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Verification, Characterization and Tissue-specific Expression of *UreG*, a Urease Accessory Protein Gene, from the Amphioxus *Branchiostoma belcheri*

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Abstract UreG genes have been found in bacteria, fungi and plants but have not yet identified in animals, although a putative UreG-like gene has been documented in sea urchin. In the course of a large-scale sequencing of amphioxus gut cDNA library, we have identified a cDNA with high similarity to UreG genes. Both reverse transcription-polymerase chain reaction and nested polymerase chain reaction, as well as *in situ* hybridization histochemistry, verified that the cDNA represented an amphioxus UreG gene (*AmphiUreG*) rather than a microbial contaminant of the cDNA library. This is further supported by the presence of urease activity in amphioxus gut, gill and ovary. *AmphiUreG* encodes a deduced protein of 200 amino acid residues including a highly conserved P-loop, bearing approximately 46%–49%, 44%–48%, and 29%–37% similarity to fungal, plant and bacterial UreG proteins, respectively. It shows a tissue-specific expression pattern in amphioxus, and is especially abundant in the digestive system. This is the first UreG gene identified in animal species.

Key words amphioxus; *Branchiostoma belcheri*; UreG; expression; urease

Urease (EC 3.5.1.5) is a nickel-containing enzyme catalyzing the hydrolysis of urea to form ammonia and carbon dioxide. Its activity has been found in bacteria, eukaryotic microorganisms, plants and some invertebrates [1–3]. Biochemically, bacterial ureases are best characterized and their activation requires the presence of several accessory proteins including UreD, UreE, UreF and UreG [2]. The specific role of each of these proteins has not yet been fully clarified, but the available data indicate that the activation process is quite complex. Most of the information on this process has been obtained from the analysis of bacterial *in vitro* activation systems. UreD binds to urease and appears to induce a conformational change for the next steps in the activation process [4]. UreF binds the UreD-urease complex and seems to facilitate carbamoylation of the nickel-bridging lysine residue and to prevent Ni^{2+} binding to the noncarbamoylated urease [5]. UreG can form a quaternary complex with UreDF-urease and appears to energize the enzyme activation process [6,7]. UreE is required for maximal enzyme activity, and is thought to bind the UreDFG-urease complex, acting as a nickel-binding protein involved in Ni^{2+} storage and possibly delivered to the active site of the enzyme [8–11].

In addition to its presence in bacteria, UreG has been identified in plants [12,13], and the homologs of bacterial UreD, UreE and UreF have recently been documented in *Arabidopsis* [14]. Sequence comparison of UreG proteins from both bacteria and plants shows that they all contain a highly conserved P-loop motif usually consisting of G-P-V-G-T-G-K-T and typically found in nucleotide-binding proteins [2,3].

Because crustacean and mollusk have been shown to possess urease activity [15], one would expect the presence of urease accessory proteins, including UreG, in invertebrates. Surprisingly, to date, no UreG has been identified in animal species, although a putative UreG-like gene (RefSeq accession No. xp_792077) has been found in sea urchin *Strongylocentrotus purpurtaus*. In this report, we document for the first time the identification of an amphioxus gene, *AmphiUreG*, which displays striking similarity to bacterial, fungal and plant UreG genes, and describe its expression pattern in adult amphioxus.

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Materials and Methods

Cloning and sequence analysis of cDNA

Gut cDNA library of adult amphioxus was constructed with the SMART cDNA library construction kit (Clontech, Palo Alto, USA) as described previously [16,17]. In a large-scale sequencing of amphioxus gut cDNA library with ABI PRISM 377XL DNA sequencer (Invitrogen, Carlsbad, USA), more than 5000 clones were analyzed for coding probability with the DNATools program (Rehm BH, Munster, Germany) [18]. Comparison against the GenBank protein database was carried out using the BLAST network server (http://www.ncbi.nih.gov/BLAST) [19]. Multiple protein sequences were aligned using the MegAlign program by the CLUSTAL method in the DNASTAR software package (DNASTAR, Madison, USA) [20]. A phylogenetic tree was constructed by the neighbor-joining method within the PHYLIP 3.5c software package, supplied by Prof. J. FELSENSTEIN (Department of Genomic Sciences, University of Washington, Seattle, USA), using 1000 bootstrap replicates.

Reverse transcription-polymerase chain reaction

Adult amphioxus *Branchiostoma belcheri* collected during their breeding season were starved for 2 d in sterilized filtered seawater before experiment to remove all food in the gut, and tissues from ovary and digestive tract were dissected. Total RNAs were prepared with Trizol (Sigma-Aldrich, St. Louis, USA) from whole organism, ovary and digestive tract. For RT-PCR, the first strand cDNA was synthesized in 50 μl of avian myeloblastosis virus (AMV)/Tfl 1×reaction buffer containing 1 μg of total RNAs, 0.1 U/μl of AMV reverse transcriptase (Promega, Madison, USA), 0.2 mM dNTP mix, 1 μM each of antisense primer NF2 (5′-CACCAGTCAGGCACATC-3′) and sense primer NS2 (5′-ACACCCTCCTGTCTCCAC-3′), 0.1 U/μl Tfl DNA polymerase (Promega) and 1 mM $MgSO_4$. The reaction was carried out at 48 ºC for 45 min. After AMV reverse transcriptase inactivation and RNA/cDNA/primer denaturation at 94 ºC for 2 min, the second-strand cDNA synthesis and PCR amplification were carried out in 30 cycles using the following parameters: denaturation at 94 ºC for 30 s, annealing at 61 ºC for 1 min, and elongation at 68 ºC for 2 min. The reaction was continued for a final extension at 68 ºC for 7 min and terminated at 4 ºC. Normalization was carried out by amplification of cytoskeletal β-actin mRNA using an antisense primer (5′-GCTGGGCTGTTGAAGGTC-3′) and a sense primer (5′-CTCCGGTATGTGCAAGGC-3′), under the same conditions as described above. The RT-PCR product from ovary RNAs was purified and ligated into pGEMT-Easy Vector (Promega), transformed into *Escherichia coli* JM109 cells, and sequenced with the ABI PRISM 377XL DNA sequencer.

Nested PCR

The digestive tracts of adult amphioxus *B. belcheri* were removed, and the genomic DNAs were isolated from the gut-free amphioxus according to Ma *et al*. [21]. The nested PCR primers used were external primers NF1 (5′-ATGGCATCTACTGATCAAGT-3′) and NS1 (5′-CCTGATAATAGCTTTCATTT-3′), that match the 20 nucleotides of both 5′ and 3′ ends of *AmphiUreG*, and internal (nested) primers NF2 (5′-CCACCAGTCAGGCACATC-3′) and NS2 (5′-ACACCCTCCTGTCTCCAC-3′), that were both designed by PRIMER 5.0. The reaction for the first step of nested PCR was carried out in 25 μl of 1×PCR buffer (Mg^{2+} plus; TaKaRa, Takara, Japan) containing 1 μg of genomic DNA, 0.2 mM dNTP mix, 0.8 μM each NF1 and NS1 and 0.025 U/μl Taq DNA polymerase (TaKaRa). The reaction conditions were: 95 ºC for 5 min; denaturating at 94 ºC for 1 min, annealing at 53.2 ºC for 1 min, and elongating at 72 ºC for 1 min, 25 cycles; and final extension at 72 ºC for 7 min. Aliquots of 5 μl of reaction mixtures were sampled and run on an agarose gel to estimate the quantity of PCR products, then the reaction mixtures were diluted to 1:1000, and used as the templates for the second step of nested PCR. The second step of nested PCR was carried out in 25 μl of 1×PCR buffer (Mg^{2+} plus) with 1 μl of diluted products from the first step of nested PCR, 0.2 mM each dNTP, 0.8 μM both NF2 and NS2, and 0.025 U/μl Taq DNA polymerase.The reaction parameters were: 95 ºC for 5 min; denaturating at 94 ºC for 1 min, 30 cycles; annealing at 61 ºC for 1 min and elongating at 72 ºC for 1 min; and final extension at 72 ºC for 7 min. A single PCR amplification product of approximately 900 bp was purified and ligated into pGEMT-Easy Vector, transformed into *E. coli* JM109 cells, and sequenced with ABI PRISM 377XL DNA sequencer.

In situ hybridization histochemistry

All reagents used for *in situ* hybridization histochemistry were prepared with 0.1% diethyl pyrocarbonate in double-distilled H_2O to rid the working solutions of RNases. Digoxigenin (Dig)-labeled *AmphiUreG* riboprobes of approximately 700 bp were synthesized *in vitro* from linearized plasmid DNA following the Dig-UTP supplier's instructions (Roche, Basel, Switzerland).

Sexually mature amphioxus were cut into two or three

pieces and fixed in freshly prepared 4% paraformaldehyde in 100 mM phosphate-buffered saline (PBS; pH 7.4) at 4 ºC for 8 h. After dehydration, they were embedded in paraffin, sectioned at 6 μm, mounted on poly-L-lysine coated slides, and dried at 42 ºC for 36 h. The sections were dewaxed in xylene for two times (10 min each time), followed by immersion in 100% ethanol for two times (5 min each time). After rehydration, they were brought to double-distilled H_2O with 0.1% diethyl pyrocarbonate, digested with 6 μg/ml proteinase K in 100 mM Tris-HCl buffer (pH 8.0) containing 50 mM EDTA at 37 ºC for 30 min, post-fixed in 4% paraformaldehyde in 100 mM PBS (pH 7.4) at room temperature for 30 min, acetylated in freshly prepared 100 mM triethanolamine-HCl (pH 8.0) with 0.25% acetic anhydrite at room temperature for 10 min, and dehydrated with graded ethanol. They were then pre-hybridized in a hybridization buffer containing 50% (*V*/*V*) deionized formamide, 100 μg/ml heparin, 5×standard saline citrate, 0.1% Tween-20, 5 mM EDTA, 1×Denhardt's solution and 1 mg/ml total yeast RNA at 55 ºC for 3 h, and hybridized in the same hybridization buffer with 1 μg/ml Dig-labeled *AmphiUreG* riboprobes at 55 ºC for 12–16 h in a humidified chamber. Subsequently, the sections were subjected to RNase A (Promega) digestion buffer (20 μg/ml in 2×standard saline citrate) at 37 ºC for 30 min, washed in 100 mM Tris-HCl (pH 7.4) with 150 mM NaCl for three times (15 min each time), pre-incubated in 1% blocking reagent (Roche) in 100 mM Tris-HCl (pH 7.4) with 150 mM NaCl for 1 h at room temperature, and incubated with anti-Dig alkaline phosphatase conjugated antibody (Roche) diluted 1:1000 in 1% blocking reagent in 100 mM Tris-HCl (pH 7.4) with 150 mM NaCl for 2 h at room temperature. The sections were washed in 100 mM Tris-HCl (pH 7.4) containing 100 mM NaCl and 50 mM $MgCl_2$ for three times (5 min each time), then incubated with a coloring solution consisting of 4.5 μg/ml NBT and 3.5 μg/ml BCIP in 100 mM Tris-HCl (pH 8.0) with 100 mM NaCl and 50 mM $MgCl_2$ (Boehringer-Ingelheim, Mannheim, Germany) for 2–24 h in the dark. The color reaction was stopped in PBS for 10 min. After rinsing in distilled water, the sections were dehydrated, mounted in Canada balsam, and photographed under a BX51 Olympus microscope (Olympus, Tokyo, Japan).

Urease activity assay

Urease activity was measured according to the instructions of the test kit (Sanqiang, Sanming, China) at room temperature. Briefly, gut, gill and ovary were dissected out of *B. belcheri*, which had been cultured for 2 d in sterilized filtered seawater to remove all food in the gut, rinsed in 50 mM Tris-HCl buffer (pH 7.2) containing 50 mM NaCl, and cut into small pieces (2–3 mm^3). The pieces were placed in the reaction solution, and the enzyme activity was demonstrated by the color change in the reaction solution from yellow to pink or red.

Results and Discussion

The cDNA clone L239 (GenBank accession No. AAT39417) obtained from the gut cDNA library of amphioxus *B. belcheri* is 966 bp long. Its longest open reading frame consists of 603 bp encoding a protein of 200 amino acid residues with a predicted molecular weight of approximately 22.41 kDa and an isoelectric point of 6.6. The 5′-untranslated region is 284 bp long with a typical oligopyrimidine motif and an in-frame stop codon (TAG), and the 3′-untranslated region is 78 bp long with a polyadenylation tail (**Fig. 1**). Therefore, the clone L239 encodes a full-length sequence protein.

The initial BLASTP search revealed that the protein encoded by clone L239 shared 70% (119/169) and 62% (95/151) identity with the UreG-like protein of sea urchin *S. purpuratus* and the UreG of fungus *Cryptococcus neoformans* (Genbank accession No. AAW41177), respectively. It was thus further compared with other members of the UreG and UreG-like family. **Fig. 2** shows an alignment of the

Fig. 1 Nucleotide and deduced amino acid sequences of amphioxus UreG cDNA (GenBank accession No. AAT39417)

The translational start site is underlined. The asterisk represents the stop codon. The oligopyrimidine signal is double underlined, and the in-frame stop codon within the 5′-untranslated region is triple underlined. Sense primer NF1 (5′-ATGGCATCTACTGATCAAGT-3′) and antisense primer NS1 (5′-CCTGATAATAGCTTTCATTT-3′) were boxed. Sense primer NF2 (5′-CCACCAGTCAGGCACATC-3′) and antisense primer NS2 (5′-ACACCCTCCTGTCTCCAC-3′) are marked by arrows.

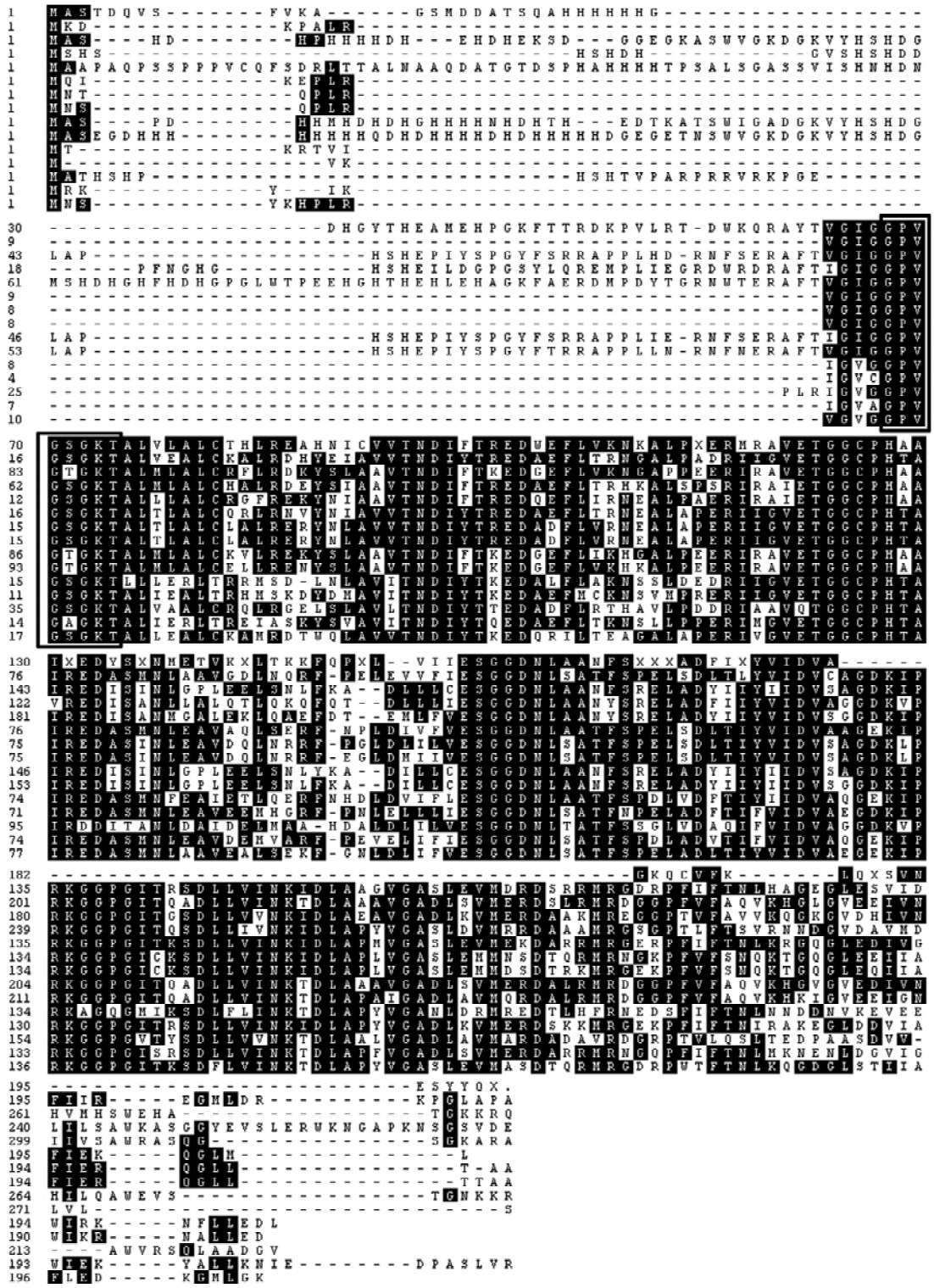

Fig. 2 Alignment of UreG and UreG-like proteins using the MegAlign program (DNASTAR) by the CLUSTAL method

Shaded (with solid black) residues are the amino acids that match the consensus. Gaps introduced into sequences to optimize alignment are represented by (–). The conserved P-loop (G-P-V-G-T-G-K-T) is boxed. UreG and UreG-like amino acid sequences were obtained from previously reported sequences: Ae (*Alkalilimnicola ehrlichei*; EAP33138), Af (*Aspergillus fumigatus*; EAL93583), Ap (*Actinobacillus pleuropneumoniae*; O54424), At (*Arabidopsis thaliana*; AAD16984), Bb (*Branchiostoma belcheri tsingtauense*; AAT39417), Cn (*Cryptococcus neoformans*; AAW41177), Gm (*Glycine max*; AAD44338), Hp (*Helicobacter pylori*; Q09066), Ka (*Klebsiella aerogenes*; P18319), Mf (*Methylobacillus flagellatus*; EAN01869), Mt (*Mycobacterium tuberculosis*; AAK46171), Pf (*Pseudomonas fluorescens*; ABA72305), Ps (*Pseudomonas syringae*; AAZ32981), Sp (*Strongylocentrotus purpuratus*; xp_792077), Ss (*Synechocystis sp.*; P72955), and St (*Solanum tuberosum*; CAC33002).

amino acid sequence of the protein with that of known UreG/UreG-like proteins from 15 species including sea urchin, fungi, plants and bacteria. The proteins encoded by clone L239 were approximately 66%, 46%–49%, 44%–48%, and 29%–37% similar to sea urchin, fungal, plant and bacterial UreG/UreG-like proteins, respectively. Moreover, they all possessed a highly conserved P-loop. Therefore, the clone L239 encodes a UreG-like protein.

To verify that the UreG-like gene is from amphioxus itself, we carried out RT-PCR and nested PCR analyses. RT-PCR, based on RNAs from digestive tract, ovary and whole organism, all formed a single amplification band of the expected size (324 bp) (**Fig. 3**), and sequencing of the RT-PCR product from ovary RNA showed that it exactly matched the expected sequence (data not shown). This suggests that the clone L239 did not represent a bacterial or fungal contaminant. Nested PCR also yielded a single amplification product of approximately 900 bp [**Fig. 4(A)**]. Sequencing of the nested PCR product produced a resulting sequence of 701 bp, which included two introns, 1 and 2, with intron 2 being complete [**Fig. 4 (B)**]. The predicted mRNA sequence of nested PCR product well agrees with that deduced from clone L239 [**Fig. 4(C)**]. It is of particular interest to note that intron 2 begins with GT and ends with an AG dinucleotide, sequences thought to be necessary for correct RNA splicing of various other eukaryotic genes [22]. The identification of the introns and consensus splice acceptor site in the nested PCR product indicates that the clone L239 represents an expressed eukaryotic gene rather than a transcribed pseudogene. In addition, *in situ* hybridization histochemistry revealed a tissue-specific expression pattern of the clone L239 in amphioxus (see below). All of these results show that the clone L239 stands for an amphioxus homolog of UreG gene, therefore designated *AmphiUreG*. This is also supported by a search of the recently completed draft assembly and automated annotation of the *Branchiostoma floridae* genome (http://shake.jgi-psf.org/Brafl1/Brafl1.home.html), which revealed the presence of *UreG*, *UreD* and *UreF* (estExt_fgenesh2_pg.C_1500097; estExt_fgenesh2_pg.C_1500095; fgenesh2_pg.scaffold_150000093). Moreover, in our experiment, the instant color change in the reaction solution containing the pieces of gut, gill and ovary demonstrated the presence of urease activity in these tissues, providing additional evidence for the existence of *UreG* in amphioxus. It is apparent that *AmphiUreG* is the first UreG gene fully identified so far in an animal species.

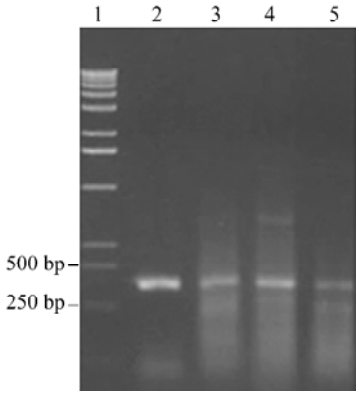

Fig. 3 Reverse transcription-polymerase chain reaction analysis of amphioxus *UreG* transcripts

1, marker; 2, control; 3, gut; 4, ovary; 5, whole organism.

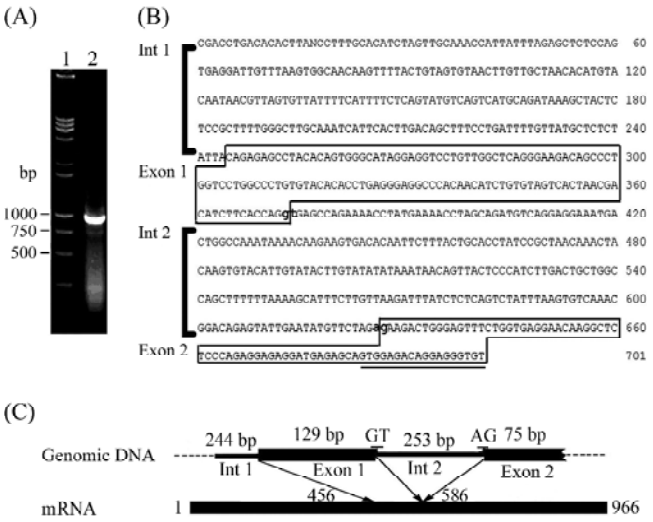

Fig. 4 Analysis of nested polymerase chain reaction (PCR) product

(A) Nest PCR analysis. 1, marker; 2, product of the second step of nested PCR. (B) The resulting sequence of the nested PCR product. The exons are boxed, the primer NS2 is underlined and the exon/intron junction sites (GT-AG) are indicated by lower case bold letters. Neither intron (Int) 1 nor exon 2 are complete. The numbering of the nucleotides is shown on the right. (C) Relation between the genomic DNA fragment from nested PCR and the corresponding mRNA from clone L239. Exons are shown by heavy lines, introns by thin lines, and parts of the gene upstream and downstream of the nested PCR fragment are shown by broken lines. Corresponding spliced sites on the mRNA are indicated by arrows. Sequences thought to be necessary for correct RNA splicing of various other eukaryotic genes GT and AG are shown just above the exon/intron junction sites.

The phylogenetic tree constructed using the amino acid sequence of *AmphiUreG* and that of other known UreG/UreG-like proteins from various species, including sea urchin, fungi, plants and bacteria, showed that *AmphiUreG* and sea urchin UreG-like protein clubbed together, forming a clade with eukaryotic UreG, whereas all bacterial UreG proteins clustered together (**Fig. 5**). Identification

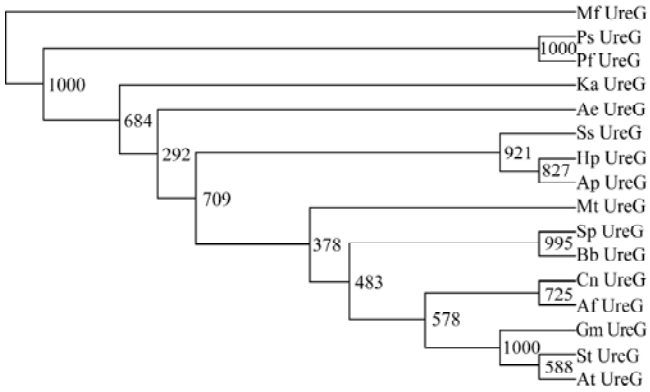

Fig. 5 Phylogenetic tree constructed from sequences of amphioxus UreG and other known UreG proteins from 15 species by the neighbor-joining method within the software package PHYLIP 3.5c

Bootstrap majority consensus values on 1000 replicates are indicated as percentages at each branch point. The sequences of UreG and UreG-like proteins refer to **Fig. 2**.

of other UreG-like genes from more animal species will shed detailed light on the evolution of UreG.

In situ hybridization histochemistry demonstrated an abundant expression of *AmphiUreG* in the digestive system, including endostyle, hepatic caecum, foregut and hindgut, and in the gill. A faint expression of *AmphiUreG* was also detected in the ovarian oocytes, but not in the muscle, notochord, neural tube, nor testis (**Fig. 6**). It is clear that *AmphiUreG* is expressed in amphioxus in a tissue-specific manner, which is in contrast to the ubiquitous expression pattern of *UerG* in plants [23,16]. Wang *et al*. [25] has recently proven the presence of allantoicase activity, one of the urate-degrading enzymes, in amphioxus [26]. Here we show the existence of urease activity and a urease accessory protein gene, UreG, in amphioxus. It is therefore highly likely that *AmphiUreG* is similar to the microbial and plant UreG and is involved in the recycling of metabolically-derived urea.

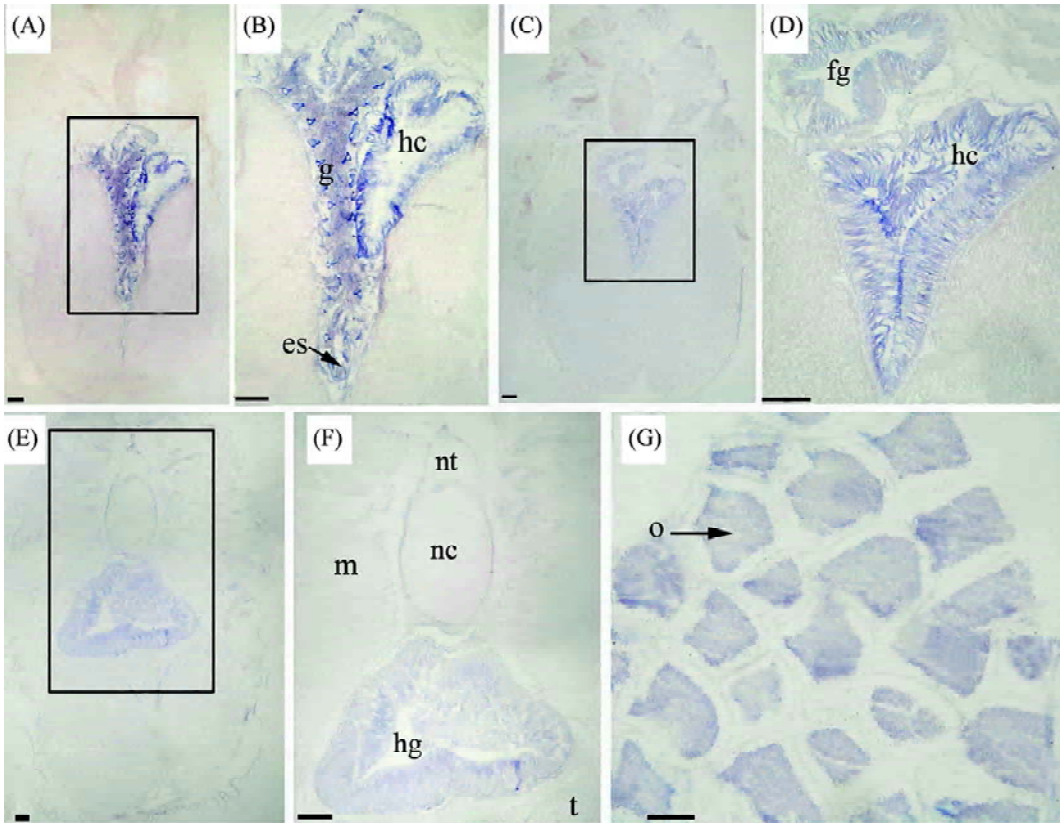

Fig. 6 Localization of amphioxus UreG transcripts in different tissues of adult amphioxus

(A) A low magnification view of tissues of a male amphioxus. *AmphiUreG* mRNAs were localized in the hepatic caecum, endostyle and gill. (B) Enlargement of the boxed section shown in (A). es, endostyle; hc, hepatic caecum; g, gill. (C) A low magnification view of tissues of a male amphioxus through foregut and hepatic caecum. (D) Enlargement of the boxed section shown in (C). fg, foregut; hc, hepatic caecum (E) A transverse section through the hindgut of a male amphioxus. *AmphiUreG* mRNAs were found in the hindgut. (F) Enlargement of the boxed section in (E). hg, hindgut; m, muscel; nc, notochord; nt, neural tube; t, testis. (G) A transverse section through the ovary of a female amphioxus. *AmphiUreG* mRNAs were seen in ovarian oocytes (o). Bar=100 μm in (A–F); bar=50 μm in (G).

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