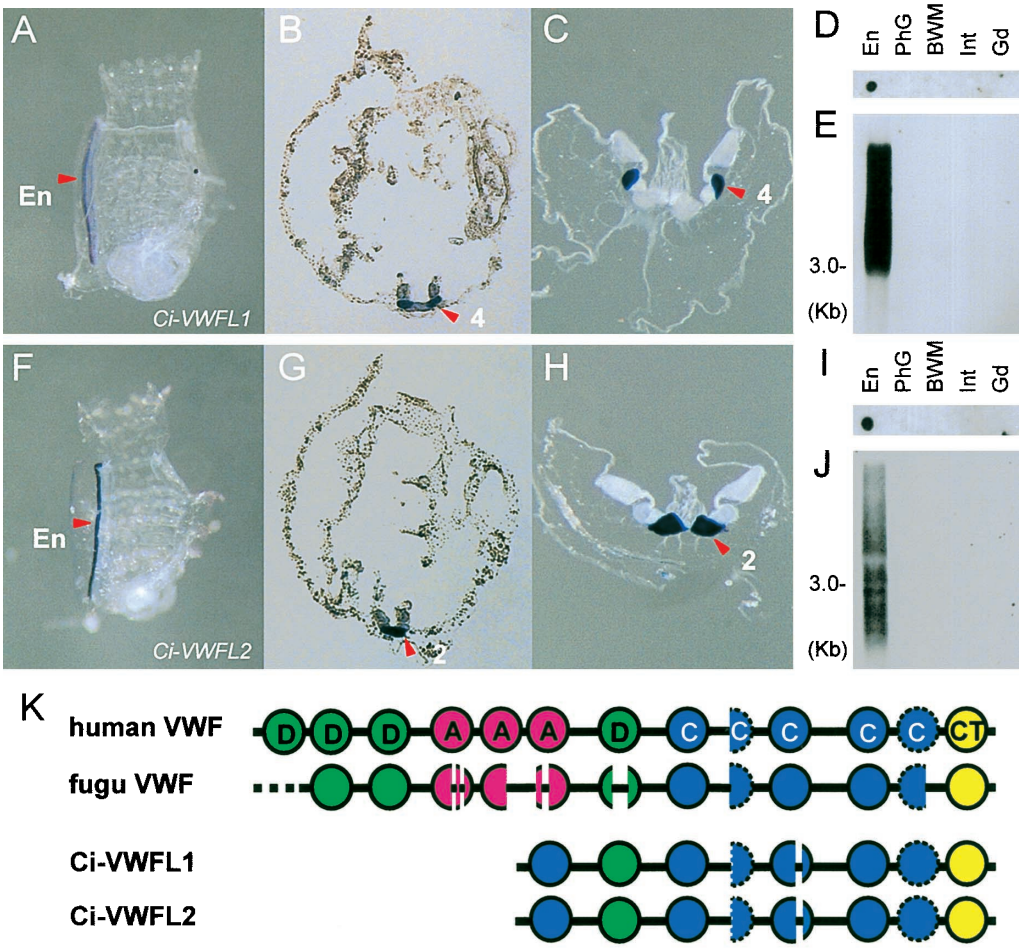

Fig. 2. VWF-like genes in *Ciona intestinalis*. (A–E) Expression of *Ci-VWFL1* assessed by *in situ* hybridization (A–C), dot blotting (D) and Northern blotting (E). (F–J) Expression of *Ci-VWFL2* assessed by *in situ* hybridization (F–H), dot blotting (I) and Northern blotting (J). Red arrowheads indicate expression signals in young adults (A, F), transverse sections of young adults (B, G) and transverse sections of the adult endostyle (C, H). *Ci-VWFL1* and *Ci-VWFL2* were expressed only in glandular zones 4 and 2 of the endostyle, respectively. (K) Domain structure of vertebrate VWF and *Ciona* VWF-like proteins. Human VWF has several A domains (red), C domains (blue) and D domains (green), and also has a cystine-knot domain (yellow). The domain structure of fugu (*Fugu rubripes*) VWF was constructed using the fugu genome database. The dotted line in fugu VWF shows the undetermined N-terminal region. The domain structures of **Ci-VWFL1** and **Ci-VWFL2** closely resemble each other. The predicted C domains found in our analysis are indicated by dotted circles. The amino acid sequence of **Ci-VWFL1** and **Ci-VWFL2** was estimated by a sequence in the DDBJ/EMBL/GenBank(accession numbers: *Ci-VWFL1*, AB112441; *Ci-VWFL2*, AB112442 and AK113188). Abbreviations: En, endostyle; PhG, pharyngeal-gill; BWM, body-wall muscle; Int, intestine and Gd, gonad.

endothelial cells. Therefore, genes ID03415 and ID05762 were named *Ci-VWFL1* (*Ciona intestinalis* von Willebrand Factor Like gene 1) and *Ci-VWFL2* (*Ciona intestinalis* von Willebrand Factor Like gene 2), respectively. *Ci-VWFL1* was expressed in the medial region of the young adult endostyle (Fig. 2A, B). In adults, expression of *Ci-VWFL1* was restricted to the entire region of zone 4, which is a medial-glandular zone of the endostyle (Fig. 2C). *Ci-VWFL2* was expressed in the ventral region of the young adult endostyle (Fig. 2F, G). In adults, *Ci-VWFL2* was expressed in the entire region of zone 2, which is the ventral-glandular zone of the endostyle (Fig. 2H). Therefore, *Ci-VWFL1* and *Ci-VWFL2* are markers specific for medial- and ventral-glandular zones of the endostyle, respectively.

Comparison between Ci-VWFL1 and Ci-VWFL2 and reported proteins in the public protein database revealed that both Ci-VWFL1 and Ci-VWFL2 have similar amino acid sequences to the C-terminal half of vertebrate VWF (Fig. 2K), which is a glycoprotein involved in the blood coagulation system (reviewed in Denis, 2002). Vertebrate VWF has several A domains, C domains and D domains, and also has a cystine-knot domain in the C-terminus. Both Ci-VWFL1 and Ci-VWFL2 have several C domains, an A domain and a cystine-knot domain with similar compositions to the corresponding vertebrate domains. Therefore, these genes encode proteins related to each other. This is a first instance of glandular-zone-specific genes with similarity to other reported proteins, and might facilitate the functional and molecular phylogenetic analysis of the endostyle. Aros and Virágh (1969) pointed out that zones 2 and 4 have identical cytoplasmic structures and secretion granules. The existence of transcripts that probably produce similar proteins in

zones 2 and 4 supports their suggestion. Although the domain compositions of *Ciona* VWF-like proteins and vertebrate VWF were basically conserved, *Ciona* VWF-like proteins have no A domain repeat or D domain repeat, but have an additional C domain in their N-termini. We could not find any other VWF-like genes or VWF orthologs (data not shown) in the *Ciona* genome (Dehal *et al.*, 2002). In reported vertebrate genomes, we found VWF orthologs in human, mouse, rat and fugu. However, we could find no vertebrate VWF-like gene which had the same domain structure as *Ci-VWFL1* and *Ci-VWFL2*. Although it is still unclear whether vertebrate VWF and *Ciona* VWF-like genes are homologous or not, further analyses in other basal chordates might provide insights into the evolution of VWF-like proteins and the endostyle. The ascidian genome contains a basic set of genes with less redundancy than the vertebrate genome, but some *Ciona* genes have been duplicated or further multiplied in the ascidian lineage (Dehal *et al.*, 2002). Analyses of these related genes, including *Ci-VWFL1* and *Ci-VWFL2,* in other ascidians closely related to *Ciona* might help us to understand the evolution of the ascidian genome.

Endostyle-specific genes which have different expression patterns between young adults and adults

In situ hybridization of the *Ciona* endostyle revealed that genes ID06909, ID02629 and ID02772 have different expression patterns between young adults (2–3 weeks after metamorphosis) and adults (2–3 months after metamorphosis). A gene ID06909, renamed *Ci-Ends8,* was expressed in the entire region of zone 6, which is a dorsal protein-secreting element in the young adult endostyle (Fig. 3A, B). In adults, *Ci-Ends8* was also expressed only in the endostyle (Fig. 3D, E), but its expression domain was changed and

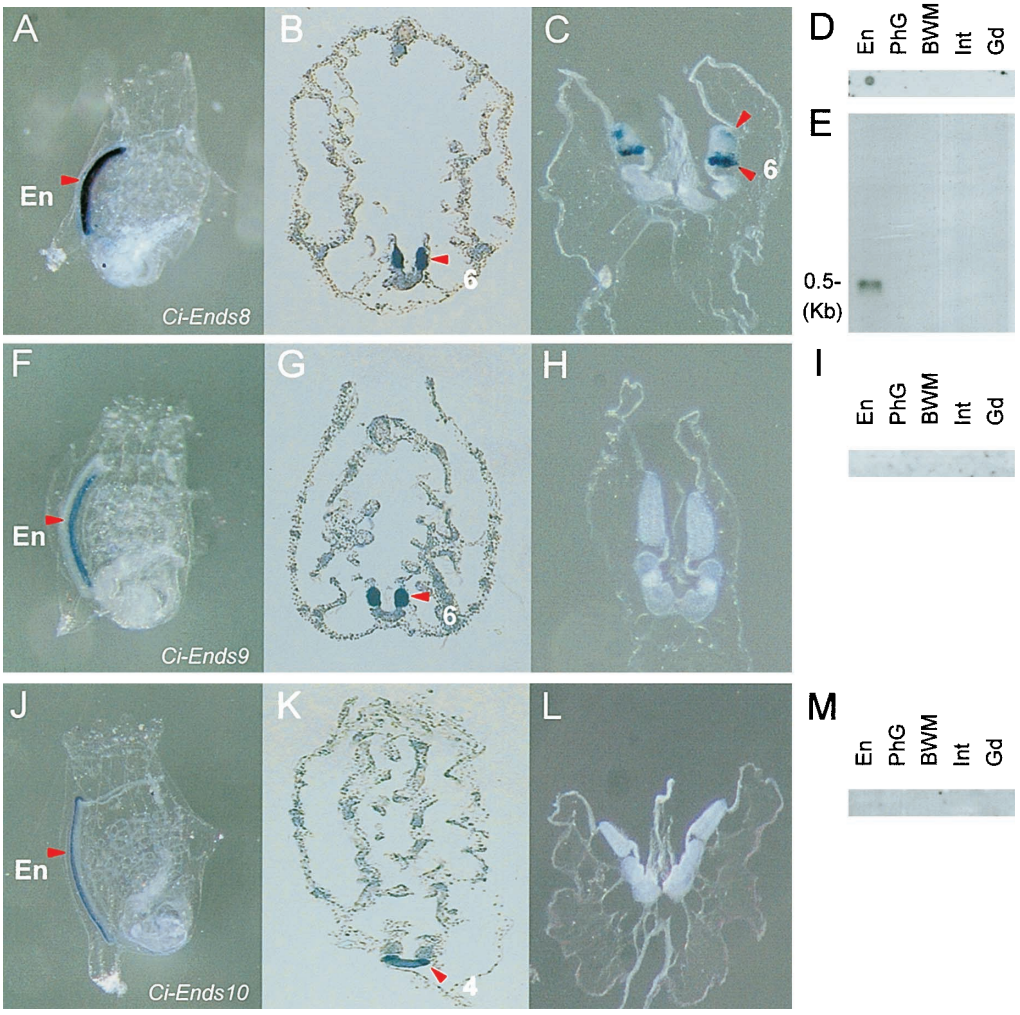

Fig. 3. Expression of *Ci-Ends8*, *Ci-Ends9* and *Ci-Ends10*. (A–E) Expression of *Ci-Ends8* detected by *in situ* hybridization (A–C), dot blotting (D) and Northern blotting (E). (F–I) Expression of *Ci-Ends9* assessed by *in situ* hybridization (F–H) and dot blotting (I). (J–M) Expression of *Ci-Ends10* assessed by *in situ* hybridization (J–L) and dot blotting (M). Red arrowheads indicate expression signals in young adults (A, F, J), transverse sections of young adults (B, G, K) and transverse sections of the adult endostyle (C, H, L). The DDBJ/EMBL/GenBank accession numbers of *Ci-Ends8*, *Ci-Ends9* and *Ci-Ends10* are AB112443, AK113034 and AB112444 respectively. Abbreviations: En, endostyle; PhG, pharyngeal-gill; BWM, body-wall muscle; Int, intestine and Gd, gonad.

restricted to the dorsal and ventral parts of zone 6 (Fig. 3C). This observation suggests that the cells in zone 6 of the adult endostyle are not uniform. The predicted amino acid sequence of Ci-Ends8 has no similarity to other reported proteins.

Genes ID02629 and ID02772, which were renamed *Ci-Ends9* and *Ci-Ends10*, were expressed in zones 6 and 4 in young adults, respectively (Fig. 3F, G, J, K). However, expression of these genes was not detected in adults under the conditions of our dot blotting and *in situ* hybridization (Fig. 3H, I, L, M). The transient expression pattern of these genes suggests that the nature of the endostyle differs between young adults and adults. The predicted amino acid sequence of Ci-Ends9 has no similarity to other reported proteins. On the other hand, *Ci-Ends10* encodes a protein with sequence similar to the binding site for thyroid hormone receptor located in the Trip230 (data not shown). The relationship between these transient expression patterns and the molecular natures of these genes are not yet clear.

Ci-Ends11 has multiple expression domains which resemble those of *CiTTF-1* expression

Gene ID06825, named *Ci-Ends11*, showed endostyle-specific expression (Fig. 4A, E) and had multiple expression domains in the endostyle (Fig. 4B, C). *Ci-Ends11* was expressed in the entire region of zone 3, which is generally thought to be a supporting element, the dorsal region of zone 2, the ventral region of zone 4, and the ventral region of zone 5 (Fig. 4D). The predicted amino acid sequence of Ci-Ends11 shows similarity to the catalytic domain of Ptp4E, a receptor-linked protein-tyrosine phosphatase (data not shown). The expression pattern of *Ci-Ends11* was similar to that of *TTF-1,* which encodes a thyroid-related transcription factor, except in the dorsal region of zone 5 (Fig. 4F-I).

Isolation of organ-specific genes based on cDNA project

Recent advances in characterizing the overall expression profiles and ESTs of *Ciona* genes (e.g., Satou *et al*., 2002) provide us opportunities for isolating tissue- or organ-specific genes in a comprehensive manner. In a previous study, only three *Ciona* endostyle-specific genes could be isolated by means of differential screening (Ogasawara *et al*., 2002). In the present study, we have characterized six novel cDNA clones for *Ciona* endostyle-specific genes based on the expression profiles of about 1,000 genes. Therefore, this approach might be more effective for isolating organ-specific genes than differential screening. Analysis of the rest of the 4,000 genes expressed in young adults should yield more endostyle-specific genes, which may be used to understand the evolution and development of the endostyle in chordates. The finding of genes which are expressed in different endostyle regions between young adults and adults suggests that a set of genes change their expression profiles during a development period from young adults to adults. Therefore, the screening of cDNAs not only from young adults but also from adults will enable us to find more endostyle-related genes, which facilitates studies of evolution and development of the endostyle globally.

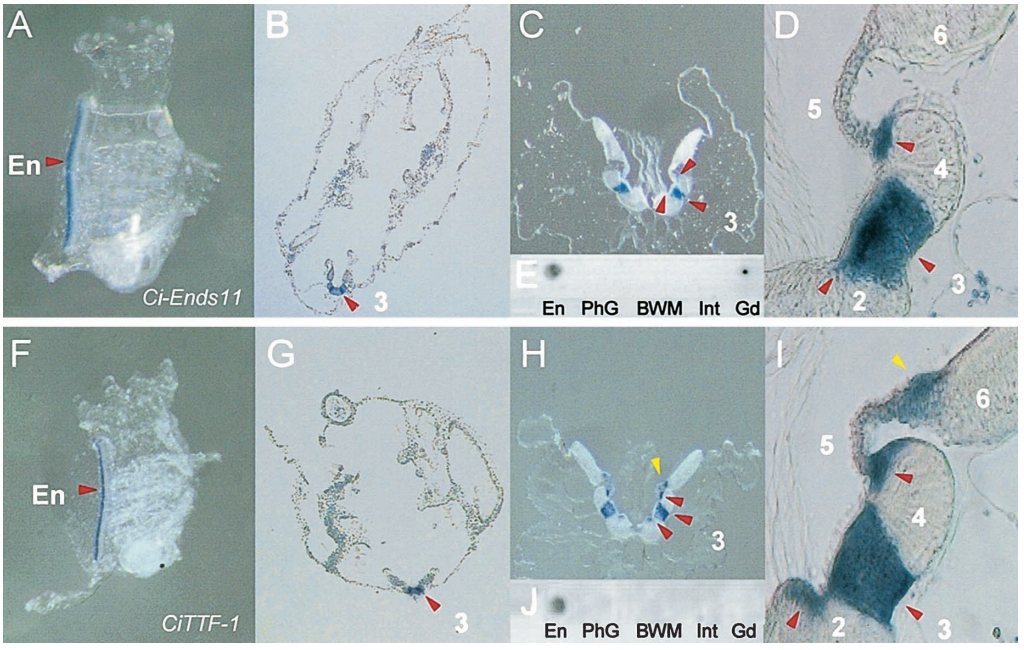

Fig. 4. Expression of *Ci-Ends11* and *CiTTF-1*. (A–E) Expression of *Ci-Ends11* assessed by *in situ* hybridization (A–D) and dot blotting (E). (F–I) Expression of *CiTTF-1* assessed by *in situ* hybridization (F–I) and dot blotting (J). Red arrowheads indicate expression signals in young adults (A, F), transverse sections of young adults (B, G), transverse sections of the adult endostyle (C, H) and at high magnifications of the adult endostyle (D, I). The expression pattern of *Ci-Ends11* was similar to that of *CiTTF-1,* except in the dorsal region of zone 5 (yellow arrowhead in H, I). The DDBJ/EMBL/GenBank accession number of *Ci-Ends11* is AB112445. Abbreviations: En, endostyle; PhG, pharyngeal-gill; BWM, body-wall muscle; Int, intestine and Gd, gonad.

ACKNOWLEDGEMENTS

We thank Kazuko Hirayama and all members in the Maizuru Fisheries Research Station, Kyoto University, Field Science Education and Research Center for their cooperation. This work was supported by a Grant-in-Aid from MEXT Japan (Grants 14034210) to M.O. We thank anonymous referees for their suggestions.

REFERENCES

Aros B, Virágh S (1969) Fine structure of the pharynx and endostyle of an ascidian (*Ciona intestinalis*). Acta Biol Acad Sci Hung 20: 281–297

Dehal P *et al*. (2002) The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science 298: 2157–2167

Denis CV (2002) Molecular and cellular biology of von Willebrand factor. Int J Hematol 75: 3–8

Dunn AD (1974) Ultrastructural autoradiography and cytochemistry of the iodine-binding cells in the ascidian endostyle. J Exp Zool 188: 103–123

Eales JG (1997) Iodine metabolism and thyroid-related functions in organisms lacking thyroid follicles: are thyroid hormones also vitamins? Proc Soc Exp Biol 214: 302–317

Ogasawara M, Tanaka KJ, Makabe KW, Satoh N (1996) Expression of endostyle-specific genes in the ascidian *Halocynthia roretzi*. Dev Genes Evol 206: 227–235

Ogasawara M, Satoh N (1998) Isolation and characterization of endostyle-specific genes in the ascidian *Ciona intestinalis*. Biol Bull 195: 60–69

Ogasawara M, Di Lauro R, Satoh N (1999a) Ascidian homologs of mammalian thyroid transcription factor-1 gene are expressed in the endostyle. Zool Sci 16: 559–565

Ogasawara M, Di Lauro R, Satoh N (1999b) Ascidian homologs of mammalian thyroid peroxidase genes are expressed in the thyroid-equivalent region of the endostyle. J Exp Zool 285: 158–169

Ogasawara M, Minokawa T, Sasakura Y, Nishida H, Makabe KW (2001) A large-scale whole-mount *in situ* hybridization system: rapid one-tube preparation of DIG-labeled RNA probes and high throughput hybridization using 96-well silent screen plates. Zool Sci 18: 187–193

Ogasawara M, Sasaki A, Metoki H, Shin-i T, Kohara Y, Satoh N, Satou Y (2002) Gene expression profiles in young adult *Ciona intestinalis*. Dev Genes Evol 212: 173–185

Ristoratore F, Spagnuolo A, Aniello F, Branno M, Fabbrini F, Di Lauro R (1999) Expression and functional analysis of *Cititf1*, an ascidian NK-2 class gene, suggest its role in endoderm development. Development 126: 5149–5159

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual. 2nd ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Satou Y, Takatori N, Fujiwara S, Nishikata T, Saiga H, Kusakabe T, Shin-i T, Kohara Y, Satoh N (2002) *Ciona intestinalis* cDNA projects: expressed sequence tag analyses and gene expression profiles during embryogenesis. Gene 287: 83–96

Simmen MW, Leitgeb S, Clark VH, Jones SJM, Bird A (1998) Gene number in an invertebrate chordate, *Ciona intestinalis*. Proc Natl Acad Sci USA 95: 4437–4440

(Received March 24, 2003 / Accepted April 28, 2003)