A novel type of urea transporter, UT-C, is highly expressed in proximal tubule of seawater eel kidney

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KIDNEY UREA TRANSPORTERS HAVE been demonstrated to play a central role in the urinary concentrating mechanism in mammals (for a review and recent studies, see Refs. 7, 26, 27, and 29). Fish nephron, however, lacks the urine-concentrating loops of Henle found in the nephron of higher vertebrates. Furthermore, in fish, urea is excreted mainly from the gill (16, 40). Because of these anatomic and functional limitations of the fish kidney, little attention has been paid to its urea transporters except those working in the kidney of marine teleosts. It is generally thought, as implied by the facts mentioned above, that urea transporters may not play important roles in the kidney and may not be expressed in physiologically significant levels in the teleost kidney. Indeed, when we identified a urea transporter in the eel gill (eUT) and performed Northern blot analysis, no band was observed in kidney samples even under low stringency conditions (17).

This view was, however, challenged by a database search combined with cellular and functional characterization. We found, in the fugu (Takifugu rubripes) genome, a novel nucleotide sequence encoding a protein that is distantly related to known urea transporters, sharing for example ~35% identity with eUT. The gene product, termed UT-C, was confirmed to be present not only in the fugu but also in the eel kidney by RT-PCR amplification of mRNA. On Northern blotting using mRNA preparations from various tissues of freshwater and seawater eels, the message was detected only in the kidney and found to be strongly induced in seawater-adapted eels. In vitro expression and immunohistochemistry indicated that the newly identified UT-C is a facilitative urea transporter highly expressed in the proximal tubules of seawater eels. In this report, based on these observations, we propose that UT-C identified here constitutes an efficient mechanism of urea excretion, in combination with the gill urea transporter, under seawater conditions where only a very small volume of urine is excreted to save body water. Namely, UT-C is thought to be involved in transepithelial reabsorption of urea across the proximal tubule, and the urea absorbed from the glomerular filtrate is then carried to the gill for excretion.

MATERIALS AND METHODS

Animals. Cultured Japanese eels, Anguilla japonica, were purchased from a local dealer. They were maintained without feeding in a 1-t freshwater tank for 1 wk to acclimate them to laboratory conditions. To prepare seawater-adapted eels, they were then transferred to a 0.5-t seawater tank and acclimated there for at least 2 wk before use. Water in the tank was continuously filtered, circulated, aerated, and thermoregulated at 18 ± 0.5°C. Before blood sampling and tissue collection, eels were anesthetized by immersion in 0.1% (wt/vol) tricaine methanesulfonate neutralized with NaHCO3.

RNA isolation. Total RNA was isolated from various tissues by acid guanidinium thiocyanate-phenol-chloroform extraction with Iso-
was stored at mRNA with T4 RNA ligase at 37°C for 60 min, and modified RNA/H11032 primer (GSP1: 5′-CTAGTACTGGCATGCAGGCTGC-TACTGC-3′) and antisense primer (GSP: 5′-GTCGCCAGGATCAACACAGA-3′). These primers were designed based on the amino acid sequence alignment of rainbow trout (Danio rerio; partial sequence) or- 

**RT-PCR and molecular cloning.** A fragment of eel urea transporter homolog (UT-C) was isolated by RT-PCR using the 5′-rapid amplification of cDNA ends (RACE) method. The 5′-RACE cDNA was synthesized with a First Choice RLM-RACE kit (Ambion) according to the manufacturer’s instructions. Briefly, 1 μg of total RNA from eel kidney was first treated with calf intestinal phosphatase (CIP) in 1× CIP buffer at 37°C for 60 min, phenol extracted, and then incubated with tobacco acid pyrophosphatase (TAP) in 1× TAP buffer at 37°C for 60 min. A single-strand 5′ RACE adapter was added to decapped mRNA with T4 RNA ligase at 37°C for 60 min, and modified RNA was stored at -20°C. Two microliters of adaptor-ligated RNA were then reverse transcribed with oligo(dT) and Moloney murine leukemia virus reverse transcriptase in 1× RT buffer with dNTPs and RNase inhibitor at 42°C for 60 min to create first-strand cDNA. The synthes- 

**Northern blot analysis.** Total RNAs were isolated from a number of tissues of freshwater and seawater eels and analyzed by Northern blotting using a [32P]dCTP-labeled (Amersham Biosciences) eUT-C cDNA probe [a partial UT-C cDNA clone of 320 bp (nucleotides 354–773) that was isolated by the RT-PCR mentioned above]. For time course analysis of eUT-C mRNA levels in total eel tissues, the total RNAs were isolated at various time points after transfer of eels from freshwater to seawater and subjected to Northern analysis. 

**cDNA cloning, sequencing, and 5′ RACE.** Construction of an eel kidney cDNA library in 17.2. Stratagene) and with a BigDye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) and the ABI 310 DNA Sequence System (Applied Biosystems). This clone was used as a probe for Northern blot analysis and for screening of the library for eel UT-C (eUT-C) cDNA clones. The coding sequences of fugu UT and UT-C cDNAs were amplified from the fugu gill and kidney cDNAs using the following primer sets (sense/antisense): 5′-CGGCCACCATTCAGTCTCCTGTGCAGGCTGC-3′/5′-TCACACGACTGCGTTTAC-3′ for UT and 5′-CGGCCACCATGAGTTGGACAACGTT-3′/5′-TCACACGACTGGCGTTTAC-3′ for UT-C. 

**Sequence analyses and comparison.** The deduced amino acid sequence of eUT-C was compared with the sequences of genomic DNA or EST clones obtained by BLAST search of the databases of GenBank (http://www.ncbi.nlm.nih.gov/BLAST), Medical Research Council (MRC, http://fugu.hgmp.mrc.ac.uk), and The DOE Joint Genome Institute (JGI, http://genome. jgi-psf.org/fugu). Identity (percentage of identical amino acids) and similarity (percentage of similar amino acids of the following groupings: STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW) were calculated with GeneTyx-Mac software (Genetyx, Tokyo, Japan).

For evolutionary analyses, the protein sequences were aligned using Clustal W software (35), and then a phylogenetic tree was constructed by the neighbor-joining method (21) using MEGA software (15) based on Poisson corrected evolutionary distances (21). Statistical analysis was performed by bootstrap methods (21).

**Antibody production.** A 117-bp fragment encoding an intracellular COOH-terminal region of eUT-C (amino acid residues 340–378) was subcloned into the SalI/EcoRI sites of the bacterial expression vector pPHAT10 (Clontech), and the construct was transformed into Esche- 

**Fig. 1.** RT-PCR amplification of eel urea transporter eUT-C mRNA. RT-PCR was performed as described in MATERIALS AND METHODS using gill and kidney total RNA preparations. The amplified products were separated by electro- 

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Western blot analysis. Eels were anesthetized and perfused with PBS to wash out the blood. The stomach and kidney were then removed for Western blot analysis. Freshwater and seawater eel stomachs and kidneys were homogenized in ice-cold buffer (12 mM HEPES and 300 mM r-mannitol, pH 7.6) containing 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 10 μg/ml apro- tinin, and 10 μg/ml pepstatin. The homogenates were initially centrifuged at 2,500 g for 20 min at 4°C to remove cell debris. The resulting supernatant was centrifuged at 200,000 g for 1 h at 4°C, and the pellets were resuspend in the same homogenization buffer. For the subcellular fractionation experiment, the suspension underwent a series of differential centrifugation steps at 4°C: 1) 1,000 g for 10 min (pellets, debris); 2) 4,000 g for 20 min (pellets containing nuclei, mitochondria, and some membranes); 3) 17,000 g for 1 h (pellets, plasma membranes); and 4) 200,000 g for 1.5 h (pellets, intracellular vesicles; supernatant, cytosolic proteins) (33). Finally the 17,000-g pellets were solubilized in TBS containing 1% Triton X-100. Total protein concentrations in the samples were measured using a Pierce BCA protein assay kit (Pierce) and adjusted to ~1 μg/μl with the solubilization solution. The membrane proteins were separated by SDS-PAGE using a 10% poly- acrylamide gel and electroblotted onto a PVDF membrane. After blocking in 10 mM Tris·HCl, pH 8.0, containing 150 mM NaCl, 0.05% Tween 20, and 5% nonfat milk for 1 h at room temperature, the PVDF membrane was incubated with anti-eUT-C antiserum, preimmune serum, or preabsorbed antiserum at 1:1,000 dilution.
overnight at 4°C. Preabsorption was carried out by incubating 10 μl of the antiserum with 400 μg of affinity-purified fusion protein (HAT-eUT-C) in 500 μl of PBS overnight at 4°C. The immune complexes on the membrane were then reacted with alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:3,000 dilution for 1 h at room temperature. The bound secondary antibody was visualized using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride as chromogenic substrates.

Functional characterization using Xenopus laevis oocytes. Fragments of eUT, eUT-C, and mUT containing the full encoding regions were subcloned into the X. laevis oocyte expression vector pGH19. Capped sense cRNAs were synthesized in vitro by using a mMessage mMachine cRNA kit (Ambion). cRNAs (10 ng) were coinjected with 10 ng secreted alkaline phosphatase (SEAP) cRNA into collagenase-treated and defolliculated X. laevis oocytes. Three days later, good cells were selected by alkaline phosphatase activity (32). Urea transport activity was measured by 1H]urea uptake as previously described (29). Oocytes were preincubated in uptake solution (200 mM mannitol, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES buffer, 5 mM Tris-HCl, pH 7.4) for 1 h, then moved to uptake solution with 74 kBq (2 μCi) [14C]urea/ml and 1 mM urea. After uptake, oocytes were washed with ice-cold uptake solution containing 1 mM urea, dissolved in 10% SDS. The radioactivity was measured by scintillation counting. In urea transport inhibitor or PKA activator experiments, oocytes were preincubated in 500 μM phloretin, 30 mM dimethylurea, 10 μM forskolin, or 10 μM vasopressin for 10 min. Urea uptake was 90 s, except for time course experiments in which the incubation time varied from 0 to 20 min.

Fig. 3. Structural model of eUT-C protein. Putative transmembrane-spanning regions were predicted using the computer program PSORT. Potential membrane-spanning regions are numbered 1 to 10. Potential PKC phosphorylation sites; potential protein kinase CK2 phosphorylation site. A potential N-glycosylation (Asn202) site, which is highly conserved in all vertebrate UTs, is present on the third relatively long extracellular loop.

Fig. 4. Amino acid sequence alignment of eUT-C and the UT family members of other species. Shading denotes identical amino acid residues. Circles indicate the positions where the coding sequences are interrupted by introns. The abbreviations, accession nos., and references are as follows: fgUT-C, fugu ortholog of eUT-C, scaffold 132; eUT, eel gill urea transporter, AB049726 (17); fgUT, fugu ortholog of eUT, scaffold 3498; hUT-A1N, NH2-terminal half of human UT-A1, NM_007163 (2); hUT-A1C, COOH-terminal half of human UT-A1, NM_007163 (2); and hUT-B, human UT-B, NM_015865 (24). Potential N-glycosylation sites are boxed. Primer sites used for initial amplification of fgUT-C are indicated by arrows. TM, transmembrane span.
Immunohistochemistry. Seawater eel kidney was fixed in 0.1 M PBS containing 2% (wt/vol) paraformaldehyde for 2 h at 4°C. After incubation in 0.1 M PBS containing 10% (wt/vol) sucrose overnight and 20% (wt/vol) sucrose for 6 h at 4°C, specimen was frozen in Tissue Tek OCT Compound on a cryostat holder. Frozen sections (6 μm) were cut in a cryostat at −20°C and mounted on 3-aminopropyltriethoxysilane (APS)-coated glass slides and dried in air for 1 h. After being washed with PBS, sections were incubated for 2 h at room temperature with primary antibody at 1:200 dilution. After washing with PBS, sections were incubated with secondary antibody at 1:200 dilution at room temperature for 1 h. After washing with PBS, sections were counterstained with hematoxylin and mounted with mowiol.

Fig. 5. Phylogenetic tree of the members of the UT family identified and sequenced so far. The protein sequences were aligned using Clustal W software (35), and a phylogenetic tree was then constructed by the neighbor-joining method (21) using MEGA software (15) based on Poisson corrected evolutionary distances (21). Statistical analysis was performed by bootstrap methods (21). The numbers for interior branches refer to bootstrap values for 5,000 replications. Accession nos. are as follows: eUT-C, AB181944; fgUT-C, AB181945; fgUT, AB181946; eUT, AB049726 (17); tUT, AF165893 (39); mtUT, AF278537 (38); Triakis UT, AB04993 (8); shUT, AF257331 (30); strUT, AF443781 (10); SkUT, AY052552 (19); fUT, Y12784 (4); hUT-A1, NM_007163 (2); hUT-A2, X96969 (23); rUT-A1, NM_019347 (29); rUT-A2, U09957 (29); rUT-A3, AF041788 (12); hUT-B, NM_015865 (24); rUT-B, NM_019346 (5). e, eel; fg, fugu; t, gulf toadfish; mt, magadi tilapia; h, human; m, mouse; r; rat; wh, short-finned pilot whale; f, edible frog; sh, spiny dogfish; str, Atlantic stingray; and Sk, winter skate.

Fig. 6. Northern blot analysis of expression of eUT-C mRNA in various tissues of eels. A: autoradiogram of a Northern filter containing RNA preparations from the indicated tissues of freshwater (F) and seawater (S) eels (n = 6, 2 replicates). An eel β-actin probe was used as an internal control. B: hybridization intensities of the 1.7-kb bands in kidney and stomach were densitometrically determined, and values (means ± SE) are expressed relative to the expression of β-actin.
containing 0.3% (vol/vol) H₂O₂ for 20 min at room temperature. After Sections were then washed with PBS and treated with methanol seawater, and their total RNA was isolated from the kidney (n = 1:1,000), or anti-Na⁺-K⁺-ATPase α-subunit antiserum (1:50,000). Sections were then washed with PBS and treated with methanol containing 0.3% (vol/vol) H₂O₂ for 20 min at room temperature. After being washed with PBS, the specimens were treated with biotinylated secondary antibody (1:2,000) and avidin-peroxidase conjugate using a Vectastain ABC kit (Vector Laboratories). Finally, bound antibodies were visualized using 3,3′-diaminobenzidine tetrahydrochloride in 50 mM Tris-HCl, pH 7.4, containing 0.02% H₂O₂ and counterstained with hematoxylin.

**Immunofluorescence microscopy.** Cryosections of seawater eel kidney were prepared as described previously (17, 18). Nonspecific reactions were blocked by preincubating them with PBS containing 5% normal goat serum in a humidified chamber at 25°C for 1 h. After being washed three times in PBS, sections were treated with either preimmune serum or primary antibody (anti-eUT-C, 1:1,000 dilution in blocking solution) for 16 h at 4°C. Sections were then rinsed thoroughly in PBS and incubated, for 1 h at 25°C, with a secondary antibody solution containing Alexa Fluor 488-conjugated anti-rabbit IgG (0.75 μg/ml, Molecular Probes) for indirect detection of eUT-C. Nuclei were labeled with Hoechst 33342 (100 ng/ml, Molecular Probes). Actin in brush border was labeled with TRITC-phalloidin (0.4 μM, Molecular Probes). Before being visualized, sections were rinsed in PBS and mounted in fluorescence mounting medium (Fluromount-G, Southern Biotechnology Associates). Fluorescence was detected using an Olympus fluorescence microscope model IX70 and filter optimized for triple-label experiments. Pictures were taken using a Princeton Instruments cooled CCD camera (MicroMAX 5 MHz; Roper Scientific) and analyzed using MetaMorph software (Universal Imaging).

**In situ hybridization histochemistry.** Anesthetized seawater eels were fixed by perfusing 10% buffered neutral formalin (Muto Pure Chemicals) through a catheter into the ventral aorta. Kidneys were removed, embedded in paraffin, and sectioned (4 μm). DNA templates used for the preparation of the digoxigenin (DIG)-labeled riboprobes were as follows: a 318-bp fragment (nucleotides 57–369, probe 1) and a 300-bp fragment (nucleotides 1298–1597, probe 2) of eUT-C cDNA. The DIG RNA Labeling Mix (Roche) was used for synthesizing DIG-labeled sense and antisense probes. Alkaline phosphatase-conjugated anti-DIG antibody and its substrates (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) were used to visualize the signal, followed by counterstaining with Kernechtrot (Muto Pure Chemicals).

**Determination of urea concentration and osmolarity.** Serum samples (1 ml) were analyzed for their urea concentrations and osmolarities by standard methods for clinical chemistry parameters at the SRL clinical chemistry laboratory (Tokyo, Japan). Namely, urea was measured by coupling the urease reaction with the NADH-NAD system, and osmolarity was measured using a freezing point osmometer.

**RESULTS**

**Identification and molecular characterization of eUT-C.** A database search revealed the presence of two UT-like genes in the *T. rubripes* genome: one in scaffold 3498, showing 65% similarity to eUT, and the other one in scaffold 132, showing only 35% similarity to eUT, which were tentatively named fgUT and fgUT-C, respectively. To confirm their existence at the message level, we performed RT-PCR using mRNA prep-
A partial sequence of eUT-C cDNA was first obtained by RT-PCR using a combination of primers corresponding to the regions highly conserved between the fugu and rainbow trout UT-C sequences but not so among the known urea transporters (see Fig. 4). Interestingly a PCR fragment of the expected size (320 bp) was obtained from the kidney but not from the gill (Fig. 1). Its sequence exhibited a low but significant similarity to the published sequences of urea transporters. Screening of an eel kidney cDNA library with this fragment yielded 10 positive clones of ~1.5 kb. The longest clone was of 1,510 bp and contained the apparent 3’-end of the sequence, including a polyadenylation signal (AATAAA) and a poly(A) tail. However, it was not clear whether the clone contained the 5’-noncoding region. We therefore performed 5’-RACE using a cap site-labeled cDNA library to determine the 5’-end of eUT-C mRNA, which yielded an additional sequence of 213 nucleotides, extending the total length of the sequence from 1510 to 1723 nucleotides (Fig. 2; GenBank accession no. AB181944). The nucleotide sequence around the initiator methionine codon ATG (GTCACC ATG T) conforms well to the consensus sequence of the common Kozak-box for translation initiation in higher eukaryotes [GCC(A/G)CC ATG G] (14).

The open reading frame encodes a protein of 378 amino acid residues with a calculated molecular mass of 39,846 Da, which is shorter than the gill urea transporter, eUT, of 486 amino acid residues. A hydropathy analysis indicated that eUT-C has 10 transmembrane-spanning hydrophobic segments (Fig. 3). There is a potential N-glycosylation site (Asn-202) on the third extracellular loop, four consensus sequences for PKC phosphorylation sites (Ser-4, Ser-51, Ser-293, and Ser-347) and a consensus casein kinase II (CK2) phosphorylation site TTPE (residues 356-359) in the cytoplasmic domains, and a consensus PDZ-binding motif at the COOH terminus of eUT-C protein. Comparison of the sequence with those of other species indicated that eUT-C shares 61% (fugu; fgUT-C), 39% (eel; eUT, AB049726), 41% (toadfish; tUT, AF165893), 41% (Magadi tilapia; mtUT, AF257331), 40% (skate; SkUT, AY052552), 39% (stingray; strUT, AF443781), 41% (dogfish; TsUT, AB094993), 43% (whale; whUT, AYO16181), 43% (human; UT-A1, NM_007163), 44% (frog; fUT, Y12784), and 41% (rat; UT-A3, AF041788) identities with the known sequences of the urea transporter (Fig. 4). This low sequence identity may help define the regions directly involved in the urea transport activity. For example, the following sequences are highly conserved among the urea transporter family members so far identified: WxLRxxxVxxxNNPxSG, GxNxxLV, DxxW-WLLxP, GxGQxYxC, SPxxxxHAxxGSxxG, YxGLxxxNx-LxCxxGG, and VxPExN. The ALE sequence typically seen in the UT-B isoform is not present in eUT-C (Fig. 4, double underline).

**Phylogenetic relationship to other members of the urea transporter family.** Phylogenetic analyses based on the amino acid sequences of the urea transporters found in the DDBJ/EMBL/GenBank database indicated an ancient separation of urea transporters into two branches, one with UT-C and the other with the members previously identified including mammalian erythrocyte UT-B, mammalian kidney UT-A, elasmobranch kidney UT, and teleost gill UT (Fig. 5). Reflecting the common origin of the UT and UT-C genes, their exon-intron

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**Fig. 10. Effects of various inhibitors on urea transport activities of eUT and eUT-C.** eUT (17) and eUT-C cRNA were microinjected into X. laevis oocytes, and urea transport activities were measured using [14C]urea in the presence of the indicated inhibitors or PKA activators, as described in MATERIALS AND METHODS.
organizations were found to be well conserved in the human and fugu genes so far determined (Fig. 4).

Tissue distribution and salinity-dependent regulation of eUT-C mRNA. To determine the multiple tissue distribution of eUT-C mRNA and to compare its expression levels in seawater and freshwater eels, we performed Northern blot analysis using total RNA preparations from various tissues of seawater and freshwater eels including the kidney, head kidney, urinary bladder, posterior intestine, anterior intestine, stomach, liver, heart, and gill. A strong signal of ~1.7 kb was detected in the kidney and also a faint band in the stomach (Fig. 6A). The other tissues examined were negative. Comparison between the seawater and freshwater samples indicated that both in the kidney and stomach, a large induction of eUT-C mRNA occurs on seawater acclimation (Fig. 6B). Figure 7 shows the time course of the induction of eUT-C mRNA expression following transfer of eels from freshwater to seawater. The adaptive alteration occurred relatively over a time course of hours to days.

Production of antiserum and immunochemical characterization of eUT-C. A rabbit polyclonal antibody was prepared against a COOH-terminal fragment of eUT-C (residues 340–378). The specificity of the antiserum was characterized by Western blot analysis using Triton extracts of membrane preparations of the eel stomach and kidney (Fig. 8). A single band of ~40 kDa was detected, which corresponds well to the calculated molecular mass of eUT-C. When freshwater and seawater samples were compared, seawater samples gave a denser band than freshwater samples, indicating a higher expression level of eUT-C in seawater eel stomachs and kidneys, confirming the result of Northern blot analysis at the protein level.

Functional characterization. We next determined whether eUT-C has a urea-transporting activity using the X. laevis oocyte expression system. A marked increase in [14C]urea uptake was seen when oocytes, injected with eUT-C cRNA, were exposed to the uptake medium (Fig. 9). Preincubation with phloretin strongly inhibited [14C]urea uptake through eUT-C (Fig. 10A). In the presence of dimethylurea, the activity was also significantly reduced (Fig. 10B). The eUT-C expressed on X. laevis oocyte was not sensitive to treatments with forskolin and vasopressin, consistent with the lack of the cAMP-dependent protein kinase (PKA) site in eUT-C. We also determined the activity of the eel gill urea transporter eUT, which had previously been identified but not functionally characterized, and obtained similar results (Fig. 10). These results suggest that both eUT and eUT-C are facilitative urea transporters.

Cellular localization of eUT-C in the kidney. The localization of eUT-C in the kidney was examined by immunohistochemistry using the antiserum characterized above. The anti-eUT-C antiserum specifically stained the basolateral membranes of proximal tubule cells (Fig. 11, B and C). The tubular epithelial cell and basolateral localization was confirmed by staining consecutive sections with an antiserum against Na⁺-K⁺-ATPase, a well-established marker protein of the basolateral membranes of renal tubule cells (Fig. 11, E and F). Localization of eUT-C (Fig. 11D, green) in renal proximal tubule was further confirmed by double staining of a single section with anti-eUT-C and phalloidin (Fig. 11D, red), a bicyclic heptapeptide toxin that specifically interacts with actin fibers in the brush border of proximal tubules (3). No specific

![Fig. 11. Immunohistochemical localization of eUT-C and phalloidin staining of actin fibers in seawater eel kidney sections. A clear dense staining was observed in renal tubular epithelial cells with anti-eUT-C antiserum (B and C) and antiserum against recombinant eel Na⁺-K⁺-ATPase α-subunit (E and F) but not with preimmune serum (A). A section of a seawater eel kidney was stained with anti-UT-C-specific rabbit antiserum, visualized with an Alexa Fluor 488-conjugated anti-rabbit IgG (D, green), and TRITC-labeled phalloidin (D, red). Localization of eUT-C in renal proximal tubule was confirmed by immunostaining of a section with a fluorescent-labeled phalloidin, a bicyclic heptapeptide toxin that specifically interacts with actin fibers in the brush border of proximal tubule (3). Nuclear counterstaining was performed with Hoechst 33342 (D, blue). Bars = 50 μm.](http://ajprenal.physiology.org/)

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staining was observed with preimmune serum or absorbed antiserum (Fig. 11A).

In situ hybridization histochemistry further confirmed the proximal tubule cell localization of eUT-C. When kidney sections of seawater eels were hybridized with an antisense cRNA probe (probe 1), a weak but significant signal was observed in proximal tubule cells but not in distal tubule cells (Fig. 12B). A similar result was obtained using another antisense probe (probe 2) corresponding to a different region of the eUT-C mRNA (data not shown). Control sense probes gave no hybridization signal (Fig. 12A).

Serum urea concentrations. Table 1 shows serum urea concentrations and osmolarities of freshwater and seawater eels. Urea concentrations in blood were about twofold higher in seawater eels than in freshwater eels. The values (2–4 mM) are comparable with those previously reported for control (0.54 ± 0.17 mM) and urea diet-fed (4.17 ± 1.60 mM) rainbow trout (13).

**DISCUSSION**

Through database mining, we identified a novel type of UT, UT-C, in fugu and cloned its eel ortholog, eUT-C, from seawater eel kidney. Its structural, functional, and histochemical characterization revealed that it is a facilitative urea transporter expressed mainly in the proximal tubule cells of the kidney. As a clue to its physiological role, we found that it is expressed much more highly in seawater than in freshwater conditions. We previously identified another type of eel urea transporter, eUT, which is expressed exclusively in the gill (the site of urea excretion) and acts as a facilitative urea transporter as demonstrated here. The fact that both eUT and eUT-C are strongly induced in seawater eels leads us to propose that eUT-C helps to stimulate reabsorption of urea from the glomerular filtrate into circulating blood, which is in turn excreted from the gill through eUT. Both transporters are therefore thought to work in harmony to eliminate urea efficiently under the seawater conditions where urine excretion is minimized. The significantly higher levels seen in the serum urea concentration of seawater eels (Table 1) may reflect the reabsorptive role of eUT-C in the proximal tubule. In some fish, mesangial cells can alter their shape to reduce glomerular filtration when the fish is in seawater. In addition to this, removal of urea from the filtrate appears to be an effective strategy to prevent water loss since urea becomes a major component of urine and increases its osmolarity and hence its volume. In fish, the kidney and gill are known to be functionally linked. The eUT/eUT-C system proposed here may represent another example of the link.

A similar system for urea handling may also be functioning in other teleost species since the counterparts of eUT and eUT-C are found in the fugu, rainbow trout, and zebrafish genome and EST databases (scaffolds 3498/132, accession nos. CA383029/CA370131, and accession nos. WA165360/CA473460, respectively). Except a few ureotelic teleost fish (6, 9, 11, 25, 38, 39), most teleost fish are ammoniotelic and excrete the majority of their nitrogenous wastes across the gills as ammonia. However, even in these ammoniotelic teleosts, a significant amount of nitrogenous waste is excreted as urea. For example, in rainbow trout, urea makes up ~10% of total nitrogen waste excretion (41). We demonstrated that eel also maintain relatively high levels of circulating urea (Table 1), which points to the necessity of a carrier-mediated urea excretion mechanism. In this context, the UT/UT-C system proposed here may contribute to a better understanding of the functional and physiological significance of urea transporters in ammoniotelic fish.

Urea transporters have been identified and characterized in a variety of organisms including bacteria, yeasts, amphibians, fish, and mammals (for a review, see Ref. 1). Their sequences are diverged and display only minimal identity (20–25%) between lower organisms and higher vertebrates. Among fish, amphibians, and mammals, however, a substantial homology

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<th>Condition</th>
<th>Urea, mM</th>
<th>Osmolality, mosmol/kgH2O</th>
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<tr>
<td>Freshwater</td>
<td>1.9±0.4</td>
<td>297±7</td>
</tr>
<tr>
<td>Seawater</td>
<td>3.7±0.1</td>
<td>343±15</td>
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Values are means ± SE; n = 4 group. Although ammoniotelic fish lack the hepatic ornithine-urea cycle, they produce substantial amounts of urea mainly from the catabolism of excess purines through the process of uricolysis.
exists that allows detailed phylogenetic analyses. According to the phylogenetic tree in Fig. 5, it is likely that an ancient urea transporter gene was duplicated into the UT and UT-C genes, the former being further duplicated into UT-A (Sle4A2) and UT-B (Sle4A1) genes in the mammalian lineage. The UT-C gene appears to be deleted in the mammals since no mammalian homologs were detected by database searches. It would be interesting to see whether the UT-C gene is maintained in elasmobranch fish and what kind of UT genes are present in lamprey and hagfish, the earliest forms of the vertebrates.

The newly identified UT-C is also unique in its proximal tubule localization. The kidney urea transporters so far cloned and characterized have been shown to be expressed in the loop of Henle, inner medullary collecting duct (IMCD), and vasa recta. For example, in mammalian kidneys, UT-A1, the largest UT-A isoform of ~100 kDa, is expressed in the apical membrane of IMCD (22, 28); UT-A2, a 55-kDa protein, in the descending thin limb of Henle's loop (37), UT-A3, a 45- to 65-kDa glycosylated protein, in the basolateral membranes of IMCD cells (31, 34); and UT-B with a molecular mass of 43–45 kDa in the descending vasa recta (36). In the kidneys of marine elasmobranchs in which urea reabsorption is essential for retaining high levels of urea in body fluid as an osmolyte, urea transporters seem to be localized in the collecting tubule of the bundle zone surrounded by the peritubular sheath as recently demonstrated in the dogfish Triakis scyllia (8). These locations and the branching pattern of the phylogenetic tree (Fig. 5) suggest that through a series of gene duplications, the urea transporter family increased the number of its members with distinct promoters and achieved the urine-concentrating mechanism in higher vertebrates and the urea retention mechanism in elasmobranchs. Among them, UT-C remained unaltered as a part of urea excretory system in teleosts, which becomes particularly important in marine environments.

A significant level of salinity-dependent expression of eUT-C was also observed in the stomach. This is a totally unexpected finding and seems to be interesting, but its physiological role is not clear at present. Identification by immunohistochemistry of stomach cell types that express UT-C will give us some clue regarding the physiological role. We hope the unusual sub-cellular localization of ornithine-urea cycle enzymes in the freshwater air-breathing teleost Heterocephalus gallus. Biochem Int 25: 1061–1069, 1991.


