Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 10997–11001, November 1994
Physiology

Cloning and expression of AQP3, a water channel from the medullary collecting duct of rat kidney

(renal water transport/major intrinsic protein family)

MIRIAM ECHEVARRIA*, ERICH E. WINDHAGER*, SURESH S. TATE†, AND GUSTAVO FRINDT*‡

Departments of *Physiology and Biophysics, and †Biochemistry, Cornell University Medical College, 1300 York Avenue, New York, NY 10021

Communicated by Edward A. Adelberg, July 25, 1994

ABSTRACT **The terminal part of the inner medullary collecting duct exhibits a high degree of water permeability that is independent of increased intracellular cAMP and not accounted for by the activity of the known renal epithelial water channels CHIP28 (28-kDa channel-forming integral protein) and WCH-CD (collecting duct water channel protein). Starting with rat kidney papilla mRNA, reverse transcription PCR was performed with degenerate primers assuming that the putative channel would be a member of the major intrinsic protein (MIP) family of proteins. A cDNA fragment was identified and used to screen a rat kidney cDNA library. A 1.9-kb cDNA clone was isolated. The open reading frame of 876 bp coded for a protein of 292 amino acids (M_r, 31,431). Aquaporin 3 (AQP3; 31.4-kDa water channel protein) is a newly discovered member of the MIP family. Northern blot analysis showed a single transcript for AQP3 of ≈1.9 kb present in the renal medulla, predominantly in the inner medulla. With *in situ* hybridization, abundant message was found in the cells of the medullary collecting ducts. Injection of the complementary RNA of AQP3 into *Xenopus* oocytes markedly increased the osmotic water permeability. This permeability had an energy of activation of 3.0 kcal/mol (1 cal = 4.184 J), it was fully blocked by 1 mM *p*-chloromercuriphenylsulfonate, and this inhibition was reversed by 5 mM dithiothreitol. cAMP did not increase this water permeability. AQP3 did not permit passage of monovalent ions (Na, K, Cl); however, it is slightly permeable to urea. The present study demonstrates the existence of an additional water channel, AQP3, in epithelial cells of the medullary collecting duct.**

Measurements of the osmotic water permeability (P_f) of *Xenopus laevis* oocytes injected with poly(A)$^+$ RNA from different portions of the rat kidney led us to conclude that the kidney contains several structurally distinct water channels (1). At present, two of these channels located in renal tubule cells have been cloned. One of them, CHIP28 or aquaporin 1 (28-kDa channel-forming integral protein), is abundant in the apical and basolateral cell membranes of proximal tubules and thin descending limbs of Henle's loop and is also found in nonrenal cells (2, 3). The other, WCH-CD or aquaporin 2 (collecting duct water channel protein), originally cloned from rat kidneys (4), is the vasopressin-regulated water channel (5). WCH-CD was immunolocalized in apical membranes of the principal cells of both the collecting tubule and the inner medullary collecting duct (IMCD) of rat kidneys (4, 6). In addition, there was heavy labeling of abundant small subapical vesicles and of membrane structures within multivesicular bodies of the principal cells. Low-density labeling was also observed in the basolateral membranes of the principal cells of the outer two-thirds of the IMCD (6). A third water channel, MIWC, that has been cloned from lung tissue has also been found in the kidney but only in vasa recta (7). However, despite these advances, the high water permeability of some nephron segments remains unknown in molecular terms.

Thus, in the terminal portion of the IMCD the transepithelial P_f is much higher than in the initial IMCD, even in the absence of detectable levels of intracellular cAMP (8, 9). This high P_f is not accounted for by the presence of WCH-CD, because immunolocalization studies have failed to detect it in this segment (6). In fact, our previous results in oocytes injected with kidney papilla mRNA have indicated the presence of a water channel different from CHIP28 (1). Furthermore, an additional water channel may be located in the basolateral cell membrane of the principal cells of the collecting duct. The P_f of this membrane is high even in the absence of vasopressin (10, 11) and WCH-CD is not abundant on this side of the principal cells (6). The goal of this study was to clone a putative medullary water channel assuming that, as are CHIP28, WCH-CD, and MIWC, it would also be a member of the MIP family (major intrinsic protein of the mammalian lens) of proteins (7, 12).§

MATERIALS AND METHODS

Reverse Transcription (RT)-PCR Cloning of a Renal Papilla cDNA Fragment. Poly(A)$^+$ RNA was prepared from cortex, medulla (without the papilla), and papilla of rat kidneys by the RNA/zol method (Cinna/Biotecx Laboratories, Friendswood, TX) as described (1). Two micrograms of papilla mRNA was used in a RT reaction mixture to synthesize the first-strand cDNA, which, in turn, served as template in a PCR. This RT-PCR was carried out with a cDNA cycle kit (Invitrogen) and two degenerate primers designed on the basis of conserved amino acid sequences in the MIP family. The sense primer [5′-(A/G)T(C/G/T)(T/A)(C/G)(A/C/T)GG(A/C/G/T)G(G/C)(A/C/G/T)CA(T/C)(A/C/G/T)T(A/C/G/T)AA(T/C)CC-3′] and the antisense primer [5′-G(A/G/C)(A/C/G/T)(C/G)C(A/C/G/T)A(A/G)(A/C/G/T)(C/G)(A/G/T)(A/C/G/T)(C/A)(G/T)(A/C/G/T)GC(A/C/G/T)GG(A/G)TT-3′] corresponded to amino acids 70–76 and 212–219, respectively, of the CHIP28 sequence. Thirty PCR cycles were carried out (each comprising 1 min at 94°C, 2 min at 50°C, and 3 min at 72°C) and the last cycle ended with 10 min at 72°C. Agarose gel electrophoresis of the PCR products showed two bands of 400–600 bp. The total PCR products were inserted into the pCR II plasmid vector, which was introduced into *Escherichia coli* (Invitrogen) for cloning.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CHIP28, 28-kDa channel-forming integral protein; WCH-CD, collecting duct water channel protein; MIP, major intrinsic protein of the mammalian lens; IMCD, inner medullary collecting duct; AQP3, aquaporin 3; cRNA, complementary RNA; pCMBS, *p*-chloromercuribenzenesulfonic acid.

‡To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L35108).

Twelve colonies containing inserts of 400–600 bp were selected, and the inserts were sequenced by the dideoxynucleotide chain-termination method with the Sequenase 2.0 kit (United States Biochemical). Two of these clones had an identical sequence of 393 bp between the end of the sense primer and the beginning of the antisense primer. A 30-nucleotide antisense probe based on this cloned sequence was synthesized (Oligos Etc., Guilford, CT) and used to screen a rat kidney cDNA library. This probe corresponded to the segment +263 to +292 of the cDNA sequence and had no homology with the rest of the members of the MIP family. The probe was ^{32}P-labeled with the DNA 5′-end-labeling system (Promega).

Screening of Rat Kidney Library. The library was constructed in the plasmid pSPORT1 using the SuperScript protocol (GIBCO/BRL) as described (13). The cDNAs were directionally inserted between the *Sal* I and *Not* I sites of the vector and were flanked by T7 and SP6 RNA polymerase promoters at their 5′ and 3′ ends, respectively. Screening was carried out as described (14). The bacterial library suspension was plated on 137-mm nitrocellulose filters (Millipore). Prehybridization was done at 37°C for 1 h in a solution containing 6× saline sodium citrate buffer (SSC), 5× Denhardt's reagent, 0.05% sodium pyrophosphate, 0.5% SDS, and 100 μg of boiled herring sperm DNA per ml. Hybridization was done overnight at 65°C in a solution containing 6× SSC, 1× Denhardt's reagents, 0.05% sodium pyrophosphate, and 100 μg of herring sperm DNA per ml. Subsequently, the filters were washed with gentle agitation in 6× SSC/0.05% sodium pyrophosphate four times at room temperature for 10 min and once for 30 min at 65°C. In the first round of screening, 10 plates were seeded with ≈25,000 clones per plate. A total of 50 positive colonies was identified and 16 of these were individually grown by seeding them at ≈200 clones per plate. Multiple positive colonies per plate were found in this second round of screening. From each plate, one positive colony was individually grown by seeding at ≈10 clones per plate. Every colony was positive in this final screening. One colony from each of these 16 plates was then individually amplified and the plasmid DNA was subsequently purified by using an anion-exchange column system (Qiagen, Chatsworth, CA). The insert in each of these plasmids was released with *Sal* I and *Not* I enzymes to measure the insert size in agarose gel electrophoresis. About 200 bp was sequenced from both ends for each clone using the Sequenase 2.0 kit (United States Biochemical) and the M13/pUC and T7 primers. In addition, two clones were fully sequenced by using synthetic oligonucleotide primers deduced from the partially determined sequence.

Complementary RNA (cRNA) Synthesis and Expression in *X. laevis* Oocytes. Capped cRNA was synthesized from one clone using T7 polymerase (mCAP mRNA capping kit; Stratagene) after linearization of the vector with *Not* I. Fifty nanoliters of water, capped cRNA, or kidney papilla mRNA solutions was microinjected into *Xenopus* oocytes. After 3–4 days of incubation at 20°C, the oocyte P_f was determined by measuring the rate of swelling of the oocyte on exposure to a hypoosmotic solution. The oocyte volume was measured every 20 s while superfused in a chamber mounted on an inverted microscope equipped with a video camera. The preparation, injection, and incubation of oocytes as well as the measurement of P_f and of the energy of activation of this P_f were carried out as described in detail (1).

Urea Permeability and Ionic Conductance of the Oocytes. The permeability to urea of oocytes injected with either cRNA or water was measured by a procedure similar to that used to measure P_f. The volume of an oocyte was measured every 20 s during superfusion with isoosmotic Barth's solution. The composition of this solution was 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.4 mM $CaCl_2$, 5 mM Hepes (pH 7.4). After 3 min, the superfusate was changed to an isoosmotic Barth's solution in which the NaCl was substituted by 165 mM urea and the oocyte volume was measured for 3 min more. The permeability to urea (P_u) was calculated

$$P_u = dV/dt \times 1/A,$$

where dV/dt is the slope of the linear fit in the plot of oocyte volume vs. time in urea-containing solution and A is the membrane area assuming that the oocyte is a sphere without microvilli. It is assumed that at time 0 the intracellular urea concentration is 0.

To measure the oocyte membrane conductance, the oocyte was impaled with two glass microelectrodes filled with 1 M KCl. The conductance was calculated from the slope of the *I–V* curve relating the current required to clamp the membrane voltage of the oocyte at values ranging between −40 and +40 mV (1).

Northern Blot Analysis. Poly(A)$^+$ RNA (2 μg) prepared from cortex, medulla, and papilla of rat kidney was fractionated on 1.4% agarose and 18% formaldehyde gels and blotted onto nitrocellulose filters as described (15). These filters were hybridized with the same probe and according to the protocol described above in the hybridization and washing steps of the library screening.

***In Situ* Hybridization.** The protocol described by Bondy *et al.* (16) was followed. Briefly, the rat kidneys were perfused *in situ* with phosphate-buffered saline (PBS) containing 4% paraformaldehyde. Coronal sections (2–3 mm thick) of the kidney were immersed in the same fixative solution for 30 min and then equilibrated in 30% sucrose/PBS overnight. The tissue was embedded in Tissue-Tek compound and frozen for sectioning (≈15 μm) on a cryostat. Serial sections were mounted on poly(L-lysine) and gelatin-coated glass slides and heated for 2 min at 55°C. The sections were then fixed for 30 min in PBS containing 4% paraformaldehyde, washed in PBS, treated with acetic anhydride (0.15% in 0.1 M triethanolamine buffer) for 10 min, and dehydrated in a series of ethanols and hybridized overnight at 55°C. The composition of the hybridization solution was 4× SSC/50% formamide/1× Denhardt's reagent/5% dextran sulfate/0.5 mg of salmon sperm DNA per ml/0.25 mg of yeast tRNA per ml/2.5 × 10^7 dpm of ^{35}S-labeled sense and antisense RNA probes per ml for the 31.4-kDa water channel protein aquaporin 3 (AQP3). Full-length RNA probes for AQP3 were prepared according to the protocol described by Ausubel *et al.* (14) and subsequently hydrolyzed for 56 min at 60°C in alkaline buffer (60 mM Na_2CO_3/40 mM $NaHCO_3$) to fragments of ≈150 bases. The slides were rinsed three times with 4× SSC containing 0.4 mM dithiothreitol and then treated for 30 min with RNase A (20 μg/ml) dissolved in 0.5 M NaCl/10 mM Tris·HCl, pH 8.0/1 mM EDTA. Subsequently, the slides were rinsed with 2× SSC, 1× SSC, and 0.5× SSC (containing 0.4 mM dithiothreitol) for 15 min each and then washed with 0.1× SSC for 30 min at 55°C and 15 min at room temperature. Slides were then dehydrated through a graded series of ethanols and an autoradiogram of the tissue was made over 72 h of exposure. Finally, the slides were dipped in Kodak NTB-2 autoradiographic emulsion at 42°C, exposed for 7 days at 4°C, and developed in Kodak D-19 developer at 14°C for 3.5 min.

RESULTS AND DISCUSSION

Analysis of the sequences of the PCR products identified three different clones; one corresponded to a fragment of CHIP28, another corresponded to a fragment of WCH-CD, and the third one was a newly determined sequence. The amino acid sequence encoded by this clone had homology to the MIP family. It contained, in addition to the 2 amino acid

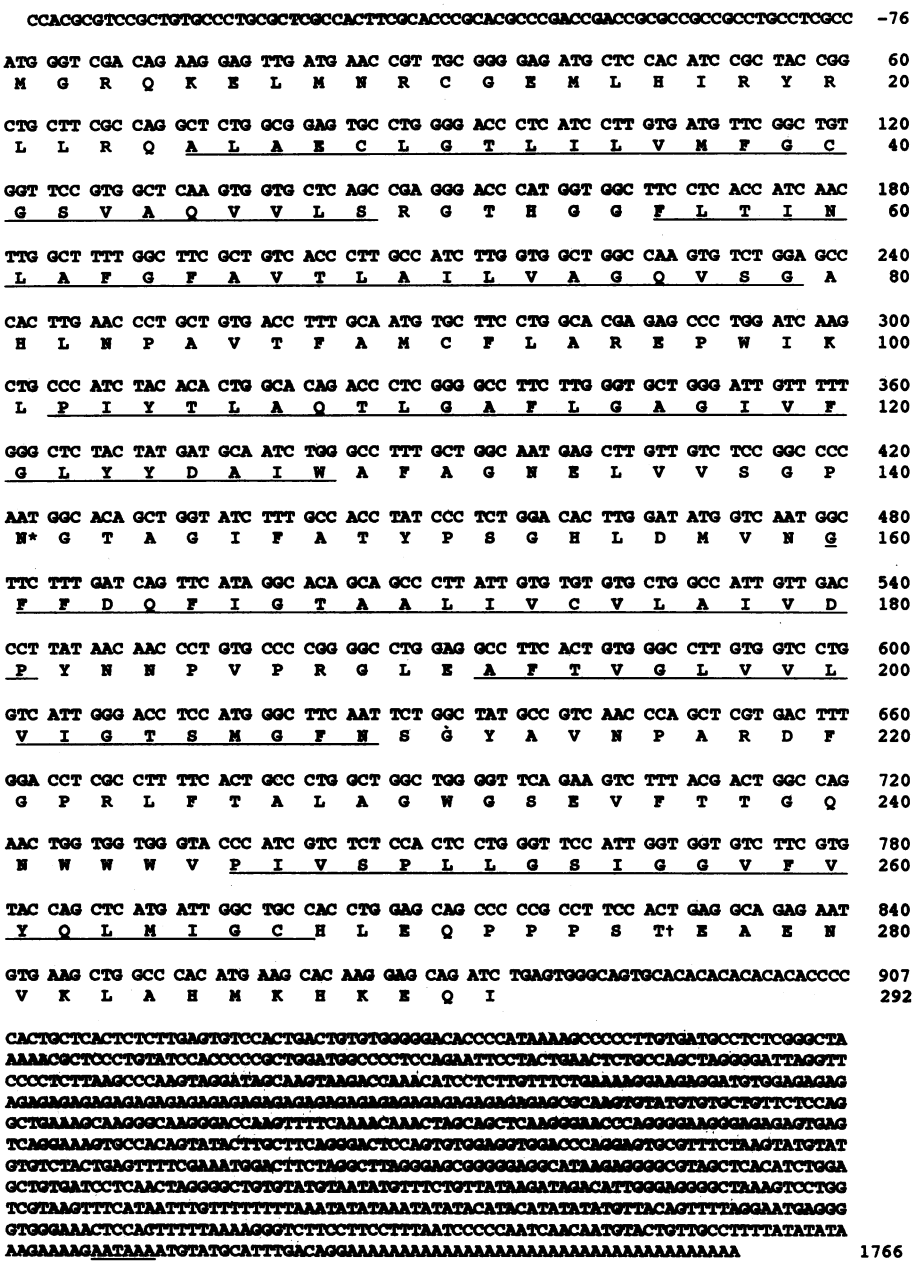

FIG. 1. (*A*) Nucleotide and deduced amino acid sequence of AQP3. The putative transmembrane domains (six) are underlined. Also underlined is the major polyadenylylation signal preceding the poly(A)$^+$ tail. Consensus sequences for the potential N-linked glycosylation site (*) and the phosphorylation site for casein kinase II (†) are indicated. (*B*) Hydrophobicity profile of the amino acid sequence of AQP3 with a window of 12 residues. Putative membrane-spanning domains are numbered from I to VI.

motifs SGAH and NPAR targeted by the primers, the sequence NPAVT, which is also highly conserved among the members of the family. The size of this clone, between the N (asparagine) codons of these conserved sequences, was 393 bp—i.e., 48 bp longer than the corresponding cDNA segments for CHIP28 and WCH-CD.

To obtain the full-length cDNA of this partial clone, a rat kidney cDNA library was screened at high stringency with a 30-nucleotide probe designed on the PCR fragment. A positive clone was isolated and sequenced. The cDNA insert was 1842 bp long, with an open reading frame of 876 bp (Fig. 1*A*). The first ATG is a likely initiation codon because it consti-

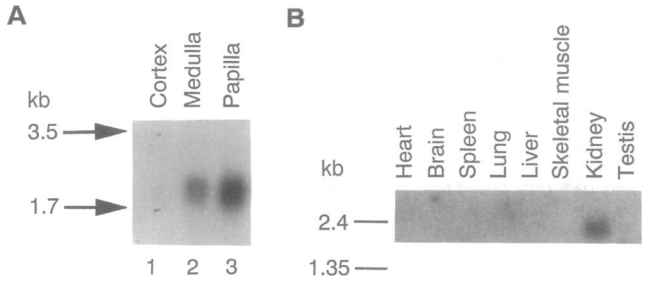

FIG. 2. Localization of AQP3 mRNA. Detection by Northern blot analysis in mRNA prepared from rat kidney superficial cortex, medulla, and papilla (*A*), and mRNA prepared from rat heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis (*B*). Positions of RNA size markers are indicated by arrows.

tutes a good Kozak initiation of translation site (CGCCATGG) (17). The two ATG codons following this site do not fit with the Kozak's consensus sequence. The poly(A)$^+$ tail comprised 37 bp, and the major polyadenylylation signal AATAAA was included in the 3′ untranslated sequence. The predicted protein had 292 amino acids and a calculated M_r of 31,431. The protein (AQP3) contained a potential N-linked glycosylation site located at N141 and a putative phosphorylation site for casein kinase II at T276. Furthermore, AQP3 had 6 cysteine residues, which are potential reaction sites with mercurial reagents.

As expected, computer search of several peptide sequence data bases, using the BLAST network service, showed that AQP3 has a good degree of homology with several of the

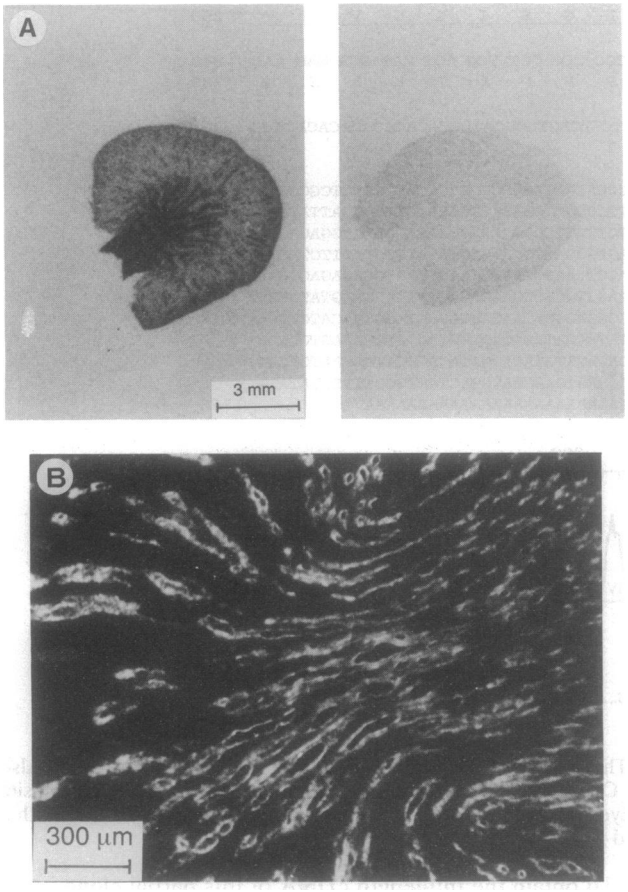

FIG. 3. Localization of AQP3 mRNA by *in situ* hybridization. (*A*) Autoradiography of a coronal rat kidney section hybridized with an antisense (*Left*) and sense (*Right*) ^{35}S-labeled RNA probe. (*B*) Micrograph of a dark-field view of the inner medulla of a kidney section hybridized with antisense RNA probe.

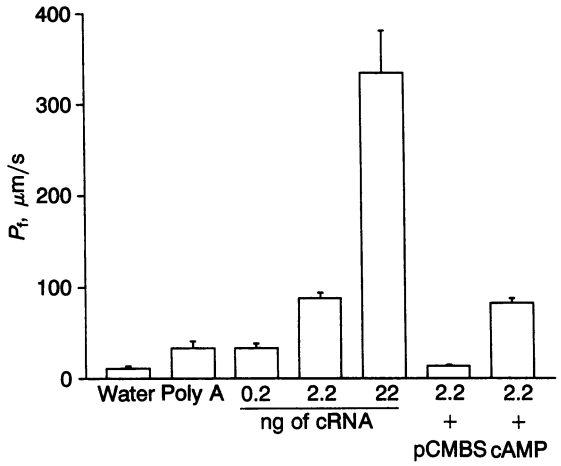

FIG. 4. Expression of water permeability (P_f) in oocytes injected with water (50 nl), papilla poly(A)$^+$ RNA (50 ng), or three different doses of cRNA for AQP3. A dose-dependent increase in P_f was observed with the cRNA. The cRNA-elicited P_f was abolished by 1 mM pCMBS and was not affected by 0.1 mM *p*-chlorophenylthio-cAMP.

members of the MIP family. The best homology, on the order of 40% identity, was with the glycerol uptake facilitator protein of *E. coli*. The homologies with CHIP28 and WCH-CD were 24% and 23%, respectively.

The hydrophobicity analysis using the hydropathy scale composed by Kyte and Doolittle (18) indicated that the translated protein had low polarity (Fig. 1*B*) and it predicted six hydrophobic segments that potentially could span the cell membrane. By analogy with the proposed conformation of CHIP28 (19) and WCH-CD (4), these segments may be arranged as transmembrane domains with the N and C termini of the protein located in the cytoplasm.

In the Northern blot analysis, it was found that the mRNA for AQP3 is predominantly located in the papilla of the kidney and to a lesser extent in the rest of the medulla. Only a single transcript of ≈1.9 kb was detected (Fig. 2*A*). The size of the transcript is the same as that of the cRNA and is in good agreement with the length of the cDNA identified in the kidney library. Although it is theoretically possible that a small part of the cDNA sequence at the 5′ end may be missing, these data make it likely that the full length of the coding region is contained in the cDNA. Additional support for this view is (*i*) that the protein encoded by the cRNA is a functional water channel when expressed in *Xenopus* oocytes as described later, and (*ii*) the similarity of the encoded protein in size and proposed membrane arrangement with respect to the other renal water channels. CHIP28 and WCH-CD are proteins constituted by 269 and 271 amino acids, respectively; their hydrophobicity profiles also predict six putative transmembrane domains (4, 19).

Fig. 2*B* shows a Northern blot in which mRNAs from various rat tissues were hybridized with the same probe used in the blot shown in Fig. 2*A*. There was a detectable signal only in the kidney, indicating that AQP3 is not broadly distributed.

A more precise localization of the message for AQP3 was obtained with *in situ* hybridization studies. The antisense RNA probe hybridized to the medullary collecting duct cells, especially toward the papilla, in good agreement with the results of Northern blot analysis. In the cortex, a small and inconstant degree of hybridization was found, whose cellular localization was uncertain. The hybridization with the sense control RNA probe produced a negligible signal (Fig. 3).

Three doses of cRNA coding for AQP3 were injected into separate groups of *Xenopus* oocytes to test for expression of

water channel activity. The synthesis and insertion of open water channels into the cell membrane should increase the P_f of the oocytes. The results of these experiments are summarized in Fig. 4. After 4 days of incubation, the control oocytes—i.e., those injected with 50 nl of water—showed a low P_f value (P_f = 14 μm/s). This is consistent with the absence of water channels in the plasma membrane of control oocytes (1). However, the oocytes injected with the cRNA of AQP3 had increased P_f relative to controls. The increment of P_f was proportional to the injected dose of transcript, reaching a P_f value of 334 ± 47 μm/s with the injection of 22 ng of the cRNA. As little as 0.2 ng of cRNA resulted in a 3-fold increase in P_f. Poly(A)$^+$ RNA increased oocyte P_f to a level similar to that obtained with 0.2 ng of cRNA (Fig. 4). The energy of activation of the P_f expressed with injection of 5 ng of AQP3 cRNA was 3.0 kcal/mol (1 cal = 4.184 J). These results are consistent with the view that the cRNA encoded a protein that conferred water channel activity on the oocyte membrane. By analogy to similar results obtained with the injection of cRNA coding for other water channels [CHIP28 (2), WCH-CD (4), and MIWC (7)], it is reasonable to conclude that the cRNA of AQP3 codes for a water channel.

Sulfhydryl modifying reagents have been shown to inhibit the water permeability of renal water channels (1, 2, 4). Therefore, the effect of incubation for 1 hr in 1 mM *p*-chloromercuribenzenesulfonic acid (pCMBS) prior to measuring P_f was examined in oocytes injected with 2.2 ng of AQP3 cRNA. The mercurial compound completely inhibited the increase in P_f observed when oocytes had not been exposed to pCMBS (Fig. 4). Complete inhibition with 1 mM pCMBS treatment was also observed in a separate experiment in which oocytes were injected with 20 ng of cRNA. In this experiment, P_f decreased from 180 ± 17 μm/s to 17 ± 3 μm/s, and this inhibition was reversed to 158 ± 23 μm/s by exposure to 5 mM dithiothreitol for 25 min after pCMBS treatment. pCMBS had no effect on water-injected oocytes, suggesting again that control oocytes lack water channels. These results indicated that the cRNA-injected oocytes had expressed water channels containing a cysteine residue(s), which upon modification with pCMBS abolished the water permeability of the pore. It is unknown which of the six cysteines present in the deduced amino acid sequence is (are) involved in the effect of pCMBS. The sensitivity to mercury in CHIP28 is given by C189 (20), which is located three residues from the second NPA motif. This has been interpreted as indicating that the NPA motif is part of the water pathway. WCH-CD also contains a C residue in the corresponding position (C181), which supports this postulate. However, in spite of the full sensitivity to a mercurial reagent, our channel does not contain a C residue near the second NPA motif. This suggests that the effect of sulfhydryl modification on water permeation through the channel may be more complex than previously thought.

The effect of exposure for 1 h to 0.1 mM *p*-chlorophenylthio-cAMP in oocytes injected with 2.2 ng of cRNA was also measured. There was no effect of cAMP (Fig. 4), in agreement with our previous observations after injection of papilla mRNA (1). Our results are therefore consistent with the view that the channel is not directly regulated by cAMP. In fact, the protein contains no consensus sequence for phosphorylation by protein kinase A.

The channel pore was not permeable to Na, K, and Cl ions, the main components of the intra- and extracellular fluids, because the membrane conductance of oocytes injected with 5 ng of cRNA (91.6 ± 2.5 nS; n = 5) was not significantly changed compared to that of oocytes injected with water (74.5 ± 6.2 nS; n = 4). The permeability to urea, a polar solute of small molecular diameter, was also evaluated in oocytes injected with 5 ng of cRNA. The urea permeability of control oocytes was low, 1.1 (±0.4) × 10^{-6} cm/s (n = 11 oocytes), and it was significantly increased to 3.1 (±0.5) × 10^{-6} cm/s (n = 12 oocytes) in oocytes injected with cRNA. Therefore, the channel is slightly permeable to urea and if the tubule cells containing AQP3 were permeable to urea, this route may account in part for this permeability.

Although the precise localization of AQP3 in the membranes of the medullary collecting duct cells is yet unknown, this water channel may be responsible for the cAMP-independent water permeability of the terminal IMCD (8, 9). Alternatively, AQP3 might render the basolateral membrane of the collecting duct cells permeable to water.

Note. Since acceptance of this manuscript, we have become aware of a paper by Ishibashi *et al.* (21). Their results are very similar to those reported here, including the amino acid sequence of the water channel protein AQP3.

We acknowledge D. Catanzaro and Dong Bei for generous advice and assistance, M. Knepper for his help with computer search of gene data bases, R. Duvoisin for teaching us the *in situ* hybridization technique, and J. Fischbarg for the use of his oocyte imaging system. This work was supported by National Institutes of Health Grant RO1 DK11489. M.E. was a Fellow of the Latin American Program of The Pew Charitable Trust.

1. Echevarria, M., Frindt, G., Preston, G. M., Agre, P., Milovanovic, S., Fischbarg, J. & Windhager, E. E. (1993) *J. Gen. Physiol.* **101**, 827–841.
2. Preston, G. M., Carrol, T. P., Guggino, W. B. & Agre, P. (1992) *Science* **256**, 385–387.
3. Nielsen, S., Smith, B. L., Christensen, E. I., Knepper, M. A. & Agre, P. (1993) *J. Cell Biol.* **120**, 371–383.
4. Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Marumo, F. & Sasaki, S. (1993) *Nature (London)* **361**, 549–552.
5. Deen, P. M. T., Verdijk, M. A. J., Knoers, N. V. A. M., Wieringa, B., Monnens, L. A. H., van Os, C. H. & van Oost, B. A. (1994) *Science* **264**, 92–95.
6. Nielsen, S., DiGiovanni, S. R., Christensen, E. I., Knepper, M. A. & Harris, H. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11663–11667.
7. Hasegawa, H., Ma, T., Skach, W., Mathay, M. & Verkman, A. S. (1994) *J. Biol. Chem.* **269**, 5497–5500.
8. Lankford, S. P., Chou, C. L., Terada, Y., Wall, S. M., Wade, J. B. & Knepper, M. A. (1991) *Am. J. Physiol.* **261**, F554–F566.
9. Han, J. S., Maeda, Y., Ecelbarger, C. & Knepper, M. (1994) *Am. J. Physiol.* **266**, F139–F146.
10. Strange, K. & Spring, K. R. (1987) *J. Membr. Biol.* **96**, 27–43.
11. Flamion, B. & Spring, K. R. (1990) *Am. J. Physiol.* **259**, F986–F999.
12. Agre, P., Preston, G. M., Smith, B. L., Jung, J. S., Raina, S., Moon, C., Guggino, W. B. & Nielsen, S. (1993) *Am. J. Physiol.* **265**, F463–F476.
13. Tate, S. S., Yang, N. & Udenfriend, S. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1–5.
14. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993) *Current Protocols in Molecular Biology* (Wiley, New York).
15. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
16. Bondy, C. A., Zhou, J. & Lee, W. H. (1993) *In Situ Hybridization Histochemistry: Handbook of Endocrine Research Techniques* (Academic, San Diego).
17. Kozak, M. (1989) *J. Cell Biol.* **108**, 229–241.
18. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
19. Preston, G. M. & Agre, P. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11110–11114.
20. Preston, G. M., Jung, J. S., Guggino, W. B. & Agre, P. (1993) *J. Biol. Chem.* **268**, 17–20.
21. Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T. & Marumo, F. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 6269–6273.