The Journal of Experimental Biology 203, 2357–2364 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2807

MOLECULAR CHARACTERIZATION OF A UREA TRANSPORTER IN THE GILL OF THE GULF TOADFISH (*OPSANUS BETA*)

PATRICK J. WALSH[1,*], MOLLY J. HEITZ[2], CATHERINE E. CAMPBELL[1], GORDON J. COOPER[2], MONICA MEDINA[1], YUXIANG S. WANG[1], GREG G. GOSS[3], VLADIMIR VINCEK[4], CHRIS M. WOOD[5] AND CRAIG P. SMITH[2]

[1]*Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science, NIEHS Marine and Freshwater Biomedical Sciences Center, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149 USA,* [2]*School of Biological Science, University of Manchester, Manchester M13 9PT, UK,* [3]*Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9,* [4]*Department of Microbiology and Immunology Sciences and Department of Pathology, School of Medicine 2E9, University of Miami, Miami, FL 33136, USA* and [5]*Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S 4K1*

*e-mail: pwalsh@rsmas.miami.edu

Accepted 8 May; published on WWW 10 July 2000

Summary

Urea excretion by the gulf toadfish (*Opsanus beta*) has been shown in previous studies to be a highly pulsatile facilitated transport, with excretion probably occurring at the gill. The present study reports the isolation of an 1800 base pair (kb) cDNA from toadfish gill with one open reading frame putatively encoding a 475-residue protein, the toadfish urea transporter (tUT). tUT, the first teleostean urea transporter cloned, has high homology with UTs (facilitated urea transporters) cloned from mammals, an amphibian and a shark, and most closely resembles the UT-A subfamily. When expressed in *Xenopus laevis* oocytes, tUT increased urea permeability (as measured by [^{14}C]urea uptake) five- to sevenfold, and this permeability increase was abolished by phloretin, a common inhibitor of other UTs. Northern analysis using the 1.8 kb clone was performed to determine the tissue distribution and dynamics of tUT mRNA expression. Of six tissues examined (gill, liver, red blood cells, kidney, skin and intestine), only gill showed expression of tUT mRNA, with a predominant band at 1.8 kb and a minor band at 3.5 kb. During several points in the urea pulse cycle of toadfish (0, 4, 6, 12 and 18 h post-pulse), measured by excretion of [^{14}C]urea into the water, gill mRNA samples were obtained. Expression of tUT mRNA was found to be largely invariant relative to expression of β-actin mRNA over the pulse cycle. These results further confirm the gill localization of urea transport in the toadfish and suggest that tUT regulation (and the regulation of pulsatile urea excretion) is probably not at the level of mRNA control. The results are discussed in the context of the mechanisms of vasopressin-regulated UT-A in mammalian kidney and morphological data for the toadfish gill.

Key words: gulf toadfish, *Opsanus beta*, urea transporter gene, UT-A2, gill, nitrogen excretion.

Introduction

The majority of adult teleost fishes excrete ammonia as their principal nitrogenous waste product. There are, however, a number of teleosts excreting a substantial proportion of waste as urea (for reviews, see Mommsen and Walsh, 1992; Wright, 1995). In addition, there is growing evidence that fish species that are ‘typical’ ammonoteles as adults may excrete a higher proportion of urea during their larval stages (Wright et al., 1995; Chadwick and Wright, 1999), suggesting that ureotely in teleosts may be a more general feature than previously thought. Adult gulf toadfish, *Opsanus beta*, excrete over 90 % of waste nitrogen as urea under selected laboratory situations (e.g. confinement stress; for a review, see Walsh, 1997), and at least one population of the species (Biscayne Bay, FL, USA) is believed to be at least 50 % ureotelic in nature (Hopkins et al., 1997), although the evolutionary and ecological significance of this phenomenon remain speculative (Walsh, 1997; Hopkins et al., 1997).

Considerably more is known about the mechanisms of urea excretion in the toadfish than in most other fish species (Walsh, 1997). Urea excretion in the gulf toadfish occurs in discrete pulses (Wood et al., 1995), with fish eliminating virtually all their daily nitrogen waste in 1–3 pulses per day, each pulse lasting 0.5–3 h (Wood et al., 1995, 1997; Gilmour et al., 1998). In the long periods between pulses, there is virtually no urea excretion, despite the persistence of high urea levels in the blood. The pulsatile excretion phenomenon is known to be due to activation of a urea transfer mechanism from the plasma to the water, with urea synthesis and accumulation in the plasma

being continuous, rather than pulsatile (Wood et al., 1997). Furthermore, divided chamber experiments have localized the site of urea excretion to the anterior end of the toadfish (Wood et al., 1995), rather than the periodic micturation of the urinary bladder hypothesized earlier (Walsh et al., 1990; Griffith, 1991). Recent experiments directly measuring expired water from the opercular chamber (Gilmour et al., 1998) have implicated the gills as the site of urea excretion.

The control of urea pulses in the toadfish appears to be at least partly under hormonal influence, but direct causative links *in vivo* have yet to be established. For example, urea pulses occur following a marked drop in plasma cortisol level, followed by an increase to pre-pulse levels (Wood et al., 1997). In addition, administration of physiological doses of the hormone arginine vasotocin (AVT), a piscine analogue of mammalian vasopressin, elicits urea pulses (Perry et al., 1998). Furthermore, the studies of Wood et al. (1998), Perry et al. (1998) and Gilmour et al. (1998) all appear to rule out a generalized increase in gill permeability (e.g. by catecholaminergic vasodilation) as the mechanism for pulsatile urea excretion. Instead, all three studies point to the activation of a highly specific, facilitated-diffusion-type transport system for urea in the gills at the time of pulse events.

Molecular studies of urea transport in mammals, particularly in the kidney, have revealed a new class of transport protein, the urea transporter (UT) family (e.g. You et al., 1993; Olives et al., 1994; Smith et al., 1995; for a review, see Sands et al., 1997). These transporters generally have a high specificity for urea and for some urea analogues and function as facilitated diffusion pathways in a variety of cell types and species. Recently, cDNAs for such transporters have also been cloned from the tissues of lower vertebrates, namely the kidney of the dogfish shark *Squalus acanthias* (Smith and Wright, 1999) and amphibians (Couriaud et al., 1999). Given the large data set implicating facilitated diffusion at the toadfish gill, we reasoned that a UT-like gene should exist in the toadfish and that this gene or the UT protein might be dynamically regulated in the gill in parallel with the observed transient nature of urea excretion *in vivo*. The primary aim of this paper was, therefore, to clone a UT cDNA from toadfish gill and to begin to examine the dynamics of its expression relative to *in vivo* patterns of urea excretion.

Materials and methods

Animals and holding conditions

Sexually mature gulf toadfish (*Opsanus beta* Goode and Bean) for molecular studies were captured by roller trawl by local fishermen in Biscayne Bay, Florida, USA, in May 1997. Toadfish for physiological studies were captured in November 1998. At the University of Miami, fish were maintained initially in glass aquaria supplied with flowing Biscayne Bay sea water (29–34 ‰, 25±1 °C). Each aquarium contained a sand/gravel substratum (2–5 cm depth) and several polyvinyl chloride tubes that acted as individual shelters. Typically, 3–4 fish inhabited a single 45 l aquarium corresponding to a density of approximately 7–10 g fish l^{-1}. On days 1 and 3 after arrival, the fish were bathed in a mixture of Malachite Green and formalin (Wood et al., 1995) as a prophylactic treatment against the ciliate *Cryptocaryon irritans* (these conditions are termed the ‘uncrowded’ treatment). Fish were maintained under these conditions for at least 1 week and were fed *ad libitum* with frozen squid on alternate days. Food was withheld for 48 h prior to experimentation.

To induce ureotelism, fish weighing 42–88 g (mean mass 63±2.0 g; mean ± S.E.M., N=32) were subjected to a standardized ‘crowding’ protocol (Walsh et al., 1994; Wood et al., 1995; Hopkins et al., 1995) for 48–72 h prior to experimentation or tissue harvest. Briefly, this procedure involved placing the fish and their tube shelters in small plastic tubs (volume approximately 6 l) to achieve densities exceeding 80 g fish l^{-1}. The tubs were aerated continuously and supplied with flowing sea water. The fish were not fed during the period of crowding.

Molecular studies

Probe and library screening

A probe to screen for urea transporter (UT) was synthesized by reverse transcriptase/polymerase chain reaction (RT PCR) with gill mRNA from gulf toadfish as template using degenerate primers based on the alignment of rat, rabbit and human UT sequences. The sense primer (Great American Gene Co., Ramona, CA, USA) corresponded to amino acid residues 84–91 (FNVQNPWW), and the antisense primer corresponded to amino acid residues 238–244 (PWTGGIF) of rat UT-A2 (Smith et al., 1995). PCR conditions were 94 °C for 4 min, then 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min, followed by 72 °C for 7 min in a Stratagene SCS-2 PCR machine using 0.5 ml GeneAmp tubes (Perkin-Elmer) with a mineral oil overlay. PCR products were separated by gel electrophoresis (1 % agarose gel in TAE buffer; TAE buffer is 40 mmol l^{-1} Tris acetate, 1 mmol l^{-1} EDTA, pH 8.0), and the major band of 484 bp was gel-purified and subcloned into the plasmid vector pGEM T (Promega, Madison, WI, USA). The resultant plasmid (PCRT1) was transfected into Epicurean Ultracompetent *Escherichia coli* (Stratagene, La Jolla, CA, USA). Standard blue/white screening on Luriabroth (LB) ampicillin–agar plates identified colonies with the potential insert, which were then liquid-cultured. The plasmid DNA was isolated by the alkaline lysis method (Qiagen Kit, Chatsworth, CA, USA). Both strands of DNA of several clones were sequenced using an automated dideoxy chain-termination sequencing method (Sanger et al., 1977) and found to share homology with the rat UT-A2 gene (see Results and discusssion). ^{32}P-labelled PCRT1 was produced by extracting the excised bands from the *Eco*RI-digested minipreps electrophoresed as above, labelling with the RadPrime kit (Gibco, Frederick, MD, USA) and [α-^{32}P]dCTP (ICN, Irvine, CA, USA) and purification on G-50 Sephadex.

Total RNA was isolated from toadfish gill by homogenization of 0.2 g of tissue in 1.2 ml of phenol-guanidinium thiocyanate (Trizol Reagent, Gibco BRL)

followed by standard chloroform extraction and isopropanol precipitation (Sambrook et al., 1989). mRNA was enriched from total RNA using an mRNA purification kit based on oligo(dT) cellulose column chromatography (Pharmacia Biotech). cDNA was synthesized using MMLV reverse transcriptase (Stratagene) and oligo-dT primers. It was then incorporated into λgt10 phage DNA (Stratagene). The cDNA library (approximately 0.5×10^6 plaque-forming units) was screened with the ^{32}P-labelled PCRT1 probe by standard methods using nitrocellulose filter lifts from agar plates and autoradiography. Conditions relating to stringency were 50 % formamide, hybridization at 42 °C and washing at 50 °C with 2× standard saline citrate (SSC; 150 mmol l^{-1} sodium chloride, 15 mmol l^{-1} sodium citrate), 0.1 % SDS. Clones were sequenced as above.

5′ RACE PCR and full-length RACE PCR

Because no full-length clones were obtained from the toadfish cDNA library, 5′ rapid amplification of cDNA ends (RACE) PCR was performed to amplify 5′ ends using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA) and adaptor-ligated gill cDNA (adaptor supplied with the kit). A gene-specific primer was synthesized on the basis of the known sequences of the toadfish cDNA library clones and was GSP1 (antisense-gene-specific primer for 5′ RACE), 5′-CCAGGTGATGTTGGGTAGCTCGGAG-3′, corresponding to base pairs 390–366 of PCRT1. The sense primer was complementary to an adaptor supplied with the kit (which was ligated to the 5′ end of the cDNA end). PCR conditions were: 94 °C for 1 min, followed by five cycles at 94 °C for 0.5 min, 72 °C for 4 min, followed by five cycles at 94 °C for 0.5 min, 70 °C for 4 min, followed by 25 cycles at 94 °C for 0.5 min, 68 °C for 4 min with machine and tubes as above. Generally, PCR reactions were performed in duplicate to exclude PCR errors. The 5′ RACE reactions produced a band of approximately 750 bp, which was purified, subcloned and sequenced as above. The sequence of this 5′ RACE product and the sequence of clones from the library screen were used to design specific primers for amplification of the full-length sequence. The resultant 1.8 kb product (tUT) was purified, subcloned into pBluescript and sequenced as above. Clones were sequenced in both directions using internal sequencing primers, and the consensus sequence was obtained.

Northern analysis

Total RNA was extracted from several toadfish tissues (gill, intestine, liver, red blood cells, kidney and skin) and poly(A)-containing RNA was enriched as described above. Equal amounts (3 μg) of toadfish poly(A)$^+$ RNA were loaded onto a formaldehyde–agarose gel, electrophoresed and transferred to a nylon membrane (Hybond-N, Amersham, Arlington Heights, IL, USA) using standard methods (Sambrook et al., 1989). Following prehybridization, the membrane was hybridized at 37 °C for low-stringency analysis or at 48 °C for high-stringency analysis with a random-primed ^{32}P-labelled full-length toadfish UT cDNA (tUT). Final washes were in 0.1×SSC/0.1 % SDS at 37 °C for low stringency and 65 °C for high stringency. Similar analyses were performed on total RNA isolated from the gills of toadfish during and following pulses of urea excretion (see below). For quantification, membranes were subsequently stripped and reprobed for β-actin mRNA using a toadfish actin probe generated by PCR. Note that, since the actin band (1.7 kb) used was of similar size to the tUT signal, stripped membranes were checked by exposure to film to ensure that all tUT probe had been removed. The signals were compared by densitometric scanning, and the ratio of tUT to β-actin signal was used to correct for any variance in RNA concentrations.

Oocyte expression

After linearization of tUT plasmid with *Xba*I or *Xho*I, cRNA was synthesized *in vitro* using T_7 polymerase (T7-mMessage mMachine, Ambion) and microinjected (30 ng) into collagenase-treated and manually defolliculated *Xenopus laevis* oocytes, as described previously (Smith et al., 1995). Other groups of oocytes were injected with water (50 nl) as a negative control or rat UT-A2 cRNA (30 ng per oocyte) as a positive control. After incubation in modified Barth's solution (Gurdon and Wakefield, 1986) containing 50 μg ml^{-1} gentamycin for 3 days at 18 °C, oocytes destined for urea flux studies were preincubated in (in mmol l^{-1}) 200 mannitol, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 Hepes buffer, 5 Tris, pH 7.4, for 1 h. To measure urea uptake, 0.1 MBq ml^{-1} of [^{14}C]urea and 1 mmol l^{-1} unlabelled urea were added to the preincubation solution. After uptake, oocytes were washed with ice-cold uptake solution containing 10 mmol l^{-1} unlabelled urea and dissolved in 10 % SDS, and the radioactivity was measured by scintillation counting. Inhibition of [^{14}C]urea uptake by 0.5 mmol l^{-1} phloretin was measured by addition of phloretin to the preincubation solution 15 min before the addition of [^{14}C]urea, as described previously (Smith et al., 1995).

In vivo *studies of UT mRNA dynamics*

Crowded toadfish were fitted with indwelling caudal artery catheters (PE-50) as described by Wood et al. (1997). After at least 24 h to recover from surgery, excretion of urea and ammonia to the water were monitored on an hourly basis by pump and fraction collection of water, as described by Wood et al. (1997), to ensure that the fish were still ureotelic and that excretion was pulsatile. Once pulsatility had been confirmed, at time zero, the fish were injected with 14.8 MBq kg^{-1} [^{14}C]urea (1480 MBq $mmol^{-1}$, New England Nuclear), and hourly water sampling was continued as described above. Water samples were subjected to immediate liquid scintillation counting (1 ml of water in 10 ml of Ecolume, ICN, Cleveland, OH, USA). Fish identified as pulsing were immediately killed by MS-222 overdose. The gills were removed, frozen in liquid nitrogen and stored at −80 °C for 2 months. Subsets of fish were similary killed and sampled at 4, 6, 12 and 18 h post-pulse, with continuous hourly monitoring of water for either

^{14}C or urea, using a chemical assay (Price and Harrison, 1987), to ensure that the fish did not pulse again.

Chemicals, analyses of sequence information and statistical analyses

All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA). Routine analyses of DNA and protein sequences was accomplished using MacVector and DNASTAR programs. Sequences for other animal UTs were obtained by a basic BLAST 2.0 search (Altschul et al., 1997) of GenBank. Statistical significance was tested by Student's *t*-test or analysis of variance (ANOVA), and *post-hoc* comparisons were made using the Student–Newman–Keuls method. Values are presented as means ± S.E.M., and statistical significance is assumed at the $P<0.05$ level.

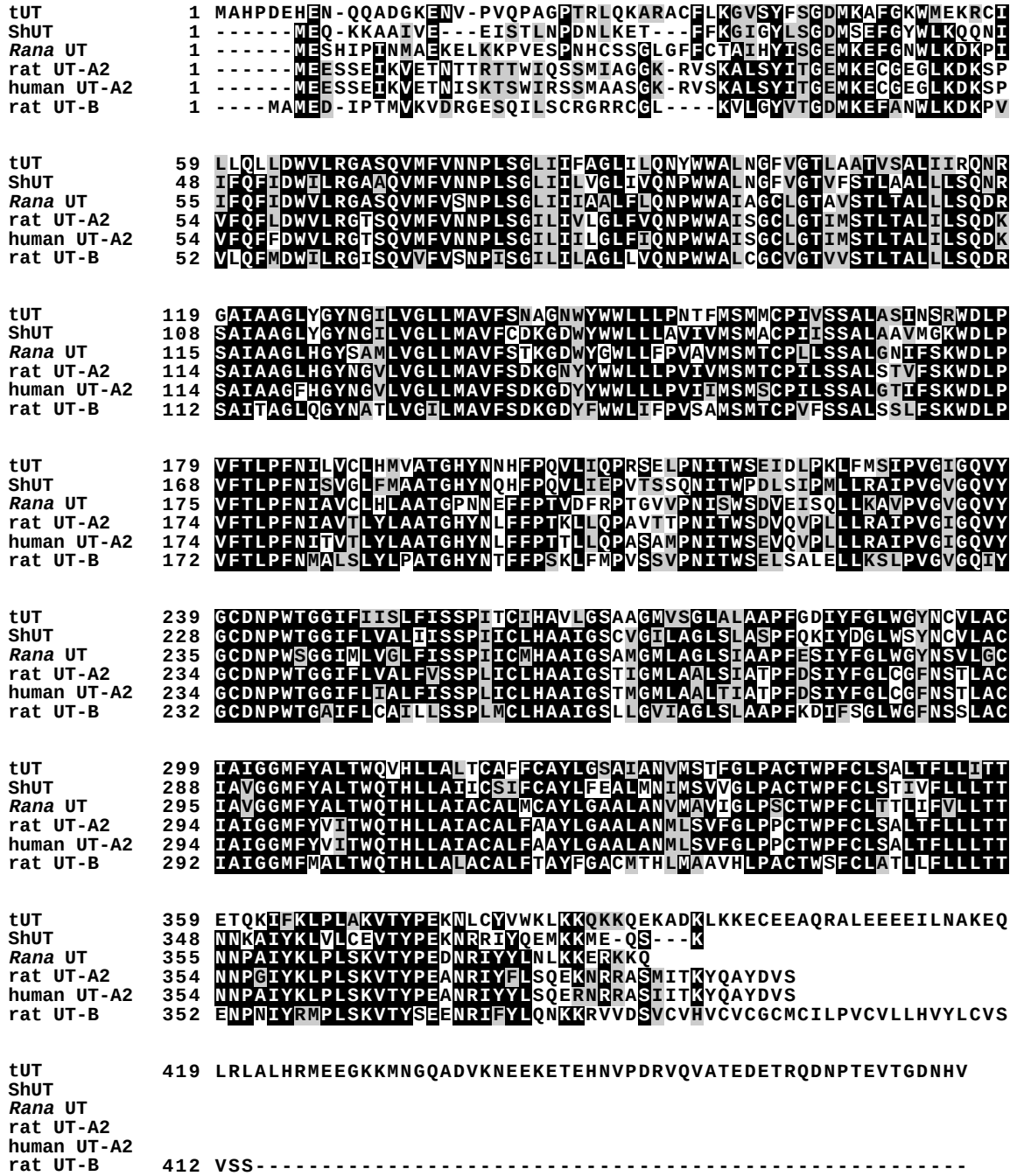

Fig. 1. Alignment of the toadfish urea transport protein (tUT) with other urea transporter (UT) proteins. References and accession numbers are as follows: ShUT, shark (Smith and Wright, 1999); *Rana* UT=fUT *Rana* (Verbavatz et al., 1996), Y12784.1; rat UT-A2=rUT (Smith et al., 1995), Q62668; human UT-A2=HUT 11 (Olives et al., 1994), Q15849; rat UT-B=UT11 (Couriaud et al., 1996), X98399.1. Black boxes denote identical amino acid residues, grey boxes denote conservative substitutions, dashes denote deletions and white areas denote non-identity.

Results and discussion

PCR yielded a 484 bp fragment (PCRT1) with high homology to published sequences for UT transporters, specifically the UT-A2 of mammals (rabbit and rat) and the shark kidney urea transporter (ShUT) (Smith et al., 1995; Smith and Wright, 1999; You et al., 1993). Screening a cDNA library with this probe yielded several clones of approximately 1 kb in length, all of which contained the apparent 3′ end of the sequence, including a poly(A) tail. 5′ RACE PCR using cDNA template yielded approximately 750 bp of additional sequence which overlapped these clones and PCRT1. The use of gene-specific primers based on these sequences then enabled amplification of a 1.8 kb product whose sequence matched the combined sequence of the PCRT1, 5′ RACE PCR product and cDNA library clones.

The full-length toadfish UT cDNA is 1814 bp cDNA (GenBank accession no. AF 165893) and has a polyadenylation sequence (ATTAAA) at nucleotides 1787–1792 and several potential open reading frames (ORFs). One possible ORF begins at nucleotide 125, as indicated by the presence of a modified Kozak site upstream from ATG (GCCACTG) and G at position +4 (whereas the consensus site is GCCA/GCC and G at position +4). This ORF ends at 1552, putatively encoding a 475-residue protein that we have named 'tUT' (toadfish UT) (Fig. 1). A second possible initiation codon in the same frame occurs at nucleotides 259–261, which is preceded by the sequence GGAGAC, and a third at nucleotides 277–279, which is preceded by the sequence TGGAAA and G at position +4. While the first 47 amino acids of the 475-residue protein do not have high homology with other vertebrate UT proteins, beginning at the second and third methionine residues (46 and 53, respectively) of the protein sequence there is higher homology with other vertebrate UT proteins (Fig. 1). Overall, tUT has 62.6 %, 59.8 %, 62.1 %, 56.1 % and 64.4 % amino acid identity with human UT-A2, *Rana* UT, rat UT-A2, rat UT-B and ShUT, respectively, approximately the level of identity of ShUT with mammalian UTs (Smith and Wright, 1999). Several signature sequences of characteristic domains of UTs (Karakashian et al., 1999), e.g. the LP box (residues 177–178), the TYPE domain (residues 372–375), which is a potential phosphorylation site, the P/YWW domain (amino acid residues 94–96), which is part of a leucine zipper motif, and the NIT potential N-glycosylation site (residues 205–207), are highly conserved. Notably, the ALE domain, which is diagnostic of UT-B proteins (residues 215–217 in rat UT-B), is absent from tUT and all other UTs compared, strongly suggesting that tUT is a UT-A. The tUT protein also exhibits a primary structure consistent with several hydrophobic regions in a pattern similar to that of other UTs. A final notable difference between tUT and other UTs is the much longer C-terminal sequence, and this stretch of tUT contains a potential amidation site (EGKK) and two potential phosphorylation sites (TEDE and TRQD) (Fig. 1). It is possible that this additional sequence is related to the unique and rapid upregulation of urea transport in the toadfish gill.

Expression of tUT cRNA in *Xenopus laevis* oocytes induced an increase in urea uptake in comparison with water-injected control oocytes and, as is characteristic of other members of the urea transporter family, the induced permeability was inhibited by phloretin; these experiments were conducted independently in two laboratories and yielded similar results. In the first experiment, tUT expression led to a fivefold enhancement of the rate of urea uptake (Fig. 2). In the second experiment, rates of uptakes were as follows: control, 6.8±2.8 pmol min^{-1} (N=14); tUT-injected, 54±6.4 pmol min^{-1} (N=12); tUT-injected + phloretin, 7.2±3.1 pmol min^{-1} (N=14); water-injected + phloretin, 6.1±2.6 pmol min^{-1} (N=14); these values are means ±1 S.E.M. These combined data indicate that the general functional characteristics of the tUT protein are similar to those previously described for elasmobranch, amphibian and mammalian urea transporters belonging to the UT-A and UT-B families. More detailed studies will be necessary to determine whether the C terminus of tUT, which is putatively unique, confers any special functional characteristics.

Of the six tissues studied (gill, liver, red blood cells, kidney, intestine and skin), only the gill showed a signal in northern analyses (Fig. 3). This was not dependent on the stringency under which the experiments were performed because low-stringency conditions gave the same results as experiments carried out at high stringency. This suggests that, in the tissues we analyzed, apart from gill, there are no other UT-A or UT-B isoforms. The latter can be concluded because UT-B isoforms are clearly detectable in other species using UT-A

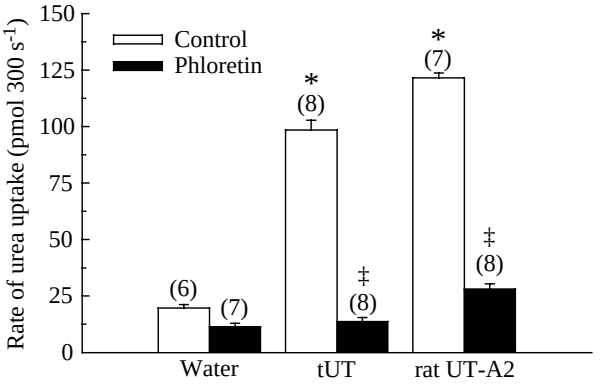

Fig. 2. Effects of toadfish urea transport protein (tUT) expression on urea uptake by oocytes. Summary of the urea accumulated over a period of 5 min by water-injected control oocytes or oocytes expressing tUT or rat UT-A2. Open columns show uptakes under control conditions. Filled columns show uptakes after pretreatment with 0.5 mmol l^{-1} phloretin for 15 min. The experiments were performed in oocytes from two animals, and the results have been normalized to the value for the water-injected control group in each case. Values are presented as means + S.E.M. with the number of oocytes tested in parentheses. An asterisk indicates a significant increase (P<0.05) in urea uptake compared with the water-injected control group, and a double dagger denotes that phloretin caused a significant reduction in uptake compared with the paired control group.

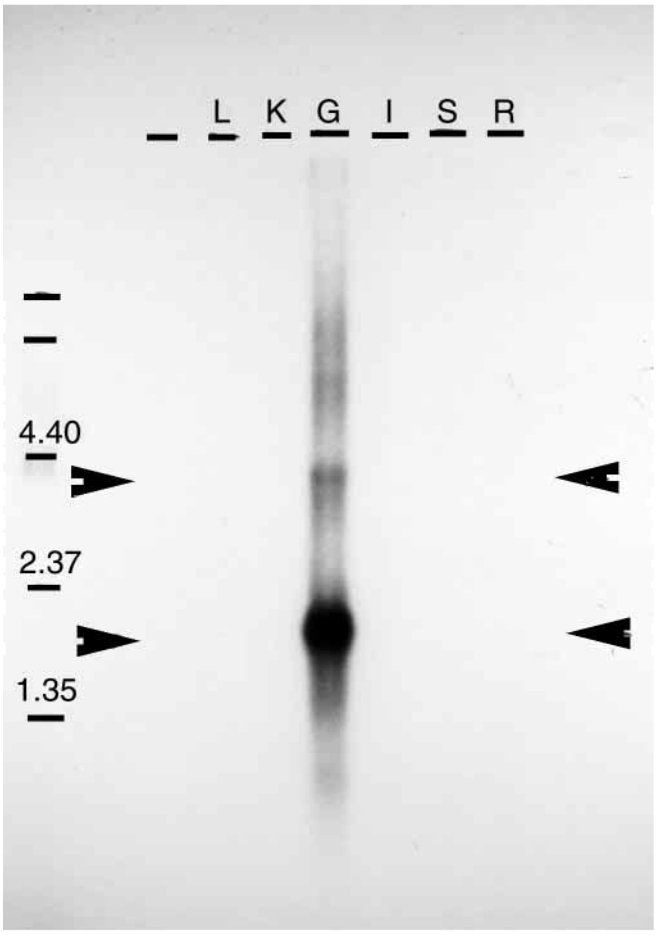

Fig. 3. Tissue distribution of the toadfish urea transport protein (tUT) using high-stringency northern analysis. Membranes were probed using ^{32}P-labelled full-length random-primed tUT cDNA probe and hybridized at 48 °C in 50 % formamide. Final washes were in 0.1×SSC, 0.1 % SDS at 65 °C. Each lane contained 3 μg of poly(A)$^+$ RNA. L, liver; K, kidney; G, gill; I, intestine; S, skin; R, red blood cells. The unlabelled lane is a negative control with no tissue. Analysis at low stringency (not shown) gave an identical pattern of distribution. Markers show relative molecular mass. Arrowheads mark bands of interest.

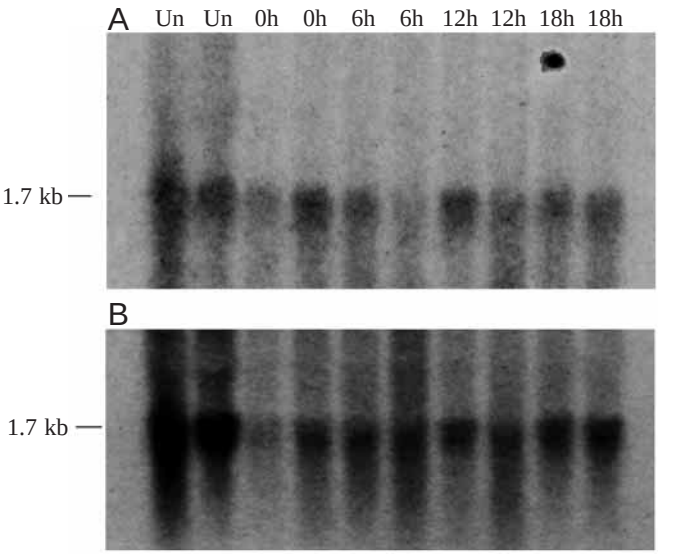

Fig. 4. Representative time course of expression of toadfish urea transport protein (tUT) mRNA in the gill of gulf toadfish *Opsanus beta* using northern analysis. (A) Membranes were probed using ^{32}P-labelled full-length random-primed tUT cDNA probe. (B) Membranes from A were stripped and reprobed with ^{32}P-labelled toadfish β-actin polymerase chain reaction product. Each lane contained 20 μg of total RNA, and membranes were hybridized at 37 °C in 50 % formamide. Final washes were in 0.1×SSC, 0.5 % SDS at 37 °C. Un, unconfined toadfish; 0 h, pulsing toadfish; 6, 12 and 18 h are times post-pulse.

Table 1. *Expression of tUT mRNA relative to β-actin mRNA in the gill of the toadfish* Opsanus beta

Experimental treatment	tUT mRNA/ β-actin mRNA	*N*
Unconfined	1.11±0.20	6
0 h (pulsing)	1.18±0.11	7
4 h post-pulse	1.12±0.34	3
6 h post-pulse	1.26±0.42	4
12 h post-pulse	1.43±0.19	6
18 h post-pulse	0.88±0.18	6

Values are means ±1 S.E.M.; no significant differences were found by one-way ANOVA; *F*-ratio=0.83, *P*=0.54.
tUT, toadfish urea transport protein.

probes. A predominant band was seen at 1.8 kb, which we propose corresponds to tUT cDNA. A far less intense signal was seen at 3.5 kb and may correspond to a larger tUT transcript. In mammals, the UT-A gene is known to give rise to at least four transcripts by alternative splicing (Sands et al., 1997); our study suggests that at least two transcripts are present in toadfish gill. The results of our northern analysis are consistent with all the physiological evidence which points to the gill as the site of urea excretion in toadfish (Wood et al., 1995, 1998; Gilmour et al., 1998; Pärt et al., 1999). The lack of a UT-A2 mRNA signal in toadfish liver is puzzling, given that this is the site of urea production, and we had previously demonstrated a phloretin-sensitive urea efflux from toadfish hepatocytes (Walsh et al., 1994; Walsh and Wood, 1996). However, the recent discovery of aquaporin 9 in human and rat liver suggests that a UT homologue may not be necessary to allow urea to exit the liver. Aquaporin 9 transports water and has a broad specificity for non-charged solutes, including urea, polyols, purines and pyrimidines, in a phloretin- and mercury-sensitive manner (Ishibashi et al., 1998; Tsukaguchi, 1998).

Examination of the dynamics of tUT mRNA expression reveal that it is statistically invariant (relative to β-actin expression) over the time course of our experiments (Fig. 4; Table 1), although there is a trend towards a peak at 12 h and a trough at 18 h. (Note that expression of the 3.5 kb band remained faint and invariant as well; results not shown.)

Nonetheless, these data suggest that, if temporal regulation of tUT is important for the control of pulsatile urea excretion in the toadfish gill, this regulation does not occur at the mRNA level. In this regard, we have recently observed that considerable membrane vesicle trafficking and fusion of vesicles with the apical membrane of gill pavement cells takes place concomitant with the pulse event (P. Laurent, P. J. Walsh, C. M. Wood, P. Pärt, K. Gilmour, S. F. Perry and Y. Wang, in preparation). These results are consistent with the view that the tUT protein is expressed in vesicles, which are then inserted into the membrane or otherwise activated following a proximal stimulus. This vesicular shuttling mode of regulation has been demonstrated for the vasopressin-sensitive water transporter aquaporin 2 (although not for UT-1A) in mammalian kidney collecting duct (Inoue et al., 1999). The cloning and characterization of tUT in this report will enable further studies (e.g. *in situ* hybridization and immunocytochemistry) to test this hypothesis more directly for the toadfish gill.

In summary, we have isolated the first cDNA encoding a teleost urea transporter and demonstrated that it has much in common with its mammalian counterparts. This marks a major step in unravelling the mechanism responsible for the rapid pulsatile increase in toadfish gill urea permeability.

This research was supported by an NSF grant to P.J.W. (IBN-9507239), an NSERC grant to C.M.W. and grants from the Royal Society and NERC to C.P.S. Part of this research was conducted by P.J.W. as a Knight Faculty Fellow awarded by the Knight Foundation administered through the Office of the UM Provost. We thank John Paupe for excellent technical assistance and Jimbo Luznar for supplying toadfish.

References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402.

Chadwick, T. D. and Wright, P. A. (1999). Nitrogen excretion and expression of urea cycle enzymes in the Atlantic cod (*Gadus morhua* L.): a comparison of early life stages with adults. *J. Exp. Biol*. **202**, 2653–2662.

Couriaud, C., Leroy, C., Simon, M., Silberstein, C., Bailly, P., Ripoche, P. and Rousselet, G. (1999). Molecular and functional characterization of an amphibian urea transporter. *Biochim. Biophys. Acta* **1421**, 347–352.

Couriaud, C., Ripoche, P. and Rousselet, G. (1996). Cloning and functional characterization of a rat urea transporter: expression in the brain. *Biochim. Biophys. Acta* **1309**, 197–199.

Gilmour, K. M., Perry, S. F., Wood, C. M., Henry, R. P., Laurent, P., Pärt, P. and Walsh, P. J. (1998). Nitrogen excretion and the cardiorespiratory physiology of the gulf toadfish, *Opsanus beta*. *Physiol. Zool.* **71**, 492–505.

Griffith, R. W. (1991). Guppies, toadfish, lungfish, coelacanths and frogs: a scenario for the evolution of urea retention in fishes. *Env. Biol. Fish* **32**, 199–218.

Gurdon, J. B. and Wakefield, L. (1986). Microinjection of amphibian oocytes and eggs for analysis of transcription. In *Microinjection and Organelle Transplantation Techniques* (ed. J. E. Celis, D. Agraessmann and A. Loytor), pp. 269–299. London: Academic Press.

Hopkins, T. E., Serafy, J. E. and Walsh, P. J. (1997). Field studies on the ureogenic gulf toadfish, in a subtropical bay. II. Nitrogen excretion physiology. *J. Fish Biol.* **50**, 1271–1284.

Hopkins, T. E., Wood, C. M. and Walsh, P. J. (1995). Interactions of cortisol and nitrogen metabolism in the ureogenic gulf toadfish *Opsanus beta*. *J. Exp. Biol.* **198**, 2229–2235.

Inoue, T. J., Terris, C. A., Ecelbarger, C. L., Chou, S. and Knepper, M. A. (1999). Vasopressin regulates apical targeting of aquaporin-2 but not of UT1 urea transporter in renal collecting duct. *Am. J. Physiol*. **276**, F559–F566.

Ishibashi, K., Kuwahara, M., Gu, Y., Tanaka, Y., Marumo, F. and Sasaki, S. (1998). Cloning and functional expression of a new aquaporin (AQP9) abundantly expressed in the peripheral leukocytes permeable to water and urea, but not to glycerol. *Biochem. Biophys. Res. Commun.* **244**, 268–274.

Karakashian, A., Timmer, R. T., Klein, J. D., Gunn, R. B., Sands, J. M. and Bagnasco, S. M. (1999). Cloning and characterization of two new isoforms of the rat kidney urea transporter: UT-A3 and UT-A4. *J. Am. Soc. Nephrol*. **10**, 230–237.

Mommsen, T. P. and Walsh, P. J. (1992). Biochemical and environmental perspectives on nitrogen metabolism in fishes. *Experientia* **48**, 583–593.

Olives, B., Neau, P., Bailly, P., Hediger, M. A., Rousselet, G., Cartron, J. P. and Ripoche, P. (1994). Cloning and functional expression of a urea transporter from human bone marrow cells. *J. Biol. Chem.* **269**, 31649–31652.

Pärt, P., Wood, C. M., Gilmour, K. M., Perry, S. F., Laurent, P., Zadunaisky J. and Walsh, P. J. (1999). Urea and water permeability in the ureotelic gulf toadfish (*Opsanus beta*). *J. Exp. Zool.* **283**, 1–12.

Perry, S. F., Gilmour, K. M., Wood, C. M., Pärt, P., Laurent, P. and Walsh, P. J. (1998). The effects of arginine vasotocin and catecholamines on nitrogen excretion and the cardiorespiratory physiology of the gulf toadfish, *Opsanus beta*. *J. Comp. Physiol.* B **168**, 461–472.

Price, N. M. and Harrison, P. J. (1987). Comparison of methods for the analysis of urea in seawater. *Mar. Biol.* **94**, 307–313.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, second edition. New York: Cold Spring Harbor Laboratory Press.

Sands, J. M., Timmer, R. T. and Gunn, R. B. (1997). Urea transporters in kidney and erythrocytes. *Am. J. Physiol.* **273**, F321–F339.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Smith, C. P., Lee, W.-S., Martial, S., Knepper, M. A., You, G., Sands, J. M. and Hediger, M. (1995). Cloning and regulation of expression of the rat kidney urea transporter (rUT2). *J. Clin. Invest.* **96**, 1556–1563.

Smith, C. P. and Wright, P. A. (1999). Molecular characterization of an elasmobranch urea transporter. *Am. J. Physiol.* **276**, R622–R626.

Tsukaguchi, H., Shayakul, C., Berger, U. V., Mackenzie, B., Devidas, S., Guggino, W. B., van Hoek, A. N. and Hediger, M. A. (1998). Molecular characterization of a broad selectivity neutral solute channel. *J. Biol. Chem*. **273**, 24737–24743.

Verbavatz, J. M., Couriaud, C., Berthonaud, V., Gobin, P., Bailly, C., Ibarra, C., Silberstein, M., Simon, M., Hediger, M., Ripoche, P., Rousselet, G. and de Rouffignac, C. (1996). Cloning, functional characterization and localization of an amphibian urea transporter (Abstract). *J. Am. Soc. Nephrol*. **7**, 1274.

Walsh, P. J. (1997). Evolution and regulation of urea synthesis and ureotely in (batrachoidid) fishes. *Annu. Rev. Physiol.* **59**, 299–323.

Walsh, P. J., Danulat, E. M. and Mommsen, T. P. (1990). Variation in urea excretion in the gulf toadfish, *Opsanus beta*. *Mar. Biol.* **106**, 323–328.

Walsh, P. J. and Wood, C. M. (1996). Interactions of urea transport and synthesis in hepatocytes of the gulf toadfish, *Opsanus beta*. *Comp. Biochem. Physiol.* **113**B, 411–416.

Walsh, P. J., Wood, C. M., Perry, S. F. and Thomas, S. (1994). Urea transport by hepatocytes and red blood cells of selected elasmobranch and teleost fishes. *J. Exp. Biol*. **193**, 321–335.

Wood, C. M., Gilmour, K. M., Perry, S. F., Pärt, P., Laurent, P. and Walsh, P. J. (1998). Pulsatile urea excretion in gulf toadfish (*Opsanus beta*): evidence for activation of a specific facilitated diffusion transport system. *J. Exp. Biol.* **201**, 805–817.

Wood, C. M., Hopkins, T. E., Hogstrand, C. and Walsh, P. J. (1995). Pulsatile urea excretion in the ureagenic toadfish *Opsanus beta*: an analysis of rates and routes. *J. Exp. Biol.* **198**, 1729–1741.

Wood, C. M., Hopkins, T. E. and Walsh, P. J. (1997). Pulsatile urea excretion in the toadfish (*Opsanus beta*) is due to a pulsatile excretion mechanism, not a pulsatile production mechanism. *J. Exp. Biol.* **200**, 1039–1046.

Wright, P. A. (1995). Nitrogen excretion: three end products, many physiological roles. *J. Exp. Biol.* **198**, 273–281.

Wright, P. A., Felskie, A. K. and Anderson, P. M. (1995). Induction of ornithine–urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. *J. Exp. Biol.* **198**, 127–135.

You, G., Smith, C. P., Kanai, Y., Lee, W.-S., Steizner, M. and Hediger, M. A. (1993). Cloning and characterization of the vasopressin-regulated urea transporter. *Nature* **365**, 844–847.