**Immunocytochemical and Phylogenetic Analyses of an Arginine Vasotocin-Dependent Aquaporin, AQP-h2K, Specifically Expressed in the Kidney of the Tree Frog, Hyla japonica**

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Water movement occurs across the plasma membrane of various cells of animals, plants, and microorganisms through specialized water-channel proteins called aquaporins (AQPs). We have identified a new member of the amphibian AQP family, AQP-h2K, from the kidneys of Hyla japonica. This protein consists of 280 amino acid residues with two NPA (Asn-Pro-Ala) sequence motifs and a mercury-sensitive cysteine residue just upstream from the second NPA motif. There are two putative N-linked glycosylation sites at Asn-120 and Asn-128 and one protein kinase A phosphorylation site at Ser-262. The AQP-h2K protein was specifically expressed in the apical membrane and/or cytoplasm of principal cells in the kidney collecting ducts. After stimulation with arginine vasotocin, it was translocated from the cytoplasmic pool to the apical membrane. Phylogenetic analysis of AQP proteins from anurans and mammals identified six clusters of anuran AQPs: types 1, 2, 3, and 5 and two anuran-specific types, designated a1 and a2. The cluster AQP2a contains Hyla AQP-h2 and AQP-h3, which are expressed in the anuran urinary bladder and ventral pelvic skin. AQP-h2K belongs to the type 2, together with mammalian (human and mouse) AQP2, suggesting that AQP-h2K is an anuran ortholog of the neurohypophysial hormone-regulated mammalian AQP2 and that the AQP2 molecule is already present in the anuran mesonephros. (Endocrinology 148: 5891–5901, 2007)

**A** NURAN AMPHIBIANS (frogs and toads), the most numerous order of the Amphibia, are widely distributed throughout the world and have adapted to a wide range of water environments. Adult anurans, with the exception of some totally aquatic species, are constantly exposed to the risk of water evaporation through their thin skin when on dry land. To adapt to their habitats, they have developed various complicated osmoregulatory systems for maintaining their water balance. Most adult anurans do not drink through their mouth; rather they absorb it through the ventral pelvic skin which, compared with that of other tetrapods, is highly permeable to water, respiratory gases, and some solutes (1, 2). Water is also reabsorbed from the urine in the adult anuran urinary bladder in response to arginine vasotocin (AVT; the nonmammalian vertebrate counterpart of vasopressin).

The amphibian kidney consists of eight morphologically distinguishable parts: a glomerulus, neck segment, proximal tubule, thin intermediate segment, early distal tubule, late distal tubule, connecting tube, and collecting duct (3). Whereas some of the water filtrated through the glomerulus is considered to be reabsorbed from the neophron in the kidney, to date no amphibian kidney has been observed to produce hyperosmotic urine, possibly because the Henle’s loop, a U-shaped medullary portion of the nephron, is not developed to the same extent as in birds and mammals (4). However, there have been reports of significant amounts of water being reabsorbed from the renal tubule of the amphibian kidney in response to AVT (5) or noradrenaline (6, 7). These results have been supported by the study by Uchiyama (8) showing the possible presence of V2-type AVT receptors in the microdissected connecting tubule of the neophron of bullfrogs based on increases in cAMP levels in response to AVT. However, it has remained an open question whether an appreciable amount of water is reabsorbed from the kidney during rehydration because the water taken up and discharged from the kidney accumulates in and is reabsorbed from the urinary bladder (9).

Aquaporin (AQP) proteins are a class of integral membrane proteins that form selective water channels (pores) in the plasma membranes of various cells of animals, plants, and microorganisms. There is, however, a subfamily of AQPs, termed aquaglyceroporins, which form membrane pores that are permeated by certain small solutes, such as glycerol and urea. To date, 13 isoforms of AQPs (AQP0–AQP12) have been identified in mammals and characterized by cloning and sequencing of their cDNA (10, 11).

We previously cloned cDNAs encoding three distinct AQPs (AQP-h1, AQP-h2, and AQP-h3) from the ventral pel-
vic skins of *Hyla japonica* (12, 13). AQP-h1 was found to be homologous to mammalian AQP1 and showed a ubiquitous tissue distribution, AQP-h2 protein was expressed in the ventral pelvic skin and urinary bladder but not in the kidney, whereas AQP-h3 displayed a specific distribution that was restricted to the ventral pelvic skins. As for *H. japonica*, therefore, water is absorbed from the apical membrane of granular cells in the ventral pelvic skins and from the urinary bladder through the mediation of AQP-h2 together with AQP-h3 or by AQP-h2 alone, respectively. In the granular cells, these proteins are translocated to the apical membrane from the cytoplasmic pools in response to stimulation by AVT (13, 14). In addition, AQP-h3BL, which was found to be homologous to mammalian AQP3, is expressed in the basolateral plasma membrane of these cells, thereby potentially forming the exit channel-proteins for the passage of water (15). Taken together, these findings provide solid evidence for both AQP-h2 and AQP-h3 dependence on AVT stimulation. However, immunoﬂuorescence and Western blot staining did not reveal the presence of AQP-h2 or AQP-h3 proteins in the kidney (12, 13).

We report here the identiﬁcation of a new member of the amphibian AQP family, AQP-h2K, which has been isolated from the kidneys of *H. japonica*. This protein is predominantly expressed in apical plasma membrane or cytoplasm of the collecting duct cells. We also examined phylogenetic relationships among the three AVT-dependent AQPs in the tree frog, AQP-h2, AQP-h3, and AQP-h2K. Bayesian and neighbor-joining analyses revealed that AQP-h2K belongs to the same cluster as mammalian AQP2, whereas AQP-h2 and AQP-h3 belong to an anuran-specific AQPα2 cluster.

**Materials and Methods**

**Animals**

Adult tree frogs (*H. japonica*) were captured in a field near our university (Shizuoka University, Shizuoka, Japan), kept under laboratory conditions, and fed crickets. The animals were 2-2.5 cm long (body length) and weighed 1.0–1.3 g; sex was not considered to be a relevant factor in our experiments. The kidneys were removed from anesthetized animals (MS 222; Nacalai tesque, Kyoto, Japan) for processing and subsequence analysis.

**Oligonucleotide design**

The sense primer and antisense primer used for cloning *Hyla* AQP-h2K are given in Table 1.

**Cloning of the partial-length cDNA**

The kidneys were used for total RNA preparation. The dissected kidneys were immediately frozen in liquid nitrogen, and total RNA was extracted with TRIZOL RNA extraction reagent (Invitrogen, Tokyo, Japan). RT-PCR was performed using Moloney-murine leukemia virus (M-MLV) reverse transcriptase (RT; Invitrogen) according to the manufacturer’s instructions. PCR was performed using primers 1 and 2, as described previously (15). The PCR products were puriﬁed after gel electrophoresis and sequenced using an Aloka DNA sequencer [Model Lic-4200LS; Aloka Co., Ltd., Tokyo, Japan].

**TABLE 1. Primers used for cloning and PCR analysis of *Hyla* AQP-h2K sequence**

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5'-3')</th>
<th>bp</th>
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<tbody>
<tr>
<td>AQP-h2K</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer 1</td>
<td>CTTATCGTGTCCATGATAGTGCT</td>
<td>32–57</td>
</tr>
<tr>
<td>Primer 2</td>
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<td>882–905</td>
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<td>oligo-dT&lt;sub&gt;1&lt;sub&gt;60&lt;/sub&gt;</td>
<td>GGATCCAGAATCTGAAAGC</td>
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<td>3' primer</td>
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<tr>
<td>5' primer</td>
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<tr>
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<tr>
<td>Primer 2</td>
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</table>

**3' and 5' rapid amplification of cDNA ends (RACE)**

The RACE technique was used to obtain the full-length *Hyla* AQP-h2K mRNA sequence. For 3'-RACE, poly(A)<sup>T</sup> RNA was isolated from the total RNA using oligo-dT<sub>19</sub>-coated beads (Oligotex<sup>®</sup>-D<sub>19</sub>) super, Takara, Kyoto, Japan) according to the manufacturer’s instructions. Isolated RNA was primed with oligo-dT<sub>19</sub> adaptor primer and reverse transcribed using M-MLV RT. A 1-μl aliquot of the RT product was then PCR amplified with primer 1 and the adaptor primer; subsequent amplification was performed using the 3' primer and the adaptor primer. After denaturation at 95° C for 5 min, PCR amplification program consisted of five cycles of 60 sec at 94° C, 60 sec at 60° C, and 90 sec at 72° C; five cycles of 60 sec at 94° C, 60 sec at 58° C, and 90 sec at 72° C; five cycles of 60 sec at 94° C, 60 sec at 56° C, and 90 sec at 72° C; and 15 cycles of 60 sec at 94° C, 60 sec at 55° C, and 90 sec at 72° C. The obtained clone was sequenced as described above.

For 3'-RACE, poly(A)<sup>T</sup> RNA was primed with primer 2 and reverse transcribed using the M-MLV-RT. This first-stand cDNA was puriﬁed with a QIA Quick PCR puriﬁcation kit (QIAGEN, Hilden, Germany), and a poly(A) tail was synthesized at the 3' terminus of the cDNA with 1 mM dATP and 0.8 μM/20 μl of terminal deoxynucleotidyl transferase (Toyobo, Osaka, Japan). After the resultant cDNA was puriﬁed once again using the same kit, the second cDNA was synthesized with Ex Taq polymerase (1.25 μl/20 μl) and oligo-dT<sub>19</sub> adaptor primer sequentially for 5 min at 93° C, 90 sec at 42° C, and 3 min at 72° C. A 1-μl aliquot of second-stand cDNA was then ampliﬁed by PCR with the 5' primer and the adaptor primer. This product was then ampliﬁed by PCR with the 5' nested primer and adaptor primer. These PCR ampliﬁcations were carried out under conditions identical with those described for the 3'-RACE-PCR. The obtained clone was sequenced as described above.

**Phylogenetic analysis**

The amino acid sequences of human, mouse, and anuran AQPs were aligned using Clustal W (http://bioweb.pasteur.fr/seqanal/interfaces/clustalw.html) (16), and alignment parameters were set according to an instruction manual by Hall (17), e.g. Gonnet 250 for protein weight matrix, 35 for the gap opening penalty, and 0.75 for the gap extension penalty in the pairwise alignment, and Gonnet series for protein weight matrix, 15 for the gap opening penalty and 0.3 for the gap extension penalty in the multiple alignment. An unrooted tree was generated by the Bayesian method (18), using the MrBayes program version 3.1.2 (http://www.mrbayes.csit.fsu.edu/index.php) (19). Metropolis-coupled Markov Chain Monte Carlo sampling was conducted for 2 million generations (four simultaneous Metropolis-coupled chains; sample frequency 100 generations; chain temperature 0.22 under the JTT (20) + I model. Among the sampled 20,000 trees, the first 5,000 trees were discarded as burnin, and the subsequent trees were used to construct a 50% majority-rule consensus tree. The credibility of clades was evaluated using Bayesian posterior probabilities. Another unrooted tree was inferred by the neighbor-joining (NJ) method (21) in the PAUP program version 4.0 b-10 (Sinauer Associates, Inc., Sunderland, MA). The mean character difference was used to estimate the evolutionary distance. Confidence in the NJ tree was assessed with 10,000 bootstrap replications (22) and used to construct a 50% majority-rule consensus tree.
Antibodies
An oligopeptide corresponding to the C-terminal amino acids 269–280 (ST-202: QTIPRSGMTEKV) of the Hyla AQP-h2K, with an amino- terminal cysteine residue, was synthesized on a model 433A synthesizer (PE Applied Biosystems, Foster City, CA.). The crude peptide was puri- fied by reverse-phase HPLC with a 0–60% linear gradient of CH3CN in 0.1% trifluoroacetic acid. Purification of the peptide was confirmed by measuring its molecular mass by mass spectrometry. The antibody was raised in a rabbit or guinea pig immunized with the ST-202 peptide coupled to keyhole limpet hemocyanin (Pierce, Rockford, IL), as de- scribed previously (23). Similarly, an antibody against Hyla AQP-h1 (12) was raised in rabbits using the C-terminal amino acids 256–266 (ST-153; CYEYELGDGARMEMK) as antigen. The rabbit anti-Hyla AQP-h3BL (15) and bullfrog vacuole proton ATPase E-subunit (V-ATPase) (24) sera had been generated and characterized previously.

RT-PCR of Hyla tissue
The tissue expression of AQP-h2K mRNA was analyzed by RT-PCR. TRIZOL reagent was used to prepare total RNA from various Hyla tissues. Total RNA (20 μg) was first treated with DNase I (4 U; Takara), after which a 10-μg aliquot of the total RNA product was reverse trans-cribed at 37°C for 1 h and then at 52°C for 30 min in 20 μl of reaction buffer containing 1 mm of each deoxynucleotide triphosphate, 9.9 U M-M-LV RT (Takara), 20 U RNase inhibitor (Toyobo), and 7.5 mm oligo-dT19 primer (Operon, Tokyo, Japan). RT-PCR was performed ba- sically by the same method described above, using primers 1 and 2. The RT-PCR products were analyzed on a 2% agarose gel containing ethidium bromide (0.5 μg/ml). Marker 6 (A/Sy1 digest; Wako Pure Chemicals, Osaka, Japan) was used as the molecular-weight marker.

Osmotic water permeability of oocytes
The full-length Hyla AQP-h2K cDNA was produced by RT-PCR using the Kozak sequence with Hyla AQP-h2K-specific primer (GCCACCAT- CATGATGTCAGAC) and Hyla AQP-h2K-specific primer with poly(A)5′ [(T)6GCGATTGCCACAGAC]. The product of the PCR amplification was cloned into the pGEM-5Z vector (Promega, Madison, WI). cRNAs were prepared from linearized pGEM-5Z vectors containing the entire open reading frame of AQP-h2K by digestion with ApaI (Takara), followed by transcription and capping with SP6 RNA polymerase (mCAP RNA cap- ping kit; Stratogene, La Jolla, CA). Stage V and VI Xenopus oocytes were defolliculated by collagenase (1 mg/ml; Roche Molecular Biochemicals, Meylan, France), microinjected with either cRNAs (50 ng) or water, and incubated for 3 d in Barth’s buffer at 18°C, after which they were transferred from 200 mOsm to 70 mOsm Barth’s buffer. The osmotically elicited in-creased water permeability in the oocyte was monitored at 24°C under a BX50 microscope (Olympus, Tokyo, Japan) with a 4× magnifying objective lens and a charge-coupled device camera connected to a computer. The coefficient of osmotic water permeability (Pf) was calculated from the initial slope of oocyte swelling according to accepted methodology (25, 26). In some experiments, AQP-cRNA-injected oocytes were incubated with 0.3 mm HgCl2 for 10 min. To confirm whether AQP-h2K protein was expressed in Xenopus oocytes after the injection of AQP-h2K cRNA, we evaluated AQP-h2K cRNA- injected or water-injected oocytes by Western blot analysis and immuno- staining as described below.

Western blot analysis
The AQP-h2K cRNA-injected oocytes were homogenized in cell lysis buffer [50 mm Tris-HCl (pH 8.0), 0.15 m NaCl, 1% Triton X-100, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin] and centrifuged at 12,000 rpm in a microcentrifuge for 10 min to remove insoluble materials. The protein (10 μg) was denatured at 70°C for 10 min in denaturation buffer comprising 3% sodium dodecyl sulfate, 70 mm Tris-HCl (pH 6.8), 11.2% glyceral, 5% 2-mercaptoethanol, and 0.01% bromophenol blue; subjected to electrophoresis on a 12% polyacryl- amide gel; and then transferred to an Immobilon-P membrane (Milli- pore, Tokyo, Japan). The proteins on the membrane were reacted se- quentially with rabbit anti-AQP-h2K (ST-202) serum diluted at 1:5000, biotinylated goat antirabbit IgG (Dako Japan, Kyoto, Japan), and streptavidin-conjugated horseradish peroxidase (Dako Japan). The reaction products on the membrane were visualized using an ECL Western blot detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). To check the specificity of the immunoreaction, we performed an absorption test by preincubating anti-AQP-h2K (ST-202) with the antigen peptide (10 μg/ml). To determine whether the immunoreactive proteins were glycosylated, we treated the extracts from the cRNA-injected oo- cytes at 37°C with peptide-N-glycosidase F (Daichi Pure Chemicals, Tokyo, Japan) before SDS-PAGE and Western blot in accordance with the manufacturer’s instructions.

Immunofluorescence
The kidney, urinary bladder, and testis were fixed overnight at 4°C in periodate-lysine-paraformaldehyde fixative, dehydrated, and embed- ded in Paraplast. Thin (4 μm) sections were cut and mounted on gelatin- coated slides, deparaffinized, and rinsed with distilled water and PBS. For single labeling of AQP-h2K or AQP-h1 protein, immunofluorescence staining was performed essentially as described previously (27). The sections were sequentially incubated with 1% BSA-PBS, rabbit anti-AQP-h2K serum (2 μg/ml, or rabbit anti-AQP-h1 serum (1:5000), and indocarboxyanine (Cy3)-labeled affinity-purified donkey antirabbit IgG (1:400; Jackson ImmunoResearch, West Grove, PA). For nuclear counterstaining, 6-diamidino-2-phenylindole (DAPI) was included in the secondary antibody solution. The sections were finally washed with PBS and then mounted in PermaFluor (Immunon, Pittsburgh, PA). The specifi- city of the immunostaining was checked using an absorption test by preincubating the anti-AQP-h2K or anti-AQP-h1 antiserum with the corresponding antigen peptide (10 μg/ml). For double-immunofluo- rescence staining for AQP-h2K and AQP-h3BL, sections were first in- cubated with a mixture of guinea pig anti-AQP-h2K (1:2000) and rabbit anti-AQP-h3BL (ST-184: 1:2000) (15) and then reacted with a mixture of Alexa488-labeled goat anti-guinea pig IgG (1:200) and Cy3-labeled don- key antirabbit IgG (1:400; Jackson), and DAPI. Tree frogs, which had been immersed in a container filled with water for 30 min, were used in the stimulation experiments with AVT. The frogs were injected with 50 μl saline or 10−7 m [Arg (8)]-AVT dissolved with saline (Peptide Insti- tute, Osaka, Japan), and 15 min after the injection, the kidneys were fixed with the same fixative solution for examination by immunofluorescence microscopy. Some sections were double stained for both AQP-h2K and bullfrog V-ATPase (24) to identify the parts of a nephron. Specimens were examined with an Olympus BX61 microscope equipped with a BX-epifluorescence attachment (Olympus Optical, Tokyo, Japan).

Statistical analysis
Student’s t test or the Cochran-Cox test for nonhomogeneous data were used for analyzing the results.

Results
cDNA cloning of Hyla AQP-h2K
Figure 1A shows the full-length cDNA sequence of Hyla AQP-h2K and deduced amino acids. The cDNA consisted of a 45-bp 5′-untranslated region and a 213-bp 3′-untrans- lated region followed by a poly(A) tail. An open reading frame encoded a protein of 280 amino acids with a relative molecular mass calculated to be 30,531 Da. Hydropathy analysis predicted six transmembrane regions with an N terminus and a C terminus located in the cytoplasm, which is similar to other major intrinsic proteins family members (Fig. 1B). There were two putative N-linked glycosylation sites at Asn-120 and Asn-128, one protein kinase C phosphorylation at Ser-236, and one protein kinase A phos-
length cDNA sequence has been deposited in DDBJ/EMBL/GenBank (accession no. AB295642).

Phylogenetic analysis of amphibian AQPs

_Hyla_ AQP-h2K showed the highest degree of amino acid sequence similarity to AQP2 (HC-2) of _H. chrysoscelis_ (97%) (28), followed by a high degree of similarity to mammalian AQP2 (69%), including that of mouse, rat, and human. In addition, _Hyla_ AQP-h2K showed sequence similarity to _Xenopus_ AQP (61%; AAN75455), _Bufo marinus_ AQP-t2 (60%; AAC6994), _Hyla_ AQP-h3 (57%) (13), AQP-h3 (57%) (12), and _B. marinus_ AQP-t3 (57%; AAC69955). Bayesian phylogenetic analysis of 25 mammalian AQPs and 19 anuran AQPs suggests that anuran AQPs are divided into six clusters, i.e. types 1, 2, 3, and 5 and two anuran-specific types, designated as a1 and a2 (the letter a represents anuran) (Fig. 2). The cluster AQPa1 comprises AQPxlo from _Xenopus laevis_ oocytes (29) and another _X. laevis_ AQP (BC090201). The cluster AQPa2 is composed of AQP-h2 (13) and AQP-h3 (12) from the tree frog, _H. japonica_, and AQP-t2 (AF020621) and AQP-t3 (AF020622) from the toad, _B. marinus_.

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**Fig. 1.** A, Nucleotide and deduced amino acid sequence of aquaporin (_Hyla_ AQP-h2K) cDNA. The predicted amino acid is shown below the nucleotide sequence (DDBJ/EMBL/GenBank accession no. AB295642). The asterisk indicates the terminal codon. NPA motifs are outlined. The solid triangle indicates a putative N-glycosylation site. The diamond, square, and open triangle indicate phosphorylation sites for protein kinase C and protein kinase A and the mercurial-inhibition site, respectively. B, Kyte-Doolittle hydropathy profile (window 11) of the deduced AQP-h2K amino acid sequence.
Interestingly, the cluster AQP2 contains not only mammalian AQP2 but also AQP-h2K from *H. japonica* and AQP HC-2 from *H. chrysoscelis* (28). Anuran AQP1 molecules cloned from several frogs, including *Hyla* (AQP-h1; AB073315) and *Rana* (FACHIPIP) (30), belong to the clade AQP1 together with mammalian AQP1. The clade AQP3 consists of *Hyla* AQP-h3BL (15), *Xenopus* AQP3 (AJ131847), and *Hyla* HC-3 (22) as well as mammalian AQP3. The clade AQP5 comprises AQPs from *Xenopus* (AQP-x5) (31) and *Bufo* (AQP-t4; AF020623) in addition to mammalian AQP5. NJ analysis corroborates the classification of anuran AQPs into six types (Fig. 3; data not shown for types 1, 3, or a1) and close relationships among AQP0, AQP2, AQP5, AQP6, and AQP2 (Fig. 3).

**Distribution of *Hyla* AQP-h2K mRNA expressed in various tissues**

The distribution of *Hyla* AQP-h2K mRNA expression in various tissues was investigated by means of RT-PCR using total RNA from these tissues. AQP-h2K mRNA was observed in the kidney, urinary bladder, and testis but not the ventral pelvic skin (Fig. 4).

**Expression of *Hyla* AQP-h2K in *Xenopus* oocytes**

Transmembrane water flow through the *Hyla* AQP-h2K protein was evaluated by an expression analysis in *Xenopus* oocytes. After 3 d of incubation at 18 °C, the oocytes were transferred from isotonic (200 mOsm) to hypoosmotic (70 mOsm) Barth’s solution: swelling was monitored by means of a microscope with an attached charge-coupled device camera, and the Pf was calculated (Fig. 5A). The Pf of AQP-h2K-injected oocytes was approximately 4-fold higher than that of water-injected oocytes. The stimulated water permeability was also significantly inhibited by 0.3 mM HgCl2 (Fig. 5, A and B). When sections of the AQP-h2K cRNA-injected oocytes were immunostained with *Hyla* AQP2, an intense immunopositive reaction was observed in the plasma membrane (Fig. 5C-1 and -2). The immunopositive sites were intense immunopositive reaction was observed in the plasma membrane (Fig. 5C-1 and -2). The immunopositive sites were
41–60 kDa became a band of 31 kDa, suggesting that the gestion with peptide-peptide used as the immunogen (Fig. 5D, lane 2). After digestion of the extracts before and after digestion of the extracts by peptide-N-glycosidase F, the smear band at 41–60 kDa became a band of 31 kDa, suggesting that the bands of apparent higher molecular mass represented glycosylated forms of the 31-kDa Hyla AQP-h2K protein (Fig. 5, lanes 3 and 4).

Localization of Hyla AQP-h2K in the kidneys

The anuran nephron is divided into eight parts, among which are the glomerulus, proximal tubule, late distal tubule, collecting duct, and neck segment (3). The collecting ducts which are the glomerulus, proximal tubule, late distal tubule, which are the glomerulus, proximal tubule, late distal tubule, collecting duct, and neck segment (3). The collecting ducts are classified into two types: type 2 and type a2. Hyla AQP-h2K belongs to the type 2 together with mammalian AQP2. The length of each branch is not proportional to the estimated number of amino acid substitutions, and the numbers in the interior branches are bootstrap probabilities (percent; 10,000 replicates). The organism (genus), name, and accession number are indicated for each AQP.

Type 2: AQP2

Human AQP0 (NM120644)
Mouse AQP0 (NM018600)

Type a2: AQPa2

Mouse AQP2 (NM009699)
Hyla japonica AQP-h2K (AB295642)
Hyla chrysoscelis HC-2 (DQ364244)

Type 5: AQP5

Hyla japonica AQP-h2 (AB107014)
Bufo marinus AQP-h2 (AF020621)
Hyla japonica AQP-h3 (AB073316)
Bufo marinus AQP-h3 (AF020622)

Type 6: AQP6

Human AQP6 (NM001651)
Mouse AQP6 (NM175087)

Type 7: AQP-x5

Hyla japonica AQP-h2K (AB295642)
M. marinus AQP-h2K (AF020621)
H. japonica AQP-h3 (AB073316)
M. marinus AQP-h3 (AF020622)

AQP-h2K (AB295642)
Hyla japonica AQP-h2K (AB295642)
M. marinus AQP-h2K (AF020621)
H. japonica AQP-h3 (AB073316)
M. marinus AQP-h3 (AF020622)

Fig. 3. Phylogenetic relationships among AQP0, AQP2, AQP5, AQP6, and AQPa2, seen in the NJ unrooted tree of AQP proteins from the human, mouse, and anurans. AQP-h2K, AQP-h2, and AQP-h3 from H. japonica are classified into two types: type 2 and type a2. Hyla AQP-h2K belongs to the type 2 together with mammalian AQP2. The length of each branch is not proportional to the estimated number of amino acid substitutions, and the numbers in the interior branches are bootstrap probabilities (percent; 10,000 replicates). The organism (genus), name, and accession number are indicated for each AQP.

Fig. 4. Expression of AQP-h2K in Xenopus oocytes. A, Time course of the osmotic swelling. Oocytes were microinjected with water or cRNAs encoding AQP-h2K. Some of the AQP-h2K-injected oocytes were incubated with 0.3 mM HgCl2. B, Pf was calculated from the initial rate of oocyte swelling. The representative data shown are given as the mean ± SE of measurements from five to six oocytes in each experimental group. *, P < 0.01 vs. water; **, P < 0.01 vs. AQP-h2K. C, Immunofluorescence images of the AQP-h2K protein in AQP-h2K-injected oocytes: after complete swelling of the oocytes, immunoreactive AQP-h2K substances are visible, predominately in the plasma membrane (1); the corresponding Nomarski differential interference image (2); in the absorption test, immunopositive substances obtained with anti-AQ-h2K are nearly abolished at background levels in the AQP-h2K-injected oocyte (3), and only background levels are observed in the water-injected oocyte with anti-AQP-h2K (4). Arrowheads indicate the plasma membrane. Bar, 50 μm. D, Western blot analysis of AQP-h2K-injected oocytes (lane 1) prepared after complete swelling. Immunoreactive bands are seen at 31 and 41–60 kDa in an extract of AQP-h2K cRNA-injected oocytes. The membrane was immunostained with the antisem preabsorbed with the antigen peptide (10 μg/ml; lane 2). Western blot analysis of extracts before and after digestion of the extracts by peptide-N-glycosidase F are shown. Specific bands with the extract of the cRNA-injected oocytes seen before and digestion (lane 3) are replaced by a signal band of 31 kDa, presumed to be the nonglycosylated form of Hyla AQP-h2K after digestion (lane 4).
serum (Fig. 8, A and B), but similar labels were seen in the sections of the preabsorbed test of antiserum (Fig. 8C). The labels were also visible in interstitial cells of the testicular stroma (Fig. 8, D and E), but the labels were not diminished in the cells of the testes (Fig. 8F) when anti-AQP-h2K is preabsorbed with the corresponding antigen peptide, indicating that these labels are nonspecific. *Hyla* AQP-h3BL, which is expressed specifically in the basolateral plasma membrane of water-permeable epithelial cells from *H. japonica* (15), was found to be expressed in a similar manner in the basolateral plasma membrane of the collecting duct principal cells in the presence and absence of AVT stimulation (Fig. 9, A and B). The cells showing V-ATPase were scattered throughout the epithelium of the collecting ducts and were unlabeled for AQP-h2K (Fig. 9, C and D).

**Fig. 6.** Immunofluorescence localization of AQP-h2K in the collecting duct of kidney. Fluorescence images of AQP-h2K (A, C, and D) and the corresponding Nomarski image (B) are shown. The labels (green) are clearly visible in the apical plasma membrane and subapical cytoplasm in principal cells of the collecting ducts (c). No labeling is seen in the other renal tubules, including the distal proximal tubules (d). C, Enlarged view of AQP-h2K-positive principal cells (green) in the collecting ducts. D, No labeling is visible in any of the kidney cells when anti-AQP-h2K is preabsorbed with the corresponding antigen peptide. Nuclei are counterstained with DAPI (blue). Bar, 50 μm (A and B); 10 μm (C and D).

**Fig. 7.** Immunofluorescence micrographs showing the principal cells in the kidney collecting ducts labeled for AQP-h2K under nonstimulated and AVT-stimulated conditions, respectively. A and C, Immunolabeling results for AQP-h2K under the nonstimulated condition are shown (green). B and F, Immunolabeling results (green) for AQP-h2K in response to AVT. Upon stimulation of AVT, the labeling for AQP-h2K (green) moved from the cytoplasm to the apical plasma membrane. C and D correspond to the Nomarski images to A and B, respectively. Nuclei are counterstained with DAPI (blue). c, Collecting duct; d, distal tubule. Bar, 50 μm (A–D); 10 μm (E and F).
and the flattened cells of the parietal layer of Bowman’s capsule but not in principal cells of the proximal tubules (Fig. 10, A, B, and D). The labels were abolished at the background levels when the antiserum was preincubated with the antigen peptide (Fig. 10C).

Discussion

We report here the full-length sequence of an mRNA encoding a novel AQP, AQP-h2K, that is specifically expressed in the principal cells of the collecting ducts of tree frogs. This AQP, denoted AQP-h2K, has been structurally characterized as having two NPA motifs, two putative N-glycosylated sites, a cysteine at a mercurial sensitivity site just upstream of the second NPA motif, and six putative transmembrane domains. AQP-h2K also has a putative phosphorylation site by protein kinase A at Ser-262, which is identical with that of mammalian AQP2 (32). The amino acid sequences of Hyla AQP-h2 and Hyla AQP-h3, both of which were cloned in previous studies, also contain a putative phosphorylation site recognized by protein kinase A (12, 13). Phosphorylations are considered to play an important role in the trafficking of mammalian AQP2 and Hyla AQP-h2 to the apical plasma membrane (14, 33, 34). Accordingly, AQP-h2K, similar to Hyla AQP-h2 and AQP-h3, might be translocated from the cytoplasmic pools to the apical plasma membrane by AVT.

The homology analysis revealed that the deduced amino acid sequence of AQP-h2K has a high degree of similarity to mammalian AQP2 and a moderate similarity to AQP-h2 and AQP-h3. The present Bayesian and NJ phylogenetic trees suggested evolutionary relationships between anuran AQPs and mammalian AQPs (Figs. 2 and 3). Previously we depicted an unrooted phylogenetic tree of these AQPs, which had been inferred by the NJ method (35). These three phylogenetic trees show partially different topologies due to the difference in the analytical algorithm, amino acid substitution model, and/or parameter for sequence alignment. For instance, amino acid substitution models and parameters for sequence alignment are different between the previously reported NJ tree and the present NJ tree. The former tree was constructed with the default setting in the Clustal W program version 1.83 (http://www.ddbj.nig.ac.jp/top-j.html) (16), i.e. Kimura’s distance (36) was adopted as amino acid substitution model in tree inference after the amino acid sequences of the AQPs were aligned with Blosum (37) for protein weight matrix and 10 for the gap opening penalty and 0.1 for the gap extension penalty in the pairwise alignment and with Blosum (37) for protein weight matrix, 10 for the gap opening penalty, and 0.2 for the gap extension penalty in the multiple alignment (35). On the other hand, the latter tree was inferred as described in Materials and Methods. The three phylogenetic trees are thus constructed under different conditions, but all the trees indicate that the hitherto reported anuran AQPs can be classified into six clusters: types 1, 2, 3, 5, a1, and a2 (Figs. 2 and 3) (35). Hyla AQP-h2K is assigned to the type 2 together with mammalian AQP2, whereas both AQP-h2 and AQP-h3 belong to the type a2, suggesting that AQP-h2K is an anuran ortholog of mamma-
lian AQP2, whereas AQP-h2 and AQP-h3 are paralogs of mammalian AQP2.

Xenopus oocytes showed a significant increase in water permeability in the presence of AQP-h2K and an inhibition in water permeation in the presence of HgCl2. Although this inhibitory action of HgCl2 is inconsistent with the results reported on AQP2 (HC-2) of *H. chrysoscelis* by Zimmerman et al. (28), they would seem to be logical, given the fact that *Hyla* AQP-h2K protein contains a mercury-sensitive cysteine residue just upstream from the second NPA motif.

Western blot analysis and digestion experiments with peptide-N-glycosidase F of AQP-h2K cRNA-injected oocytes showed one band at approximately 31 kDa and smear band at 41–60 kDa, indicating that the smear band is glycosylated forms. In contrast, Western blot analysis of the extract of *Hyla* kidneys did not produce a positive reaction, even when the membrane extracts of the kidneys, prepared with the ultracentrifuge or the Native membrane protein extract kit, were used (data not shown). This may be because the principal collecting duct cells expressing AQP-h2K protein occupy a very small area of the kidney.

RT-PCR analyses on the expression of AQP-h2K mRNA in various *Hyla* tissues revealed that AQP-h2K was expressed in the kidney, urinary bladder, and testis. The immunofluorescence study demonstrated that AQP-h2K protein was localized in the apical plasma membrane or cytoplasm of the principal cells of the kidneys in situ but that it was not present in the urinary bladder and testes, even though RT-PCR had detected AQP-h2K mRNA in the latter two organs. This finding suggests that AQP-h2K protein is not translated in the urinary bladder and testes. Because mammalian AQP1 is located in the principal cells of the kidney proximal tubules, which consequently reabsorb water constitutively (33, 34, 38, 39), we investigated the localization of *Hyla* AQP-h1 in the anuran kidney by immunolocalization. *Hyla* AQP-h1 was not detectable in any of the cells of the proximal tubules. Anuran amphibian kidney is mesonephric, whereas the mammalian kidney is metanephric, and this difference may be derived from a shift from the mesonephros to metanephros during development. Two water transport pathways have been proposed for the salivary acinar cells of mammals: the paracellular and transcellular routes (40). However, because water is considered not to be reabsorbed in the proximal tubules of anuran kidney and most of the reabsorbed water is stored in the urinary bladder (9), only AVT-dependent AQP, AQP-h2K, may be active in reabsorbing water in the kidney of anuran amphibians.

Immunolocalization of *Hyla* AQP-h2K in the frog kidneys after AVT treatment resulted in enhanced signals for AQP-h2K on the apical plasma membrane of the principal cells of the kidney collecting ducts.

In mammals, AQP2 is involved in the reabsorption of water in the collecting duct of the kidney (25, 41) and is expressed in the apical plasma membrane and cytoplasm just beneath the apical membrane (25, 42). In response to antidiuretic hormone (ADH), mammalian AQP2 is translocated from the cytoplasmic pool to the apical membrane, thereby enhancing the water permeability of the membrane (42, 43); conversely, in the nonstimulated condition, AQP2 is removed from the apical membrane by endocytosis, thereby decreasing water permeability (43). Thus, mammalian AQP2 is considered to be an ADH-regulated AQP. In the present study, we obtained data indicating that AQP-h2K resides in the vesicles distributed throughout the entire cytoplasm in the nonstimulated condition and that it is translocated to the

![Fig. 10. Immunofluorescence localization of AQP-h1 in the kidney.](https://academic.oup.com/endo/article/148/12/5891/2501790)
apical plasma membrane in response to AVT. Based on these results, we propose that Hyla AQP-h2K is a water-channel molecule that is regulated in response to AVT.

Several lines of evidence have shown that protein kinase A phosphorylation at the consensus Ser-256 is a necessary condition for the migration of mammalian AQP2 from the intracellular membrane vesicles to the apical plasma membrane (44–46). Because there is a protein kinase A site at Ser-262 in AQP-h2K, phosphorylation at this site may be necessary for the translocation of AQP-h2K protein to the apical plasma membrane. We have previously shown that in the granular cells of the urinary bladder, Hyla AQP-h2 is phosphorylated in the vesicles within 2 min of AVT stimulation and subsequently translocated to the apical membrane of these same cells (14). Similar events may occur with Hyla AQP-h2K.

Taken together, our present and previous data (12, 13) demonstrated that an AVT-dependent AQP is expressed in the collecting ducts of the kidney of H. japonica and that there are three distinct types of AVT-dependent AQPs in this tree frog: AQP-h2, AQP-h3, and AQP-h2K. These AQPs show different tissue distribution and seem to play important roles in the hormone-dependent water transport in three anuran osmoregulatory organs: AQP-h2 and AQP-h3 in the ventral pelvic skin, AQP-h2 in the urinary bladder, and AQP-h2K in the kidney.

Acknowledgments

We are grateful to Dr. S. Hyodo (Ocean Research Institute, University of Tokyo) for his valuable advice on phylogenetic analysis.

Received May 9, 2007. Accepted August 31, 2007.

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This work was supported in part by a grant-in-aid for science research from the Ministry of Education, Science, Sports, and Culture of Japan (to T.S.).

Disclosure Statement: The authors have nothing to disclose.

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