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Localization and regulation of a facilitative urea transporter in the kidney of the red-eared slider turtle (*Trachemys scripta elegans*)

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SUMMARY

Urea is the major excretory end product of nitrogen metabolism in most chelonian reptiles. In the present study, we report the isolation of a 1632 base pair cDNA from turtle kidney with one open reading frame putatively encoding a 403-residue protein, the turtle urea transporter (turtle UT). The first cloned reptilian UT has high homology with UTs (facilitated urea transporters) cloned from vertebrates, and most closely resembles the UT-A subfamily. Injection of turtle UT cRNA into *Xenopus* oocytes induced a 6-fold increase in [^{14}C]urea uptake that was inhibited by phloretin. The turtle UT mRNA expression and tissue distribution were examined by RT-PCR with total RNA from various tissues. Expression of turtle UT mRNA was restricted to the kidney, and no signal was detected in the other tissues, such as brain, heart, alimentary tract and urinary bladder. An approximately 58 kDa protein band was detected in membrane fractions of the kidney by western blot using an affinity-purified antibody that recognized turtle UT expressed in *Xenopus* oocytes. In an immunohistochemical study using the anti-turtle UT antibody, UT-immunopositive cells were observed along the distal tubule but not in the collecting duct. In turtles under dry conditions, plasma osmolality and urea concentration increased, and using semi-quantitative RT-PCR the UT mRNA expression level in the kidney was found to increase 2-fold compared with control. The present results, taken together, suggest that the turtle UT probably contributes to urea transport in the distal tubule segments of the kidney in response to hyperosmotic stress under dry conditions.

Key words: cloning, urea transporter (UT), kidney, osmoregulation, turtle.

INTRODUCTION

Reptiles exhibit a high degree of adaptability for excretory functions. Nitrogenous wastes excreted by the reptilian kidney include variable amounts of uric acid, urea and ammonia, depending on the animal's natural environments. Although urea was previously proposed to cross cell membranes by lipid-phase permeation, it has been found that passive urea movement occurs through facilitative urea transporters (UTs), which have been identified and characterized in cell membranes from the kidney of mammals (e.g. Smith et al., 1995; Shayakul et al., 1996; Karakashian et al., 1999; Smith and Rousselet, 2001; Bagnasco, 2005) and several non-mammalian vertebrates (Smith and Wright, 1999; Hyodo et al., 2004; Konno et al., 2006; McDonald et al., 2006). In mammals, the UT family includes two main groups; the renal urea transporters (UT-A) and the erythrocyte urea transporters (UT-B). The UT-A family includes six isoforms: UT-A1 (Shayakul et al., 1996), UT-A2 (Smith et al., 1995), UT-A3 (Karakashian et al., 1999), UT-A4 (Karakashian et al., 1999), UT-A5 (Fenton et al., 2000) and UT-A6 (Smith et al., 2004), which are encoded by a single gene (*Slc14a2*). Four mammalian UT-As (UT-A1 to UT-A4) are expressed in the renal medulla of the kidney and are regulated by several hormones, such as arginine vasopressin (AVP), glucocorticoids and mineralocorticoids (Shayakul et al., 2000; Wade et al., 2000; Peng et al., 2002; Gertner et al., 2004), and also in various physiological and pathological states (Shayakul et al., 2000; Sands, 2003). UT-A5 and UT-A6 are found, respectively, in the testis and the colon (Fenton et al., 2000; Smith et al., 2004). The UT-B family so far comprises two transporters, UT-B1 and UT-B2, encoded by a single gene (*Slc14a1*) (Lucien et al., 1998). UT-B1 and/or UT-B2 are widely expressed and participate in urea recycling in the descending vasa recta (Timmer et al., 2001; Sands, 2003).

In non-mammalian vertebrates, several cDNAs encoding the UTs have been isolated and characterized from the kidneys and extrarenal tissues (Couriaud et al., 1999; Smith and Wright, 1999; Mistry et al., 2001; Hyodo et al., 2004; Mistry et al., 2005; Konno et al., 2006). These UTs were proposed to play key roles in body fluid homeostasis rather than in the urinary concentrating mechanism in elasmobranchs, teleost fish and amphibians. In the dogfish, a facilitative UT was localized in the renal collecting tubule (Hyodo et al., 2004). Eel UT-C and *Bufo* UT were found to be highly expressed in the renal proximal tubule of eel (Mistry et al., 2005) and distal tubule of *Bufo marinus* (Konno et al., 2006), respectively. The other UTs were also found in the gills of elasmobranchs (Fines et al., 2001), teleost fish (Walsh et al., 2000; Mistry et al., 2001) and frog urinary bladder (Couriaud et al., 1999).

Freshwater turtles that spend much of their life in water excrete equal amounts of ammonia and urea, whereas those with amphibious habitats excrete more urea (Davies, 1981; Campbell, 1995). It has also been reported that semi-aquatic turtles accumulate plasma urea under some physiological conditions, such as desiccation under dry conditions, acclimation to salt environments and winter hibernation. When turtles were exposed to a high salinity, plasma osmolality and concentrations of Na^+, Cl^- and urea increased significantly and urea synthesis also increased (Lee et al., 2006). During hibernation plasma osmolality increased, largely due to the retention of urea, in *Chrysemis picta* (Costanzo et al., 1995). In hatchling turtles, accumulation of urea might be associated with cold hardiness in the winter (Costanzo et al., 2000). In terrestrial chelonians, especially desert species, plasma urea nitrogen concentrations normally vary from 5 to 16.7 $mmol\,l^{-1}$ and the concentrations (range 18 to 62 $mmol\,l^{-1}$) increase during dry seasons (Christopher et al., 2003). These are considered to be mechanisms to elevate plasma osmolality for reducing water loss from the body under severe environments.

Thus, urea probably functions as an osmolyte in cells and extracellular fluids in the chelonians as well as in the elasmobranchs, amphibians and mammals (e.g. Wright, 1995; Bentley, 2002). However, reptilian UT has, so far, not been detected in the osmoregulatory organs, such as kidney, urinary bladder and gastrointestinal tracts. Thus, elucidation of the molecular structure and protein expression of UTs in reptiles may greatly contribute to tracing the evolution of UT systems. Here we report the cloning, molecular characterization, mRNA expression under desiccation and immunohistochemical localization in the kidney of a UT from the red-eared slider turtle, *Trachemys scripta elegans*.

MATERIALS AND METHODS

Animals

Adult male and female specimens, 700–1000 g body weight, of the red-eared slider turtle, *Trachemys scripta elegans* (Seidel 2002), were collected from ponds in Takaoka City, Toyama Prefecture, Japan. The semi-aquatic turtles were housed in plastic containers (45 cm×50 cm×40 cm) containing freshwater at the laboratory and maintained on a 12L:12D cycle at *ca*. 25°C. They were fed goldfish once a week. All tissue samples were taken under deep anesthesia with 5% pentobarbital sodium (80 $mg\,kg^{-1}$ body weight; Dainippon Sumitomo Pharm., Osaka, Japan) and samples except those for histological observation were frozen quickly in liquid nitrogen and stored at –80°C until use. All animal experiments were conducted according to the regulations of the ethics committee of the University of Toyama.

Molecular cloning of turtle UT cDNA

Total RNA was extracted from the kidney using Isogen (Nippon Gene, Tokyo, Japan). First-strand kidney cDNA was synthesized with a First-Strand cDNA synthesis kit (Roche Diagnostics, Basel, Switzerland). Degenerate primers for the UT were designed based on the UT cDNA sequences of the dogfish (GenBank accession no. AB094993), the marine toad (AB212931) and rat UT-A2 (U09957; Table 1). PCR was performed using Biotaq DNA polymerase (Bioline Inc., London, UK) using the following schedule: 94°C for 2 min, 35 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 1 min, and finally 72°C for 10 min. The PCR products were separated electrophoretically in 3% agarose gel containing ethidium bromide (0.5 $\mu g\,ml^{-1}$), and the major band of the predicted size was cut from the gel. The cDNA fragment purified from the gel slice was ligated into pT7Blue T-Vector (Novagen, San Diego, CA, USA), and the plasmid was transformed into XL1-Blue competent cells (Invitrogen, Carlsbad, CA, USA). The plasmid DNA was isolated by a modified alkaline/SDS method (Rapid Plasmid Purification Systems, Marligen Bioscience, MD, USA). The sequencing reaction was performed with a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The nucleotide sequence was determined using an ABI Prism 310 genetic analyzer (Applied Biosystems). The full-length turtle UT cDNA was obtained by 5′- or 3′-rapid amplification of cDNA ends (RACE) with adaptor primers (Takara Bio, Otsu, Japan) and UT gene-specific primers (Table 1), which were designed on the basis of the sequences of cDNA fragments obtained by degenerate PCR.

Tissue distribution of turtle UT mRNA

Tissue expression of turtle UT mRNA was examined by RT-PCR. Total RNA was isolated from various tissues (brain, heart, lung, liver, stomach, small and large intestines, kidney, urinary bladder and cloaca) using Isogen. To prepare the first-strand cDNA, 1 μg of total RNA was reverse-transcribed with the First Strand cDNA synthesis kit. The specific PCR primers (Table 1) were synthesized based on nucleotides 844–864 and 1434–1456 of the turtle UT cDNA sequence (DDBJ accession no. AB308450). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal standard to estimate relative levels of turtle UT mRNA expression. The GAPDH primers (Table 1) were designed based on the partial GAPDH cDNA sequences identified in the kidney of the red-eared slider turtles. PCR was performed with 40 cycles (UT) and 28 cycles (GAPDH) of denaturation (94°C, 40 s), annealing (57°C, 40 s) and extension (72°C, 1 min). The PCR products were separated electrophoretically in 3% agarose gel containing ethidium bromide, and detected by a gel photograph instrument (Printgraph, ATTO, Tokyo, Japan). Band densities were analyzed using Scion Image software (Scion Corporation, Frederick, MD, USA).

Functional characterization using *Xenopus laevis* oocytes

cRNA was prepared from linearized pT7Blue T-Vector (Novagen) containing the entire open reading frame of the turtle UT with *Eco*RI (TOYOBO, Osaka, Japan) and transcribed/capped with T7 RNA (mMESSAGE mMACHINE T7 Ultra; Ambion, Austin, TX, USA). Stage V and VI *Xenopus* oocytes were defolliculated by collagenase (Worthington, Lakewood, NJ, USA) and each oocyte was microinjected with 30 ng cRNA in 50 nl water. After a 3 day incubation in modified Barth's solution (MBS; 88 $mmol\,l^{-1}$ NaCl, 1 $mmol\,l^{-1}$ KCl, 0.7 $mmol\,l^{-1}$ $CaCl_2$, 1 $mmol\,l^{-1}$ $MgSO_4$, 2.5 $mmol\,l^{-1}$ $NaHCO_3$, 5 $mmol\,l^{-1}$ Hepes, pH 7.5) at 18°C, urea transport activity was measured by [^{14}C]urea uptake as previously described (Janech et al., 2003; Konno et al., 2007). Urea uptake was determined for individual oocytes by incubation for 10 min in 2 ml of Barth's medium containing 8 $\mu Ci\,ml^{-1}$ (1.3 $mmol\,l^{-1}$) [^{14}C]urea (GE Healthcare Biosciences, Piscataway, NJ, USA) and 1 $mmol\,l^{-1}$ urea at room temperature. After uptake, oocytes were washed with an ice-cold uptake solution containing 1 $mmol\,l^{-1}$ urea, dissolved in 10% SDS. The radioactivity

Table 1. PCR primers for cDNA cloning and semi-quantitative RT-PCR

Target genes	Application	Sequences
Urea transporter	Degenerate PCR	Sense: 5′-GTNCARAAYCCNTGGTGGRC-3′
		Antisense: 5′-CCANGGRTTRTCRCANCCRTA-3′
	5′-RACE PCR	Sense: 5′-ACATTCTAGAGGCCGAGGCG-3′
		Antisense: 5′-CCACCAATAATAATCTCCTTTGTC-3′
	3′-RACE PCR	Sense: 5′-TTCTTCCCTACGACGCTCATT-3′
		Antisense:5′-ATTCTAGAGGCCGAGGCGGCCGACA-d(T)30N-3′
	Semiquantitative RT-PCR	Sense: 5′-TTCTTCCCTACGACGCTCATT-3′
		Antisense: 5′-TGCATTAGGAAGCCTAGTTAGAC-3′
GAPDH (internal standard)	cRNA synthesis	Sense: 5′-ACAGATATGGAAGACAGTGAGTTTG-3′
		Antisense: 5′-TGCATTAGGAAGCCTAGTTAGAC-3′
	Semiquantitative RT-PCR	Sense: 5′-TCCACTCACGGTCGTTTTC-3′
		Antisense: 59-TGACACCCATCACAAACATAGG-39

was measured by scintillation counting (LSC-5100, Aloka, Tokyo, Japan). Phloretin sensitivity of the UT-mediated [^{14}C]urea uptake was determined by preincubation of oocytes in MBS containing 0.5 mmol l^{-1} phloretin for 20 min and then by incubation in the uptake solution containing radiolabeled urea and 0.5 mmol l^{-1} phloretin dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in the incubation medium was 0.2% or less. As a control, oocytes were incubated in Barth's medium with radioisotope and ethanol. To confirm whether turtle protein was expressed in *Xenopus* oocytes after injection of turtle UT cRNA, cRNA- or water-injected oocytes were evaluated by western blot analysis and immunohistochemical study as described below.

UT polyclonal antibody

A UT polyclonal antiserum was raised by immunizing Japanese white rabbits subcutaneously with a synthetic peptide that included cysteine at the COOH terminus of the amino acid sequence, NH_2-LSKVTYPEC-COOH, conserved in the other UTs. The antiserum was collected and purified using an affinity column bearing the immobilized synthetic peptide with affinity gel beads (Affi-Gel 10, Bio-Rad Laboratories, Tokyo, Japan). The specificity of the antibody raised against the synthetic peptide was confirmed by western blot analysis of an oocyte expressing the turtle UT.

Western blotting of turtle UT expressed in *Xenopus* oocytes and in turtle kidney

cRNA- or water-injected oocytes were incubated in MBS for 3 days, and 20 oocytes each were homogenized in ice-cold membrane isolation solution (250 mmol l^{-1} sucrose, 10 mmol l^{-1} triethanolamine containing 1 μg ml^{-1} leupeptin, and 0.1 mg ml^{-1} phenylmethylsulfonyl fluoride, adjusted to pH 7.6) using a tissue homogenizer (Physcotron NS-310E, Microtech Nition, Chiba, Japan). The homogenates were centrifuged at 2000 ***g*** for 20 min at 4°C to remove unbroken cells, nuclei and mitochondria, and the supernatant was centrifuged at 17,000 ***g*** for 1 h at 4°C to collect a pellet containing the plasma membrane fractions. The pellet was suspended in ice-cold membrane isolation solution containing 1% Triton X-100. Total protein concentration in the samples was measured with a Bio-Rad protein assay reagent utilizing the Bradford method (Bradford, 1976). The samples were solubilized at 60°C for 15 min in Laemmli buffer. Denatured sample (oocyte; 40 μg protein) was loaded on 12.5% polyacrylamide gel for electrophoresis, and the proteins were then transferred from the gel to a nitrocellulose membrane (Hybond-C, GE Healthcare Biosciences). To prevent non-specific binding, the membranes were blocked with 5% skimed milk for 2 h at room temperature and then incubated overnight at 4°C with the UT polyclonal antibody (dilution 1:2000 with 1% BSA-PBS) raised against turtle UT. The membranes were washed with TBS-Tween 20 then incubated with horseradish peroxidase-conjugated anti-rabbit IgG (ECL plus western blotting detection system, GE Healthcare Biosciences) for 2 h at room temperature. After further washing of the membranes with TBS-Tween 20, secondary antibody binding was visualized using the ECL kit.

Western blot analysis of tissue samples from the kidney, heart and liver using the UT polyclonal antibody was performed as stated above.

Immunofluorohistochemistry of turtle UT expressed in *Xenopus* oocytes

Three days after being injected with turtle UT cRNA, *Xenopus* oocytes were fixed with Bouin's fixative overnight at 4°C. The tissue was dehydrated and embedded in paraffin, and sectioned (8 μm). Deparaffinized sections were incubated overnight at 4°C with the UT antibody (dilution 1:2000 with 1% BSA-PBS). After washing in PBS, the sections were incubated with Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen) for 2 h at room temperature in blocking buffer (each for 24 h) and mounted in Mount Quick Aquous (Daido Sangyo, Saitama, Japan). Pictures were taken with a confocal laser-scanning microscope (Nikon Eclipse C1, Nikon, Tokyo, Japan).

Immunohistochemistry of turtle UT in the kidney

The kidney was perfusion fixed *in situ via* the cardiac ventricle with Bouin's fixative, and then removed and post-fixed in the same solution overnight at 4°C. The tissue was dehydrated and embedded in paraffin, and then tissue sections (6 μm) were cut and stained by the immunoperoxidase technique (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). The sections were incubated overnight at 4°C with the UT antibody (dilution 1:2000 with 1% BSA-PBS). Adjacent sections were stained with anti-vacuolar-type H^+-ATPase antibody (dilution 1:10,000 with 1% BSA-PBS) to specifically recognize intercalated cells of the late distal tubule and the collecting duct. Anti-Na^+,K^+-ATPase antibody (dilution 1:4000 with 1% BSA-PBS) was also used to recognize distal nephron in the adjacent sections (Uchiyama and Yoshizawa, 2002; Konno et al., 2006). Immunoreactivity for the UT was visualized with DAB solution (3,3′-diaminobenzidine; Sigma-Aldrich Japan, Tokyo, Japan) containing 0.02% H_2O_2. Sites showing immunoreactivity for the UT, the vacuolar-type H^+-ATPase or the Na^+,K^+-ATPase were confirmed by omitting the primary antibodies, replacing the respective antibodies with rabbit preimmune sera, and immunoreabsorption of antibodies with the synthetic antigens (5 μg ml^{-1}) for 24 h at 4°C. All control preparations were negative for immunostaining. To investigate the distribution of the turtle UT along the nephron of the kidney, we carried out immunohistochemical analysis of turtle kidney sections using an affinity-purified antibody raised against the C-terminal peptide of UT. In order to identify localization of the UT immunoreactive cells in the nephron, two adjacent sections were stained with anti-UT antibody and antibodies against either Na^+,K^+-ATPase or H^+-ATPase.

Plasma components and UT mRNA expression under dry conditions

In the dry acclimation group, turtles were kept in arid conditions and were not allowed access to freshwater during the 7 days of the experiment. In the control group, turtles were maintained in freshwater (0.18 mmol l^{-1} Na^+ and 0.03 mmol l^{-1} K^+). Blood samples were collected by cardiac puncture using heparinized 1 ml syringes. Plasma osmolality and Na^+ concentrations were measured with an osmometer (Osmostat OM-6020, Kyoto Daiichi Kagaku, Kyoto, Japan) and an atomic absorption spectrophotometer (180-70, Hitachi Instruments Service, Tokyo, Japan), respectively. Plasma urea concentration was measured using the Wako Urea NB test (Wako Pure Chemical Industries, Osaka, Japan) *in vitro* enzymatic colorimetric method.

The kidneys of both groups were excised and the total RNA was extracted using the Isogen kit. Semi-quantitative RT-PCR analysis was performed to detect UT mRNA expression levels in the kidneys under dry and wet conditions. RT-PCR analysis was performed as described above.

Statistical analysis

Data are represented as means ± s.e.m. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test, and by Mann–Whitney's *U*-test and Student's *t*-test. Differences at $P<0.05$ were considered statistically significant.

A

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                                 TM1                              TM2
Turtle UT: MEDSEFVRTDNNGERKMYNEKSTRDQRPVGSGKRRICKTLGYLTGEMKECGDWLKDKPLV
Rat UT-A2: MEESSEIKVETNTTR------TTWIQSSMIAGGKRVSKALSYITGEMKECGEGLKDKSPV
Rat UT-B2: MEDIPTMVKVDRGES----------QILSCRGRRWGLKVLGYVTGDMKEFANWLKDEPVG
           **:   :    .                 *     * :   *.*.*:**:***  .: ***:.

Turtle UT: VQFIDWVLRGISQVMFVNNPLSGLLILAGLLIQNPWWTLTGCSGTVVSTLTALILSQDRS
Rat UT-A2: FQFLDWVLRGTSQVMFVNNPLSGILIVLGLFVQNPWWAISGCLGTIMSTLTALILSQDKS
Rat UT-B2: LQFMDWILRGISQVVFVSNPISGILILAGLLVQNPWWALCGCVGTVVSTLTALLLSQDRS
           .**:**:*** ***:**.**:**:**: **::*****:: ** **::******:****:*
                TM3                            TM4
Turtle UT: AIAAGLFGYNGVLVGLLMAVFSDKGDYYWWLLLPVVVTSMACPVLSSALGSVFRKWDLPV
Rat UT-A2: AIAAGLHGYNGVLVGLLMAVFSDKGNYYWWLLLPVIVMSMTCPILSSALSTVFSKWDLPV
Rat UT-B2: AIAAGLQGYNATLVGILMAVFSDKGDYFWWLIFPVSAMSMTCPVFSSALSSLFSKWDLPV
           ****** ***..***:*********:*:***::**  . **:**::****.::* ******
              TM5
Turtle UT: FTLPFNIAVSLYLAATGHYNLFFPTTLIQPVTSVPNITWSEIEVPLLLQSIPVGIGQVYG
Rat UT-A2: FTLPFNIAVTLYLAATGHYNLFFPTKLLQPAVTTPNITWSDVQVPLLLRAIPVGIGQVYG
Rat UT-B2: FTLPFNMALALYLSATGHYNTFFPSKLFMPVSSVPNITWSELSALELLKSLPVGVGQIYG
           ******:*::***:****** ***:.*: *.  :.******::..  **:::***:**:**
                  TM6                  TM7                   TM8
Turtle UT: CDNPWTGGIFLVALLISSPLICLHAAIGSAVGMLAALTIAMPFDRLYFGLASYNCVLACI
Rat UT-A2: CDNPWTGGIFLVALFVSSPLICLHAAIGSTIGMLAALSIATPFDSIYFGLCGFNSTLACI
Rat UT-B2: CDNPWTGAIFLCAILLSSPLMCLHAAIGSLLGVIARLSLAAPFKDIYSGLWGFNSSLACI
           *******.*** *:::****:******** :*::* *::* **. :* **  .:*. ****
                          TM9                        TM10
Turtle UT: AIGGMFYAPTWQTHLLAVACAFFGAYLGAALANSLSVFGLPACTWPFCFSAFLFLLLTTD
Rat UT-A2: AIGGMFYVITWQTHLLAIACALFAAYLGAALANMLSVFGLPPCTWPFCLSALTFLLLTTN
Rat UT-B2: AIGGMFMALTWQTHLLALACALFTAYFGACMTHLIAAVHLPACTWSFCFATLLFLLLTTE
           ****** . ********:***:* **:**.::: ::.. **.***.**:::: ******:

Turtle UT: NSAIYKLPLCKVTYPEANRVYYLAVERNRKATVMTKQPSNMAS
Rat UT-A2: NPGIYKLPLSKVTYPEANRIYFLSQEKNRRASMITKYQAYDVS
Rat UT-B2: NPNIYRMPLSKVTYSEENRIFYLQ---NKKSAVDRPL------
           *. **::**.****.* **:::*    *:::::
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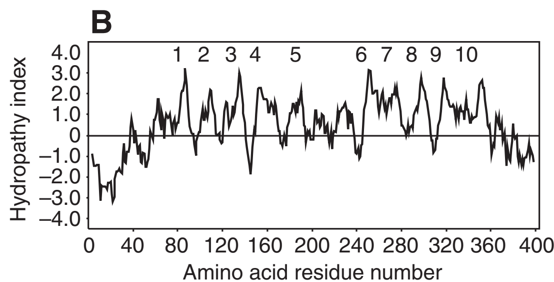

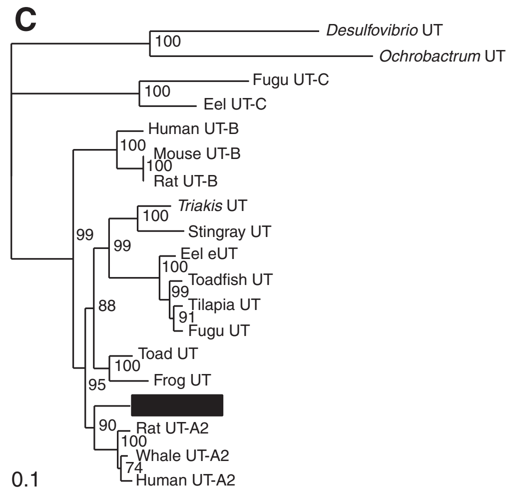

Fig. 1. (A) Primary structure of the urea transporter (UT) isolated from the kidney of the red-eared slider turtle, *Trachemys scripta elegans*. The deduced amino acid sequence is aligned with those of rat UT-A2 (U09957) and rat UT-B2 (U81518) using the Clustal algorithm. Asterisks denote identical amino acid residues to turtle UT. The horizontal bars indicate the predicted transmembrane regions. The box indicates putative *N*-glycosylation sites (NIT). The ALE domain, which is considered to be a signature sequence for the UT-B, is underlined. (B) Kyte–Doolittle hydropathy profile of the deduced Turtle UT amino acid sequence predicts the presence of transmembrane regions (1–10). (C) Phylogenetic tree showing the relationship between vertebrate UTs. The tree was constructed by the neighbor-joining method using ClustalW based on UT sequences. Numbers at branch points are derived from bootstrap analysis (1000 repetitions). UTs of bacteria, *Desulfovibrio* and *Ochrobactrum,* were regarded as outgroups. Scale bar represents a phylogenetic distance of 0.1 amino acid substitutions per site. The position of turtle UT is boxed. Each sequence appears in the protein database with the following accession nos: *Desulfovibrio* UT,YP-010379; *Ochrobactrum* UT, YP-001370983; fugu (pufferfish) UT-C, NP001033079; eel UT-C, BAD66672; human UT-B, AAH50539; mouse UT-B, AAI00571; rat UT-B, EDL84685; *Triakis* UT, BAC75980; stingray UT, AAQ23382; eel eUT, BAC53976; toadfish UT, AAD53976; tilapia UT, AAG49891; fugu UT, NP001027896; toad UT, BAF16706; frog UT, CAA73322; turtle UT, AB308450; rat UT-A2, AAA84392; whale UT-A2, BAF46914; and human UT-A2, CAA65657.

RESULTS

Molecular cloning of turtle UT

The full-length turtle UT cDNA sequence is 1632 bp long, with a putative open reading frame from nucleotides 241 to 1452 encoding a protein of 403 amino acids (DDBJ accession no. AB308450; Fig. 1A). The turtle UT does not contain an ALE domain, which is considered to be a signature sequence for UT-B. Hydropathy analysis using the Kyte–Doolittle algorithm predicted 10 putative transmembrane regions with the N-terminus and C-terminus located in the cytoplasm (Fig. 1B). There is one putative *N*-glycosylation

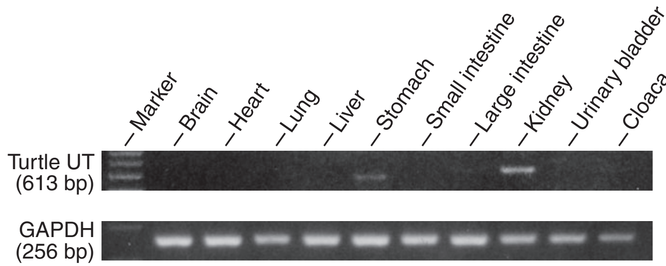

Fig. 2. Tissue-specific expression of turtle UT mRNA determined using RT-PCR. PCR was performed using specific primers for turtle UT and turtle glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Turtle UT mRNA was expressed in kidney but was not detected in the other tissues examined. A smaller band that was shown by sequencing to be non-specific was found in the stomach. In this experiment the expression level of turtle UT was normalized by RT-PCR amplification of the same cDNA templates using the GAPDH primer.

site at amino acids 216–218 (NIT) in the putative central extracellular loop (Fig. 1A). A phylogenetic tree of the UT family proteins including turtle UT is shown in Fig. 1C. The phylogenetic tree indicates that there are three main branches supported by a bootstrap value of 100%: (1) teleost UT-C, (2) mammalian UT-B and (3) lower vertebrate UT and mammalian UT-As. Moreover, the third group is divided into four subgroups: (1) elasmobranchs UT, (2) teleost UT, (3) amphibian UT and (4) turtle UT and mammalian UT-As. The turtle UT is closely related to mouse and rat UT-A2 (75% identity), and *Bufo* UT (70% identity).

Turtle UT mRNA expression and tissue distribution

The turtle UT mRNA expression and tissue distribution were examined by RT-PCR with total RNA from various tissues. As shown in Fig. 2, the expression of turtle UT mRNA was restricted to the kidney, and no signal was detected in the other tissues (brain, heart, lung, liver, stomach, large intestine, urinary bladder and cloacae). A smaller band that was shown by sequencing to be non-specific was found in the stomach sample.

Urea transport through turtle UT

In a *Xenopus* oocyte cRNA expression study, we evaluated whether the turtle UT functions as a urea transporter. The [^{14}C]urea uptake was measured in oocytes injected with turtle UT cRNA or water. After a 10 min incubation, uptake of [^{14}C]urea in oocytes injected with the turtle UT cRNA was significantly increased, being 6-fold greater than that in water-injected control oocytes (Fig. 3). As is the case with the other members of the UT family, the increase of urea uptake through the turtle UT was fully inhibited by 0.5 mmol l^{-1} phloretin, the urea transport inhibitor (Fig. 3).

Identification of immunoreactive turtle UT proteins

To verify turtle UT antibody specificity, turtle UT protein was expressed in the *Xenopus* oocyte system, and immunohistochemical and western blot analyses were performed using the affinity-purified turtle UT antibody. Immunoreactive proteins were detected in the plasma membrane of turtle cRNA-injected oocytes but not in that of water-injected oocytes (Fig. 4A). According to western blot analysis of the membrane fractions prepared from *Xenopus* oocytes, an immunoreactive band was detected at *ca*. 58 kDa in extract from the UT-injected oocytes but not in that of the water-injected oocytes (Fig. 4B). It was subsequently verified that the antibody recognized turtle UT protein.

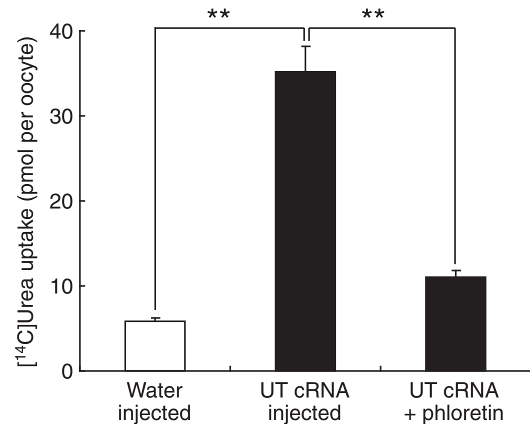

Fig. 3. Analysis of urea uptake in *Xenopus* oocytes injected with turtle UT cRNA or water. Summary of [^{14}C]urea accumulated over a 10 min period by water-injected control oocytes or oocytes expressing turtle UT. Oocytes injected with turtle UT cRNA showed significant increases in urea uptake compared with the water-injected control oocytes; phloretin significantly inhibited turtle UT-mediated urea influx. Open bar represents urea uptake in the water-injected oocyte group after incubation in Barth's medium for 10 min. Filled bars show uptake after incubation in the solution with or without 0.5 mmol l^{-1} phloretin for 10 min. Values are presented as means ± s.e.m. of six oocytes in each group. $^{**}P<0.01$ *versus* control or UT with phloretin treatment (ANOVA followed by Bonferroni's test).

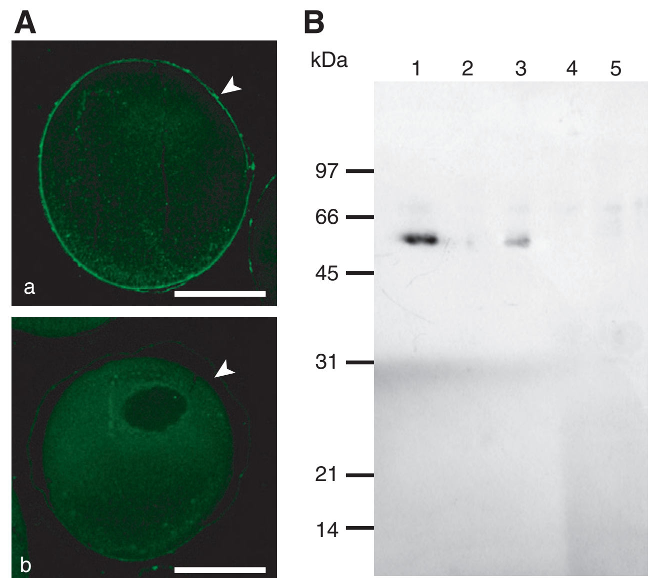

Fig. 4. Confocal microscopic and western blot analyses of *Xenopus* oocytes injected with turtle UT cRNA. (A) Immunofluorescence images for turtle UT protein in turtle UT cRNA-injected oocytes using affinity-purified turtle UT antibody. Immunoreactive proteins were detected in the plasma membrane (arrowheads) of turtle cRNA-injected oocytes (a). The protein was not detected in the plasma membrane (arrowheads) of water-injected oocytes (b). Scale bars, 500 µm. (B) Western blot analysis of the membrane fractions prepared from the turtle UT-injected *Xenopus* oocytes, and those from the desiccated turtle kidney, heart and liver using the anti-turtle UT antibody. An immunoreactive band was detected at an approximately 58 kDa in extract of the UT-injected oocytes (lane 1) and no band was observed in the water-injected oocytes (lane 2). The 58 kDa band was detected in extract of the kidney (lane 3). No band was observed in either heart (lane 4) or liver (lane 5).

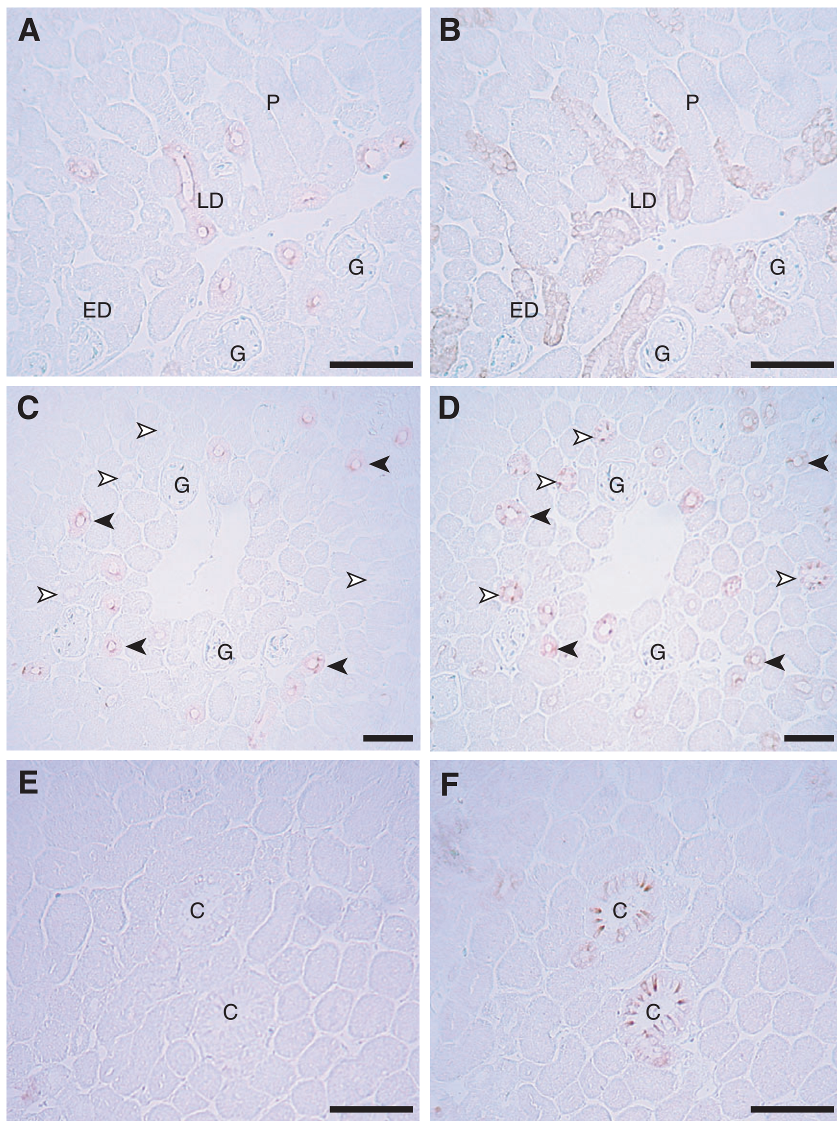

Fig. 5. Immunohistochemical localization of the UT (stained brown) in the turtle kidney using the affinity-purified UT antibody. Using the anti-turtle UT and anti-Na^{+},K^{+}-ATPase antibodies in adjacent mirror sections, immunoreactivity for UT and Na^{+},K^{+}-ATPase was detected on the apical cell membrane of epithelia along the late distal tubule (A) and the distal nephron (B), respectively. In contrast, in adjacent mirror sections immunolabeling with the turtle UT and the H^{+}-ATPase antibodies was observed in the apical membrane of some epithelial cells in the late distal tubules (C) and in the intercalated cells along the late distal tubules (D), respectively. In C and D, filled and open arrowheads show the UT and H^{+}-ATPase immunopositive tubules, and H^{+}-ATPase immunopositive cells along the distal tubules, respectively. In adjacent mirror sections, no UT immunoreactivity was detected in the collecting duct (E) where H^{+}-ATPase was expressed (F). Abbreviations: G, glomerulus; ED, early distal tubule; LD, late distal tubule; P, proximal tubule. Scale bars for A–F, 50 μm.

To detect expression of UT protein in the kidney, we performed western blot analysis using extracts from the kidney of dehydrated turtle. In the membrane fraction, the affinity-purified UT antibody demonstrated prominent labeling of a single band at 58 kDa (Fig. 4B, lane 3). In contrast, an immunopositive band was not detected in extracts from the kidney of normal turtle (data not shown). The molecular mass of the turtle UT protein was similar to that of mammalian UT-A2 (about 55 kDa). As shown in Fig. 4B (lane 4), no immunopositive band was observed when the antibody was preincubated with an excess of the immunizing peptide (1 $\mu g\,ml^{-1}$). In addition, no immunoreactive band was observed in the extract from liver used as a negative control.

Localization of UT in the turtle kidney

UT immunolabeled tubules were not clustered in a bundle but were sparsely distributed among unlabeled tubules. When adjacent mirror sections were treated with anti-turtle UT antibody or with anti-Na^{+},K^{+}-ATPase antibody, the turtle UT antibody predominantly labeled the apical membrane of the cells in the late distal tubule (Fig. 5A), whereas anti-Na^{+},K^{+}-ATPase antibody labeled the distal nephron (Fig. 5B). We also performed the immunohistochemical analysis using both anti-turtle UT and anti-H^{+}-ATPase antibodies in adjacent mirror sections (Fig. 5C–F). The anti-H^{+}-ATPase antibody sharply labeled the intercalated cells along the late distal tubule, connecting tubule and collecting duct (Fig. 5D,F), whereas immunolabeling for the turtle UT was observed in the apical membrane of some epithelial cells in the late distal tubules but not the collecting ducts (Fig. 5C,E). An absorption test showed that the peptide blocked labeling by the anti-turtle UT antibody.

Changes of plasma components during dry acclimation

Following acclimation to the dry conditions for 7 days, the turtles showed a significant loss of body weight, the percentage change in body weight after treatment being –9.4% (Table 2). The decrease in the dry acclimated group was significantly different from that in the control group ($P<0.05$, Mann–Whitney *U*-test). Hematocrit as an indicator of plasma volume was significantly increased relative to that of the control group ($P<0.01$), suggesting a decrease in plasma volume. In desiccated turtles, plasma osmolality, urea and Na^{+} concentrations were significantly increased relative to control ($P<0.01$). Na^{+} and urea concentrations in the bladder urine were also significantly increased compared with values of control groups after 7 days acclimation. The ratio of urine per plasma concentration of Na^{+} ($U/P_{Na^{+}}$) was 0.04 and 0.05 in control and desiccated groups,

Table 2. Plasma parameters in control and dry conditions

Parameter	Control	Dry	Increase (%)
Change in body mass (%)	–4.3±0.8	–9.4±1.7*	–
Hematocrit (%)	20.4±1.0	28.6±2.2**	–
Plasma			
Osmolality (mosmol kg^{-1} H_2O)	286.2±2.6	338.7±14.4**	118
Na^+ (mmol l^{-1})	100.3±8.5	154.9±6.0**	155
Urea (mmol l^{-1})	16.5±1.0	50.6±12.4**	307
Urine			
Na^+ (mmol l^{-1})	3.9±0.3	7.2±0.2**	185
Urea (mmol l^{-1})	61.1±12.9	278.7±61.8**	471

Values are means ± s.e.m. *P<0.05 and **P<0.01, significantly different from control values (Mann–Whitney U-test). All groups, N=6.

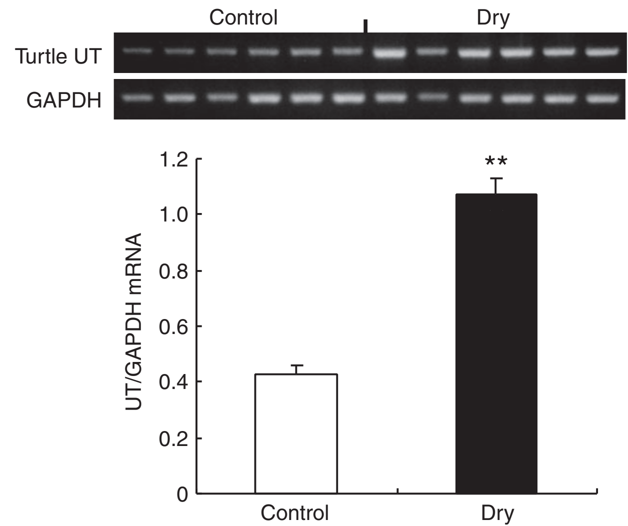

Fig. 6. Expression of turtle UT mRNA relative to GAPDH mRNA in the kidney of control turtles and experimental turtles acclimated to dry conditions for 7 days. The signal for each band was measured by densitometry and is presented as a ratio (turtle UT/GAPDH). The UT mRNA expression was significantly increased more than 2-fold following dry acclimation. Values are means ± s.e.m., N=6. **Significant difference from control (Student's t-test, P<0.01).

respectively. The ratio of urine per plasma concentration of urea (U/P_{urea}) was 3.70 and 5.50 in control and desiccated groups, respectively. This indicates that over 95% of filtered Na^+ was reabsorbed, while filtered urea was reabsorbed passively but net tubular secretion occurred in the renal tubules and/or the urinary bladder.

Effects of dry acclimation on UT mRNA expression in the kidney

Semi-quantitative RT-PCR analysis was examined to clarify whether changes in the levels of UT mRNA expression in the kidney could be responsible for dry acclimation (Fig. 6). When the level of UT mRNA expression was normalized to the expression of GAPDH mRNA, the UT mRNA expression level was found to be two times greater in the kidney of dry acclimated turtles than in control (P<0.01).

DISCUSSION

In the present study, we identified a full-length cDNA encoding a UT from the kidney of the red-eared slider turtle, *Trachemys scripta elegans*, and investigated changes of the UT mRNA expression in the kidney of the turtle under desiccation stress. We also clarified cellular localization of the turtle UT along the nephron segments of the kidney.

Sequence analysis indicated that the turtle UT consists of 403 amino acid residues and lacks the ALE sequence that is diagnostic of UT-B (Timmer et al., 2001; Sands, 2003). The turtle UT, which is a facilitative urea transporter, is similar to those of other species (Couriaud et al., 1999; Smith and Wright, 1999; Mistry et al., 2001; Hyodo et al., 2004; Konno et al., 2006) and closely related to both mammalian UT-A2 (75% identity) and *Bufo* UT (70% identity) (Smith et al., 1995; Konno et al., 2006). On the basis of structural analysis, it seems that non-mammalian UTs show a higher homology to mammalian UT-A2 than the other UT-As and UT-B. Thus, the present result seems to confirm the previous hypothesis that the non-mammalian UTs and the mammalian UT-A2 may all derive from a common ancestral form and that, among the mammalian UTs, UT-A2 may be the most representative of the common ancestral form (see Sands, 2003; Bagnasco, 2005).

According to the tissue-specific expression study of the turtle UT mRNA using RT-PCR, only the kidney exhibited a strong signal for the UT, while no signal was observed in the other tissues including brain, lung, alimentary tracts and urinary bladder. In our previous study, a facilitative *Bufo* UT was expressed in the urinary bladder as well as in the kidney of the marine toad (Konno et al., 2006). Although urea accumulation in the bladder of desiccated turtle was observed in the present study, the turtle UT was not present in the urinary bladder, colon and cloaca of the red-eared slider turtle.

In mammals, it is known that the urea reabsorbed from the collecting ducts is trapped to a large extent in the medulla where it plays a role in the urine-concentrating process of the kidney. UT-A1, UT-A2, UT-A3 and UT-B1 are the major renal isoforms, and it has been shown that these proteins play a vital role in the urinary concentration mechanism in mammals. Two isoforms, UT-A1 and UT-A3, are expressed exclusively in the inner medulla collecting duct cells. In contrast, UT-A2 is localized to the thin descending limbs of Henle's loop in both the inner medulla and the inner stripe of the outer medulla in the mammalian kidney (see Sands, 2003). In an immunohistochemical study using affinity-purified anti-turtle UT antibody, the turtle UT was observed in the apical membrane of some epithelial cells in the late distal tubule segments but not the collecting ducts. As reptilian kidney lacks a countercurrent multiplication system and cannot produce hypertonic urine compared with plasma, the urine-concentrating process known as medullary urea recycling *via* the UTs is certainly not present in the kidney. Although the site or sites of passive reabsorption and/or secretion of urea in the renal tubules of reptiles have not yet been clearly defined, the late distal tubule must play an important role in the red-eared slider turtle.

In the present study, acclimation of red-eared slider turtles to dry conditions induced hyperuremia in addition to hypovolemia and hypernatremia. An increase in urea excretion and a decrease in urinary volume were also observed in dehydrated turtles. Baze and Horne (Baze and Horne, 1970) have reported that dehydrated turtles show high activity of both argininosuccinate synthase and arginosuccinate lyase, which are urea cycle enzymes, and this metabolic change induces the increase in ureogenic activity. Lee and colleagues (Lee et al., 2006) suggested that urea was retained for osmoregulatory purposes in the aquatic soft-shelled turtle, *Pelodiscus sinensis*, acclimated to brackish water. Thus, urea synthesis and excretion may be regulated in response to the turtle's current needs. That is, an increase of plasma urea concentration would be required to keep pace

with the rate of hepatic urea production, and the large fraction of urea filtered by the glomerulus may account for the enhancement of urea transport in the excretory organs. In the present study, the U/P ratios for urea in turtles under control and dry conditions were 3.7 and 5.5, respectively. If the turtle UT acted in urea retention and if urea was just reabsorbed by the kidney, its U/P ratio would be <1. Thus, the present results show that either urea is secreted *via* the UT across the late distal tubule, which would not make sense based on the concentration gradient and increased UT expression during desiccation, or it is trapped further down the kidney tubule or within the urinary bladder as water gets reabsorbed.

Although the collecting duct and urinary bladder seem to have lower urea permeability due to the lack of the turtle UT, it has been reported that the urinary bladder is a substantial site of fluid absorption in turtles and tortoises (see Bentley, 2002). The regulation of urea accumulation under osmotic stress has also been observed in amphibians (Funkhouser and Goldstein, 1973; Jørgensen, 1997; Konno et al., 2006). In our previous study, tissue expression of *Bufo* UT was observed in the urinary bladder as well as the kidney of the marine toad, *Bufo marinus* (Konno et al., 2006). Under acclimation to dry and hypersaline conditions, the plasma urea concentration and osmolality were significantly increased, and these physiological changes were correlated with significant increases in the levels of *Bufo* UT mRNA in both the kidney and urinary bladder (Konno et al., 2006). On the other hand, it is known that some types of water channel, AQP3, AQP7 and AQP9, are certainly permeable to small neutral solutes such as urea and might function as a urea transporter in mammals (e.g. Ishibashi et al., 1994). In turtles, thus, there is a possibility that some channels or transporters like AQPs might be present and function as the urea transporter in the urinary bladder, which accumulates abundant urea.

In conclusion, we have demonstrated that the first reptilian UT cloned from the kidney of turtle belongs to the UT-A2 family of facilitative urea transporter proteins. The present study immunohistochemically shows that turtle UT is located in the apical membrane of epithelia along the distal tubule of the kidney. Under acclimation to dry conditions, the plasma urea concentration and osmolality were significantly elevated, and these physiological changes were correlated with significant increases in the levels of turtle UT mRNA in the kidney. Taken together, the putative facilitative urea transporter expressed in the kidney probably plays a role in the urea retention response to dry conditions in the red-eared slider turtle.

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