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Expression of pharyngeal gill-specific genes in the ascidian *Halocynthia roretzi*

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Abstract The most primitive chordates may have arisen with a shift to internal feeding through the use of the pharyngeal gill slits and endostyle for extracting suspended food from the water. Therefore, the pharyngeal gill and endostyle, in addition to notochord and nerve cord, are structures key to an understanding of the molecular developmental mechanisms underlying the origin and evolution of chordates. In this and a following study, isolation of cDNA clones for genes that are specifically expressed in the pharyngeal gill or endostyle in the ascidian *Halocynthia roretzi* was attempted. Differential screening of a pharyngeal gill cDNA library and an endostyle cDNA library with total pharyngeal-gill cDNA probes yielded cDNA clones for two pharyngeal gill-specific genes, *HrPhG1* and *HrPhG2*. Northern blot analysis showed a 3.0-kb transcript of *HrPhG1* and a 2.0-kb transcript of *HrPhG2*. Predicted amino acid sequences of the gene products suggested that both genes encode secretory proteins with no significant match to known proteins. In adults, both *HrPhG1* and *HrPhG2* genes were only expressed in the pharyngeal gill and not in other tissues including the endostyle, body-wall muscle, gonad, gut and digestive gland. *HrPhG1* and *HrPhG2* transcripts were undetectable in embryos and larvae, and were first detected in juveniles 3 days after initiation of metamorphosis. In situ hybridization revealed that the expression of *HrPhG1* and *HrPhG2* was restricted to differentiating pharyngeal-wall epithelium, with intense signals in the area surrounding the stigma or gill slit. These genes may serve as probes for further analyses of molecular mechanisms underlying the occurrence of pharyngeal gill and formation of gill slits during chordate evolution.

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Introduction

We are interested in molecular developmental mechanisms that permitted and/or accelerated the occurrence of chordates. Many extant invertebrate groups and several hypothetical forms have been considered as potential chordate ancestors, and evolutionary pathways leading to an emergence of chordates have been a subject of extensive investigation and vigorous discussion for more than a century (e.g. Haeckel 1868; Garstang 1928; Berrill 1955; Jollie 1973; Løvtrup 1977; Jefferies 1986; Brusca and Brusca 1990; Willmer 1990). This classical and fundamental research subject of evolutionary biology has recently attracted the renewed attention of molecular developmental biologists (e.g. Holland et al. 1994; Satoh and Jeffery 1995).

The phylum Chordata consists of the subphyla Urochordata (tunicates), Cephalochordata (amphioxus) and Vertebrata. They are categorized as deuterostomes, along with two other invertebrate groups, echinoderms and hemichordates (e.g. Brusca and Brusca 1990). Deuterostomes share many embryological and anatomical features, which include radial cleavage, a blastopore not fated to form a mouth and an enterocoelic coelom (e.g. Willmer 1990). Recent molecular phylogenic studies (Wada and Satoh 1994; Turbeville et al. 1994) as well as a cladistic analysis (Schaeffer 1987) support a monophyletic origin of deuterostomes. In addition to the deuterostomy, chordates share several characteristic features including a notochord, a dorsal hollow nerve cord and pharyngeal gill slits, which are hallmarks of the chordate body plan.

These characteristic features of chordates seem to have evolved with an emergence of tadpole larva-like creatures. Invertebrates develop various types of larvae. Most of the larvae form cilia on their surface, which they use for movement to places appropriate for settlement. In

contrast, primitive chordates develop tadpole larvae with a motile tail, in which notochord, muscle and dorsal nerve cord are formed. In other words, the notochord, muscle and nerve cord might have evolved along with this change in the mode of larval locomotion. The hypothesis proposed by Garstang (1928) states that chordates arose by evolution of an invertebrate larva, like the ascidian tadpole. Therefore, investigation of the organization of these structures is of salient importance in attempts to understand the origin of chordates. This subject, therefore, has already been attacked by recent molecular embryological studies (Satoh and Jeffery 1995; Yasuo et al. 1995; Satoh 1995).

Coincidently with this change in the mode of larval locomotion, the most primitive chordates or chordate ancestors may have 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 (e.g. Brusca and Brusca 1990). This possibility suggests that, in addition to the notochord, muscle and nerve cord, the pharyngeal gill and endostyle are key organs to explore molecular mechanisms involved in an emergence of chordates. As the first step to discover these mechanisms, isolation and characterization of pharyngeal gill-specific genes or endostyle-specific genes in the ascidian *Halocynthia roretzi* have been attempted in this and a following investigation (Ogasawara et al. 1996).

Materials and methods

Biological materials

Halocynthia roretzi was purchased during the spawning season from fishermen near the Otsuchi Marine Research Centre, Ocean Research Institute, University of Tokyo, Iwate, Japan. After dissection of adults, tissues and organs were removed and quickly frozen in liquid nitrogen. The specimens were kept at –80°C until use.

H. roretzi is hermaphroditic and self-sterile. Naturally spawned eggs were fertilized with a suspension of non-self sperm, and raised in filtered seawater at about 12°C. Embryogenesis proceeded synchronously in various batches of eggs, becoming gastrulae about 12 h after fertilization and tailbud embryos at about 24 h. Tadpole larvae hatched at about 40 h of development. They were allowed to accomplish metamorphosis naturally. Juveniles that adhered to plastic dishes were cultured for more than 2 months in aquaria with circulating natural seawater. Samples at appropriate developmental stages were frozen in liquid nitrogen for Northern blot analysis, or fixed for in situ hybridization.

Isolation of RNAs and construction of cDNA libraries

Total RNA was extracted from frozen samples of pharyngeal gill or endostyle by the acid guanidinium-thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi 1987). Poly(A)$^+$ RNAs were purified by use of Oligotex dT30 beads (Roche Japan, Tokyo). Complementary DNA was synthesized from the poly(A)$^+$ RNA with a ZAP cDNA kit (Stratagene, La Jolla, Calif.). Double-stranded cDNA was size-fractionated on a column of sepharose CL-4B (Sigma, St Louis, Mo.), and fractions that contained fragments of more than 300 bp in length were collected. Double-stranded cDNA prepared from pharyngeal gill mRNA or from endostyle mRNA was cloned directly into the *Eco*RI-*Xho*I site of a Uni-ZAP-II vector (Stratagene).

Isolation of cDNA clones for pharyngeal gill-specific genes and sequencing

The two cDNA libraries were screened differentially. Duplicate filters of the libraries were hybridized with [^{32}P]-labelled total cDNA probes prepared from 5 μg of poly(A)$^+$ RNA of pharyngeal gill under high stringency conditions [hybridization: 5 × SSPE (0.9 M NaCl, 50 mM Sodium phosphate pH 7.7 and 10 mM EDTA), 0.1% SDS (sodium dodecyl sulphate), 1 × Denhardt's solution, 0.1 mg/ml salmon sperm DNA, 50% formamide at 42°C; washing: 2 × SSC (sodium chloride/sodium citrate), 0.1% SDS at 65°C]. Plaques which showed positive hybridization in the pharyngeal gill library but negative in the endostyle library were selected and isolated by two rounds of screening. Specificity of the positive clones to the pharyngeal gill was confirmed by Northern blot analysis. These 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 with ABI PRISM labelling kits (Perkin Elmer).

Northern blot analysis

After extraction of total RNA by the AGPC method (Chomczynski and Sacchi 1987), poly(A)$^+$ RNAs were purified by use of Oligotex dT30 beads. Northern blot hybridization was carried out by the standard procedure (Sambrook et al. 1989) and filters were washed under high stringency conditions. DNA probes for blot hybridizations were labelled with [^{32}P]-dCTP using a random primed labelling kit (Boehringer Mannheim, Heidelberg).

Genomic Southern analysis

High-molecular weight genomic DNA was extracted from a single adult by the standard procedure (Sambrook et al. 1989). After exhaustive digestion with *Bam*HI, *Eco*RI, *Hin*dIII and *Pst*I, and 0.7% agarose gel electrophoresis, the DNA fragments were blotted onto Hybond-N+ nylon membrane (Amersham). The blots were hybridized with random-primed [^{32}P]-labelled DNA probes at 42°C for 16 h and washed under high-stringency conditions.

In situ hybridization

Juveniles were fixed as whole-mount specimens, in 4% paraformaldehyde in 0.5 M NaCl and, 0.1 M MOPS (3-[N-morpholino] propansesulfonic acid) buffer at (pH 7.5) 4°C for 12 h. For adults, the tunic was stripped off with tungsten needles prior to fixation as above. After dehydration with a graded series of alcohol, specimens were embedded in polyester wax (BDH Chemicals) and sectioned at 6 μm. In situ hybridization of whole-mount and sectioned specimens was carried out basically as described previously (Yasuo and Satoh 1994), except for the antibody reaction (Wada et al. 1995). That is, after blocking in 0.1 M TRIS-HCl (pH 7.5), 0.15 M NaCl supplemented with 0.5% blocking reagent (blocking buffer) at room temperature for 30 min, the specimens were incubated with 1: 2000 alkaline phosphatase-conjugated anti-DIG (Digoxigenin) antibody (Boehringer Mannheim) in the blocking buffer for 1 h. The specimens were washed with PBT (phosphate-buffer saline containing 0.1% Tween 20) four times (10 min each) and alkaline phosphatase buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM TRIS-HCl, pH 8.0) three times (10 min each). Signal detection was carried out in the alkaline phosphatase buffer with 4.5 μl NBT (Nitroblue tetrazolium chloride)/ml and 3.5 μl BCIP (5-Bromo-4-chloro-3-indolylphosphate)/ml at room temperature. Probes were synthesized by following the instructions from the supplier of kit (DIG RNA labelling kit; Boehringer Mannheim).

Results

Differential screenings of an *H. roretzi* pharyngeal-gill cDNA library and an endostyle cDNA library with total cDNA probes of the pharyngeal gill yielded ten cDNA clones specific to or enriched in the pharyngeal gill library. A preliminary Northern blot analysis showed that nine of them were specific to the pharyngeal gill, and from those, we selected five clones exhibiting distinct hybridization signals. Examination of cross-reactivity of the five clones revealed three independent clones. The corresponding genes were named *HrPhG1* (*Halocynthia roretzi* pharyngeal gill gene 1), *HrPhG2* and *HrPhG3*, respectively. We report here characterization and expression of the *HrPhG1* and *HrPhG2* genes.

Characterization of cDNA clone for the *HrPhG1* gene

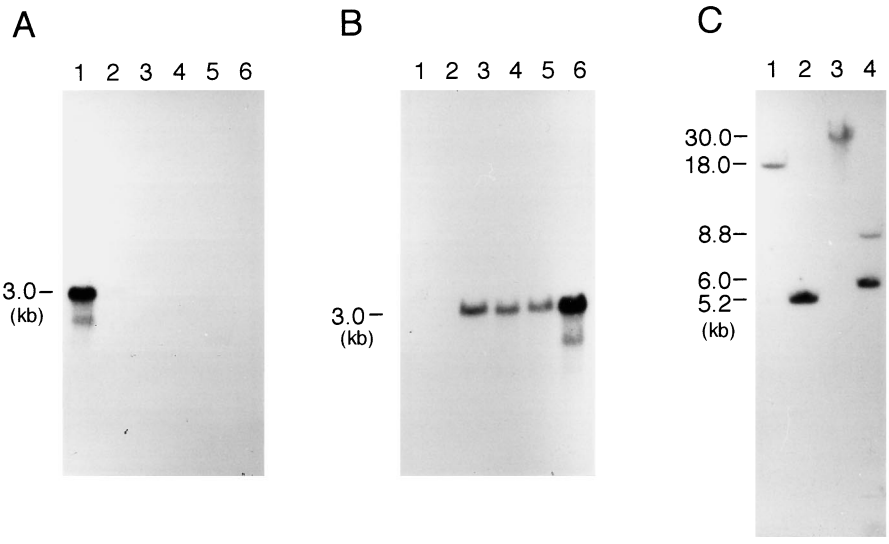
As shown in Fig. 1A, Northern blot analysis of the *HrPhG1* transcript in various tissues and organs of an *H. roretzi* adult showed the occurrence of the transcript only in the pharyngeal gill. The blot revealed a major band of about 3.0 kb and a minor band. At present, it is uncertain whether the minor band represents a true transcript or is an artifact of the experimental procedures. The *HrPhG1* transcript was not detected in other organs including the endostyle, body-wall muscle, gonad, digestive gland and intestine (Fig. 1A), suggesting that the expression of *HrPhG1* is specific to the pharyngeal gill.

The nucleotide and deduced amino acid sequences of a cDNA clone for *HrPhG1* are shown in Fig. 2A. The insert encompassed 2,901 bp including 18 adenyly residues at the 3' end. The similarity in size to the 3.0 kb transcript seen in our Northern blot suggests that the clone is close to full length. The ATG position 64–66 represented a putative start codon of the HrPHG1 protein. A single open reading frame (ORF) extended from there to nucleotide 2,286, encoding a protein that consisted of 741 amino acids with a calculated molecular mass (Mr) of 84.8 kDa.

Examination of consensus or characteristic sequence motifs in HrPHG1 revealed a proline-rich region in the centre of the protein (Fig. 2A), and many *N*-linked glycosylation sites. Beside this region, the HrPHG1 amino acid sequence did not show any significant matches with motifs of transcriptional factors, transmembrane domains, nuclear localization signals or growth factor proteins.

As shown in Fig. 2B, however, mean hydropathy profiles of HrPHG1 suggest a characteristic feature of the polypeptide. The *N*-terminus was highly hydrophobic, with a typical signal peptide sequence that consisted of a positive charged residue (amino acid position 3; R, Arg), a hydrophobic (4–13) region of 10–15 residues, a

Fig. 1 **A** Distribution of *HrPhG1* (*Halocynthia roretzi* pharyngeal gill gene 1) transcript in tissues and organs of *Halocynthia roretzi* adults. Northern blots of poly(A)$^+$ RNA prepared from the pharyngeal gill (*lane 1*), endostyle (*lane 2*), body-wall muscle (*lane 3*), gonad (*lane 4*), digestive gland (*lane 5*), and intestine (*lane 6*) were hybridized with random-primed [^{32}P]-labelled DNA probes and the membrane was washed under high-stringency conditions. The *HrPhG1* transcript of about 3.0 kb in length was found only in the pharyngeal gill. Each lane was loaded with 10 μg of poly(A)$^+$ RNA. **B** Occurrence of *HrPhG1* transcript during development of *H. roretzi*. Northern blots of poly(A)$^+$ RNA prepared from gastrulae (*lane 1*), swimming larvae (*lane 2*), juveniles at 3 (*lane 3*), 5 (*lane 4*), and 7 days (*lane 5*) after initiation of metamorphosis, and adult pharyngeal gill (*lane 6*) were hybridized with random-primed [^{32}P]-labelled DNA probes and the membrane was washed under high-stringency conditions. The *HrPhG1* transcript was not detected in embryos and larvae, but the transcript became evident in juveniles at 3 days after initiation of metamorphosis. Each lane was loaded with 10 μg of poly(A)$^+$ RNA. **C** Genomic Southern blot analysis of the *HrPhG1* gene. Genomic DNA was isolated from a single adult and aliquots were digested separately with *Bam*HI (*lane 1*), *Eco*RI (*lane 2*), *Hin*dIII (*lane 3*) and *Pst*I (*lane 4*). The blots were hybridized with random-primed [^{32}P]-labelled DNA probes and the filter was washed under high-stringency conditions. Ten micrograms of digested genomic DNA were loaded per lane. The *numbers* indicate sizes (in kb) of the signals



A

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   1    M  I  R  F  F  V  L  A  L  T  I  W  I  S  S  A  A  G  Q    19
 121 ACCGAATTGCCAACAAAAGAACCTGCTACCACCCGTTTTCCAATTACAGTCTCTCCCCAC  180
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  40  V  T  S  S  A  G  N  F  S  T  T  W  N  Y  T  T  F  P  P  R   59
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 301 ATCACCTATGTGCAGTGTCCAGTGGGTGTACGTGGATTAACTACGACATGGGTGATATCT  360
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 361 TTCCCGACAACGCTGGGGTACAACGCGGTCGCAACTCTACAAATTCTCCTCTGCTCTCCG  420
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 140  D  V  I  R  C  S  A  D  C  C  S  Y  D  Y  C  R  S  Y  H  S  159
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 160  T  Y  S  S  P  Q  Q  F  N  I  P  E  K  D  L  L  D  V  K  V  179
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1081 GTTGTCTACAACTTTTTGAATATCTCGACTGATACTTGCAAGCTGAACGATACCATCAAC 1140
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 420  T  I  L  E  T  W  T  C  E  A  I  F  T  K  S  T  P  Y  E  T  439
1381 CCAGATGCATGCAAGACAAATGTGAACCGGCTCATGAATTGCGTCGCAAATCGTTTCGAA 1440
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1681 AAGGAGTTAATCAATCGTACAAGACATCTGTACTACGAATATGTCGGAGCTAGAAACTCC 1740
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B

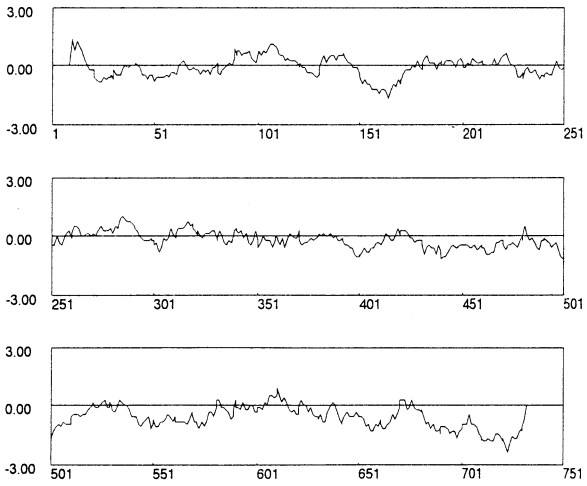


Fig. 2 **A** The nucleotide and predicted amino acid sequences of a cDNA clone for *HrPhG1*, a pharyngeal gill-specific gene of *H. roretzi*. The insert encompasses 2,901 bp including 18 adenyly residues at the 3' end. The ATG at position 64–66 represents the putative start codon of the *HrPhG1*-encoded protein, which consists of 741 amino acids. An *asterisk* indicates the termination codon. The potential signal sequence for polyadenylation is *underlined*. An *arrowhead* indicates a predicted cleavage site. A proline-rich region is *enclosed*, and putative *N*-linked glycosylation sites are shown by *broken lines*. **B** Mean hydropathy profiles of the *HrPhG1* gene product (HrPHG1). The mean hydropathy index was calculated across a window of 19 residues according to the method of Kyte and Doolittle (1982). The *N*-terminus of the protein is characterized by a 17-amino-acid-long hydrophobic region that contains a predicted signal peptide sequence. This suggests that HrPHG1 is a secreted protein

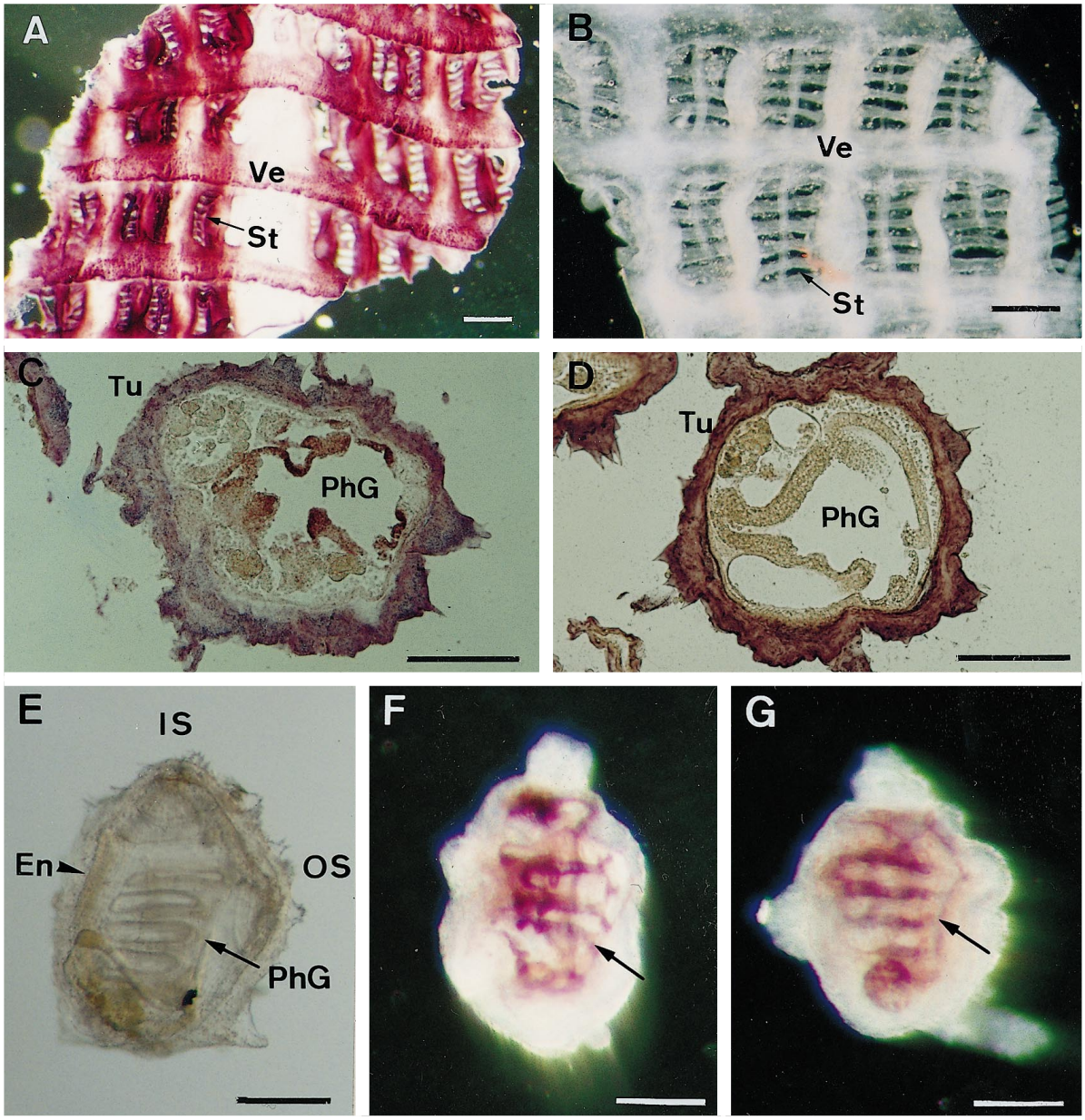


Fig. 3A–G Localization of *HrPhG1* and *HrPhG2* transcripts, as revealed by in situ hybridization. **A, B** Pharyngeal gills dissected from adults were hybridized with *HrPhG1* antisense (**A**) and sense (**B**) probes. **A** Distinct signals are evident in the entire organ, particularly, in cells of pharyngeal wall that surround the stigma (*St*) or gill slits. Vessels (*Ve*) do not show signals. **B** A control hybridized with sense probe showing no signals above background. *Scale bar* = 100 μm. **C, D** The *HrPhG1* gene expression in 7-day-old juveniles. **C** A cross-section hybridized with antisense probe showing signals in differentiating pharyngeal gill (*PhG*). Tunic (*Tu*) showed high background staining. *Scale bar* = 100 μm. **D** A cross-section hybridized with sense probe showing no signals in the internal organs. **E** A living 2-month-old young adult showing development of the pharyngeal gill (*PhG*) with four stigmata (*En* endostyle, *IS* incurrent siphon, *OS* outcurrent siphon, *scale bar* = 50 μm). **F, G** *HrPhG1* (**F**) and *HrPhG2* (**G**) gene expression in 2-month-old young adults. Whole-mount specimens hybridized with antisense probes showing signals in the pharyngeal gill (*arrows*). *Scale bar* = 50 μm

charged residue (positions 14 and 15; S, Ser) and a residue containing a short side chain (position 18; G, Gly). A predicted cleavage site of the signal peptide was evident behind the Gly (position 18). This sequence motif strongly suggests that HrPHG1 is a secreted protein, with a probability of 43% as determined by PSORT Program (Netscape: PSORT WWW Server, http://psort.nibb.ac.jp).

We determined the number of different sequences that correspond to *HrPhG1* in the ascidian genome by genomic Southern hybridization. As shown in Fig. 1C, only one band was detected in the lanes of *Bam*HI (about 18 kb), *Eco*RI (about 5.2 kb) and *Hin*dIII (about 30 kb), whilst in the lane of *Pst*I a major (about 6.0 kb) and a minor band (about 8.8 kb) were detected. This result suggests that *HrPhG1* is present as a single copy per haploid genome of *H. roretzi*.

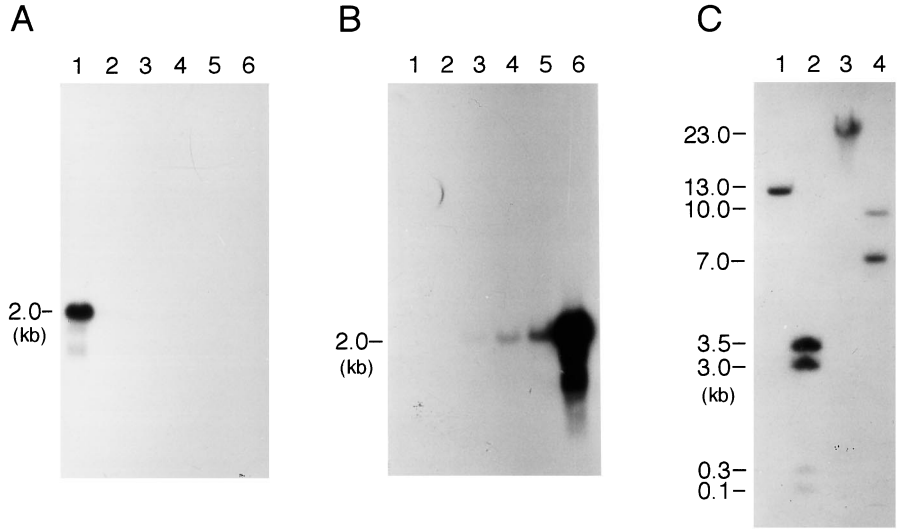


Fig. 4 **A** Distribution of *HrPhG2* transcript in tissues and organs of *H. roretzi* adults. Northern blots of poly(A)$^+$ RNA prepared from the pharyngeal gill (*lane 1*), endostyle (*lane 2*), body-wall muscle (*lane 3*), gonad (*lane 4*), digestive gland (*lane 5*), and intestine (*lane 6*) were hybridized with random-primed [^{32}P]-labelled DNA probes and the membrane was washed under high-stringency conditions. The *HrPhG2* transcript of about 2.0 kb in length was detected only in the pharyngeal gill. Each lane was loaded with 10 μg of poly(A)$^+$ RNA. **B** Occurrence of *HrPhG2* transcript during development of *H. roretzi*. Northern blots of poly(A)$^+$ RNA prepared from gastrulae (*lane 1*), swimming larvae (*lane 2*), juveniles at 3 (*lane 3*), 5 (*lane 4*), and 7 days (*lane 5*) after initiation of metamorphosis and adult pharyngeal gill (*lane 6*) were hybridized with random-primed [^{32}P]-labelled DNA probes and the membrane was washed under high-stringency conditions. The *HrPhG2* transcript was not detected in embryos and larvae, but the transcript became detectable in juveniles at 3 days after initiation of metamorphosis, although intensity of bands of 3-, 5- and 7-days-old juveniles was weaker than that of adult pharyngeal gill. Each lane was loaded with 10 μg of poly(A)$^+$ RNA. **C** Genomic Southern blot analysis of the *HrPhG2* gene. Genomic DNA was isolated from a single adult and aliquots were digested separately with *Bam*HI (*lane 1*), *Eco*RI (*lane 2*), *Hin*dIII (*lane 3*) and *Pst*I (*lane 4*). The blots were hybridized with random-primed [^{32}P]-labelled DNA probes and the filter was washed under high-stringency conditions. Ten micrograms of digested genomic DNA were loaded per lane. The *numbers* indicate sizes (in kb) of the signals

Expression of the *HrPhG1* gene

Temporal expression of *HrPhG1* was examined by Northern blot analysis. As shown in Fig. 1B, hybridization signals were not detected in embryos and larvae. A distinct band that corresponded to a transcript with an approximate length of 3.0 kb was first detected in 3-day-old juveniles. The intensity of the band was nearly the same in 3-, 5- and 7-day-old juveniles. Because the transcript was not detected in the gonad (Fig. 1A), the expression of *HrPhG1* must be zygotic and it initiates after metamorphosis.

In situ hybridization demonstrated that the expression of *HrPhG1* is restricted to differentiating pharyngeal gill. As shown in Fig. 3A,B, hybridization signals were only distributed in epithelial cells of the pharyngeal gill. Signals were intense near the stigma or gill slits (Fig. 3A).

By in situ hybridization, the occurrence of *HrPhG1* transcript was evident in 7-day-old juveniles. As shown in Fig. 3C, hybridization signals were restricted to differentiating pharyngeal wall, although the juvenile tunic showed intense non-specific signals (Fig. 3D). Figure 3E shows a living 2-month-old young adult, in which formation of four rows of stigma is evident. A whole-mount specimen hybridized with *HrPhG1* antisense probe clearly showed signals in the pharyngeal-wall epithelium (Fig. 3F).

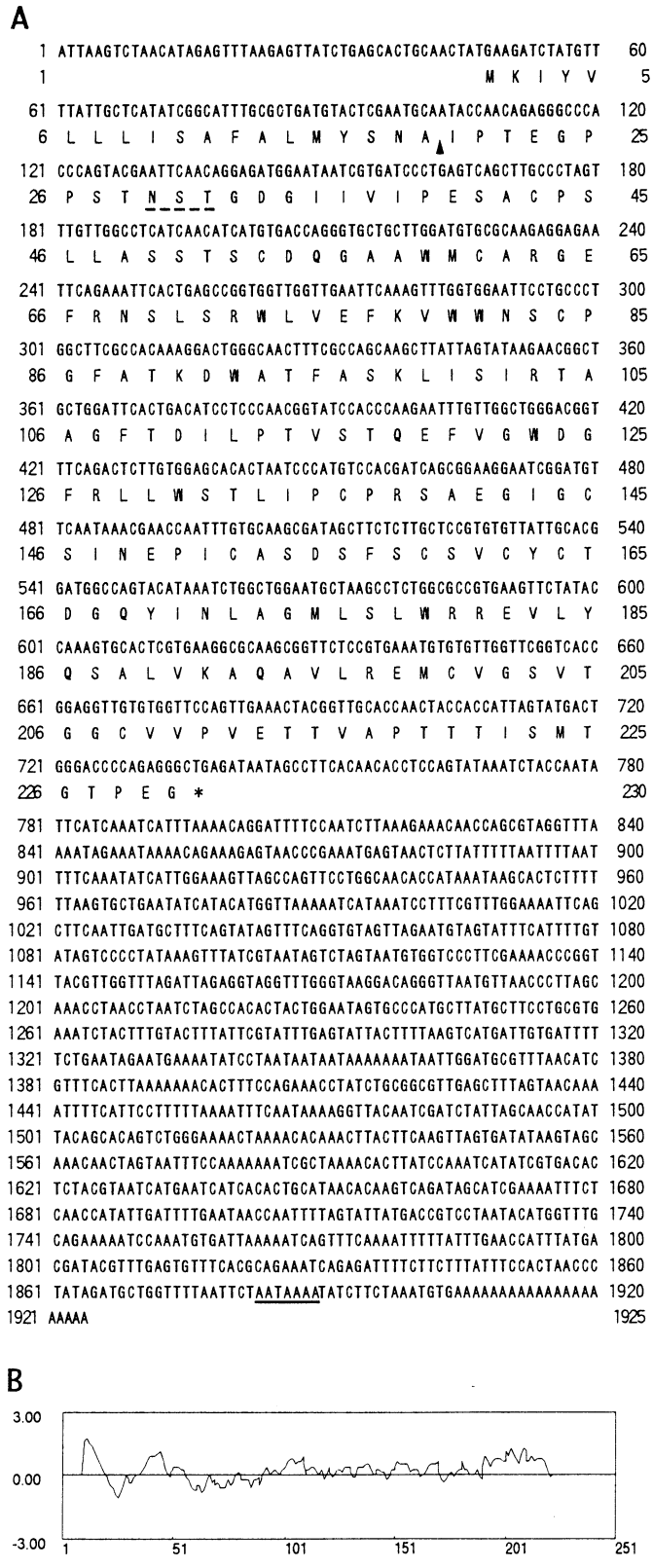
Characterization of cDNA clone for the *HrPhG2* gene

As shown in Fig. 4A, Northern blot analysis revealed a 2.0-kb transcript of *HrPhG2* only in the pharyngeal gill and not in other organs including the endostyle, body-wall muscle, gonad, digestive gland and intestine, suggesting that the *HrPhG2* gene is also specific to the pharyngeal gill.

As shown in Fig. 5A, the nucleotide and deduced amino acid sequences of a cDNA clone for *HrPhG2* indicated that the insert consisted of 1,925 bp including 21 adenyly residues at the 3’ end. The clone was therefore close to full length. The clone contained a single ORF of 690 nucleotides that encoded a polypeptide (HrPHG2) of 230 amino acids, with a calculated molecular mass (Mr) of 24.7 kDa.

Although HrPHG2 contained an *N*-linked glycosylation site, it did not show any sequence similarity to reported proteins. However, its mean hydropathy profiles suggest that HrPHG2 is a secreted protein (Fig. 5B). The *N*-terminus was highly hydrophobic. This region had a typical signal peptide sequence that consisted of a positive charged residue (amino acid position 2; K, Lys), a hydrophobic (3–15) region of 10–15 residues, a charged residue (positions 16, 17 and 18; Y, Tyr; S, Ser; and N, Asn) and a residue containing the short side chain (position 19; A, Ala). A predicted cleavage site of the signal peptide was evident behind the Ala (position 19). This

sequence motif strongly suggests that HrPHG2 is a secreted protein, with a probability of 56% as determined by PSORT Program (Netscape: PSORT WWW Server, http://psort.nibb.ac.jp).



The number of different sequences that corresponded to *HrPhG2* in the *H. roretzi* genome was examined by genomic Southern hybridization. As shown in Fig. 4C, only one band was detected with *Bam*HI (about 13 kb) and *Hin*dIII (about 23 kb) digestion. With *Eco*RI, two distinct bands (about 3.5 and 3.0 kb) were evident, whilst with *Pst*I, a major (about 7 kb) and a minor band (about 10 kb) were seen. This result suggests that *HrPhG*2 is also present as a single copy, or two at most, per haploid *H. roretzi* genome.

Expression of the *HrPhG2* gene

Northern blot analysis showed that *HrPhG2* was expressed after metamorphosis. As shown in Fig. 4B, a weak band that corresponded to a transcript with an approximate length of 2.0 kb was first detected in 3-day-old juveniles. The intensity of the band of 7-day-old juveniles was stronger than that of 3-day-old juveniles. Since the transcript was not detected in the gonad (Fig. 4A), *HrPhG2* expression must be zygotic and initiates after metamorphosis.

Spatial distribution of *HrPhG2* transcripts resembled that of *HrPhG1* transcripts. In situ hybridization of whole-mount as well as sectioned specimens showed that, in adults, the *HrPhG2* transcript was restricted to epithelial cells of pharyngeal wall (data not shown). In 7-day-old juveniles signals were found in the differentiating pharyngeal wall (data not shown). Figure 3G shows a whole-mount specimen of a 2-month-old young adult, with hybridization signals evident only in differentiating pharyngeal wall.

Discussion

The chordate body-plan is characterized by several common features, including a notochord, a dorsal nerve cord and pharyngeal gill slits. An approach to the molecular mechanisms that permitted the emergence of chordates is to isolate developmental genes that are responsible for the formation of these shared organs. As to the notochord, the *Brachyury* gene is implicated in its formation (reviewed by Herrmann and Kispert 1994; Yasuo et al.

Fig. 5 **A** The nucleotide and predicted amino acid sequences of a cDNA clone for *HrPhG2*, a pharyngeal gill-specific gene of *H. roretzi*. The insert encompasses 1,925 bp including 21 adenyly residues at the 3' end. The ATG at position 46–48 represents the putative start codon of the *HrPhG2*-encoded protein, which consists of 230 amino acids. An *asterisk* indicates the termination codon and the potential signal sequence for polyadenylation is *underlined*. An *arrowhead* indicates a predicted cleavage site and a putative *N*-linked glycosylation site is shown by a *broken line*. **B** Mean hydropathy profiles of the *HrPhG2* gene product (HrPHG2). The mean hydropathy index was calculated across a window of 19 residues according to the method of Kyte and Doolittle (1982). The *N*-terminus of the protein is characterized by a 20-amino-acid-long hydrophobic region that contains predicted signal peptide sequence, suggesting that HrPHG2 is a secretory protein

1995). The gene has been characterized in mouse (Herrmann et al. 1990), chick (*Ch-T*; Kispert et al. 1995), *Xenopus* (*Xbra*; Smith et al. 1991), zebrafish (*Zf-T* or *no tail*; Schulte-Merker et al. 1992, 1994), amphioxus (Holland et al. 1995; Terazawa and Satoh 1995), ascidian (Yasuo and Satoh 1993, 1994) and sea urchin (Harada et al. 1995). In addition, recent studies have aimed to explore molecular mechanisms involved in nerve cord formation in relation to development of the central nervous system. In ascidians, a cDNA clone for a neural-specific gene was isolated (Okamura et al. 1994), and a LIM-class homeobox gene is expressed in the ascidian nervous system (Wada et al. 1995). In addition, amphioxus contains a Hox gene cluster with the sequential order of homeobox genes closely resembling that of vertebrates (Garcia-Fernàndez and Holland 1994).

The molecular mechanism responsible for the formation of gill slits or stigma is unknown. As the first step to answer this question, we isolated cDNA clones for genes that are specifically expressed in the pharyngeal gill. Our two pharyngeal gill-specific genes, *HrPhG1* and *HrPhG2*, are not expressed in embryos and larvae, but begin to be expressed about 3 days after initiation of metamorphosis. The amount of *HrPhG1* transcripts seems constant during later development. HrPHG1 has many *N*-linked glycosylation sites and seems to be a secreted protein. On the other hand, the amount of *HrPhG2* transcripts increased as development proceeded. HrPHG2 may also be a secreted protein. Therefore, both genes are expressed when the pharyngeal gill or branchial sac is formed.

According to a description of Willey (1893), the pharyngeal gill or branchial sac of *Ciona intestinalis* is formed as follows. The dorsal ectoderm of the larva forms paired atrial invaginations that fuse with the rudiment of the pharynx to produce the gill slits, and each of these gives rise simultaneously to two gill slits, the first and fourth gill slits. These gill slits gradually elongate laterally as the pharynx grows and develops into the branchial sac and the atrial cavity. Finally, their two ends at the endostyle side bend toward each other. These bent portions eventually separate off, forming two new slits (2nd and 3rd) in the space between the first two. Later two more pairs (5th and 6th) are formed posterior to these. Temporal expression of these genes suggests that *HrPhG1* is expressed in the first and/or fourth gill slits that are formed one day after metamorphosis, whilst *HrPhG2* is expressed in the second and/or third gill slits that are formed later. However, as revealed by in situ hybridization of the pharyngeal gill of 2-month-old young and adults, both genes are expressed in all of the pharyngeal gill and there was no difference in their expression patterns. Therefore, the role of the *HrPhG1* and *HrPhG2* genes in the formation of the pharyngeal gill and/or stigma is presently uncertain.

A feature characteristic to primitive vertebrates is aquatic respiration. Many invertebrates have some kind of gills outside their body. On the other hand, animals leading to vertebrates have an entobranchia or pharyngeal gill. Pharyngeal gill slits are found in certain hemichordates, tunicates, cephalochordates and vertebrates, and their ontogeny has been carefully examined (e.g. Bone 1979). In these species, the pharyngeal gill has two functions: branchial respiration and collection of food particles from water. Barrington (1979) points out that the ciliary filter-feeding mechanisms of all deuterostomes form a logical evolutionary sequence, with external ciliated grooves visible in early fossil echinoderms and in hemichordates gradually giving way to ciliated gill slits and internal food collection (e.g. Willmer 1990). Therefore, the *HrPhG1* and *HrPhG2* gene probes may be useful to determine whether the pharyngeal gills of these different animal groups are homologous or convergent organs. Such studies are now underway.

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