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Postnatal Expression of Aquaporins in Epithelial Cells of the Rat Epididymis[1]

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ABSTRACT

The mammalian aquaporins (AQPs) are a family of 13 transmembrane channel proteins that are involved in the transport of water in numerous organs. In the male excurrent duct, the movement of fluid and solutes across the epithelium is essential for establishing the proper luminal environment in which sperm mature and are stored. AQP9 is abundantly expressed in the efferent ducts, the epididymis, and the vas deferens, where it could represent an important apical pathway for transmembrane water and solute movement. However, other organs in which water transport is critical, including the kidney, the lung, or the eye, express several different AQPs with a cell-specific pattern. To undertake a systematic analysis of the expression of known AQPs in the postnatal and adult rat epididymis, we examined the expression of their respective mRNAs in epithelial cells isolated by laser capture microdissection (LCM), and we determined their corresponding protein expression pattern by immunofluorescence and Western blotting. Our data show that, whereas AQP9 is the main AQP of the epididymis, the mRNA specific for *Aqp2*, *5*, *7,* and *11* are also expressed in epididymal epithelial cells. AQP5 protein colocalizes with AQP9 in the apical membrane of a subpopulation of principal cells in the corpus and cauda regions. *Aqp2* mRNA was detected in epithelial cells after the second postnatal week and the amount significantly increased up to adulthood. However, AQP2 protein was detected only in the distal cauda of young rats (between the second and fourth postnatal week). No AQP2 protein was detected in the adult epididymis, indicating that posttranscriptional mechanisms are involved in the regulation of AQP2 expression. In addition, epididymal epithelial cells express significant amounts of the mRNAs coding for AQP7 and 11. No mRNA or protein for AQPs 0, 4, 6, and 8 were detectable in epithelial cells, and Aqp1 was detected in whole epididymal samples, but not in epithelial cells. Thanks to the recent development of microdissection technologies, our observations suggest that epididymal epithelial cells express several members of the AQP family with a region-specific pattern. AQPs may be involved not only in the transepithelial transport of water in the epididymis but also in the postnatal development of this organ, as suggested by the differential expression of AQP2.

epididymis, male reproductive tract

INTRODUCTION

The absorptive and secretory capacity of the epithelial cells lining the lumen of the male excurrent duct creates the appropriate environment for spermatozoa as they mature and are stored in the epididymis [1–3]. Fluid movement across the excurrent duct epithelium is required to achieve proper sperm concentration, which is important for fertility. Between 50% to 90% of the testicular luminal fluid is reabsorbed in the efferent ducts [4–6], but a considerable fluid reabsorption also occurs in the epididymis. This is reflected by a significant increase in sperm concentration as fluid transits toward the distal regions of the epididymis, and by the establishment of a hypertonic luminal fluid [6–8].

While water can be transported across the plasma membrane by simple diffusion through the lipid bilayer, our understanding of the basis of water transport has been revolutionized during the last decade with the discovery of aquaporins (AQPs), which greatly facilitate water movement across membranes [9]. APQs are small, intrinsic membrane proteins that are present in many cell types involved in fluid transport [9]. In mammals, the AQP family consists of 13 known genes. Two subgroups of mammalian AQPs have been defined on the basis of their permeability characteristics: the AQPs (major intrinsic protein, i.e., MIP or AQP0, AQP1, 2, 4, 5, 6, and 8) are highly selective for water, and the aquaglyceroporins are also permeated by glycerol (AQP3, 7, 10) or even larger solutes (AQP9), in addition to water. AQPs are widespread in many tissues. At least seven distinct AQPs are expressed in the mammalian kidney [10], five in the eye [11] and four in the respiratory tract [12, 13], further demonstrating the complexity of their physiology. AQPs have also been shown to be directly involved in disease states, including nephrogenic diabetes insipidus (AQP2) [14–18] or cataracts (AQP0) [19]. Multiple AQPs can be segregated on the apical or basolateral plasma membrane of epithelial cells, but some can also be present within the same membrane domain. For example, the basolateral membrane of principal cells in the kidney contains AQP2, 3, and 4 [20]. Whereas the majority of the AQPs are constitutively expressed at the cell surface, AQP2 is accumulated in the apical membrane of kidney collecting duct epithelial cells after binding of vasopressin to its receptor, thus increasing the water permeability of the collecting duct [18].

So far, AQPs 1, 2, 3, 8, 9, and 10 have been found in the epithelium of the male excurrent duct. AQP1 (previously known as CHIP28) is responsible for bulk flow across erythrocytes and the plasma membrane of the kidney proximal tubule [21–23]. Our laboratory and others have shown that it is also expressed in the efferent ducts, which are embryologically related to the proximal tubule [24–28]. Moreover, while AQP1

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is absent from the epithelium of the epididymis, it is present in adjacent smooth muscle and endothelial cells [26, 27]. This illustrates the need for careful isolation of specific cell types when searching for novel proteins. AQP2 is a major water channel in the kidney and is also expressed in the inner ear and in the colon. We have shown that AQP2 is expressed in the distal vas deferens of adult rats [29]. AQP9 allows the passage of a wide range of structurally unrelated molecules, including urea, glycerol, mannitol, and sorbitol, as well as water [30]. It is expressed in the liver, leukocytes, the brain, the spleen, and the testis [30–32]. We and others have localized AQP9 in the efferent ducts, the epididymis, and the vas deferens [26, 32–34]. In addition, recent studies have revealed that AQP3 [35] and AQP8 [36] can be immunologically detected in basal cells. However, no systematic analysis of the expression of all known AQPs in epididymal epithelial cells has been undertaken. The purpose of this study was, therefore, to examine the expression of AQP mRNAs in epithelial cells of rat epididymis by RT-PCR analysis of microdissected epithelial cell samples, and to determine the corresponding protein expression patterns by immunofluorescence and Western blotting. In addition, we analyzed the evolution of AQP expression in epididymal epithelial cells during the first weeks of postnatal development by real-time quantitative PCR, Western blotting, and immunofluorescence.

MATERIALS AND METHODS

Experimental Animals

Experiments were conducted using newborn, postnatal (1–4 wk) and mature (350–450 g) male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). These rats were acquired, retained, and used in compliance with the U.S. National Research Council's recommendations. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (6.5 mg per 100 g body weight).

Laser Capture Microdissection

Laser capture microdissection (LCM) was developed at the National Cancer Institute at the National Institutes of Health, Bethesda, MD, and initially described by Emmert-Buck et al. [37]. Epididymides were dissected, placed in cryomolds, embedded in Tissue-Tek OCT medium, immediately frozen on dry ice, and stored at −80°C. Tissue was sectioned at 6 μm on a Reichert-Jung 2800 Frigocut cryostat (Spencer Scientific, Derry, NH). Sections were mounted on uncoated glass slides and stored at −80°C. Before use, slides were stained for 30 sec in Mayer hematoxylin and dehydrated in 70%, 95%, and 100% ethanol. Slides were dipped in xylene twice for 5 min and air dried. Epithelial cells were harvested by using the PixCell II LCM system (Arcturus Engineering, Mountain View, CA) with the following parameters: spot size 7.5 μm, pulse duration 1.5 msec, power 40–60 mW.

As shown in Figure 1, LCM allows for the precise isolation of epithelial cells from the rat epididymis with minimal contamination by surrounding cell types. In the newborn rat epididymis (Fig. 1, A and B), 15–30 immature epithelial cells were isolated from each tubule undergoing development. In the adult rat (Fig. 1, C–E), mature epithelial cells were isolated with limited contamination from the surrounding smooth-muscle cells and spermatozoa. Moreover, using this procedure, contamination by endothelial and blood cells is extremely unlikely. Thus, the subsequent gene expression profiles reflect mostly the epithelial cell population. For this study, we did not concentrate on any specific area of the epididymis: the LCM was performed in caput, corpus, and cauda epididymidis and, consequently, our LCM samples contain epithelial cells from the whole organ. Each sample used 2000–4000 laser pulses, which corresponded to approximately 2000–4000 epithelial cells. Each time point is represented by at least three samples.

Total RNA Extraction, DNase Treatment, and RNA Amplification

LCM samples were incubated for 30 min at 42°C with 10 μl RNA extraction buffer, and total RNA was extracted following the PicoPure RNA Isolation kit protocol (Arcturus). DNase treatment was performed within the purification column using the RNase-Free DNase set (Qiagen, Valencia, CA). Because LCM limits the starting amount of mRNA available for RT-PCR analysis, the RNA samples were amplified by a T7-based process derived from the Eberwine method [38], using the RiboAmp RNA Amplification kit (Arcturus). After two rounds of amplification, the amplified antisense RNA (aRNA) was quantified by measuring the absorbance of a 1:50 dilution at 260 nm. The quality of aRNA was assessed by gel electrophoresis. After two rounds of amplification, the bulk of the aRNA product is 200–600 bases in length. We generated on average 70 μg of aRNA from each LCM sample. Whereas this technique is capable of linearly amplifying small amounts of RNA without significantly distorting the information content of the sample, the aRNA product is shorter and contains only the 3′ region of the starting mRNA template. Consequently, our sequence-specific primer sets were designed to amplify a short (<130 bp) region of the 3′ end of the rat *Aqp0–9* and *Aqp11* mRNAs (Table 1).

To isolate total RNA from whole adult epididymides, tissues were frozen in liquid nitrogen immediately after dissection, then powdered in a pestle and mortar, and homogenized in RNAwiz (Ambion, Austin, TX). RNA was isolated following the manufacturer's protocol and genomic DNA contamination was eliminated using the DNA-free kit (Ambion). DNA-free aRNA and total RNA samples were aliquoted and stored at −80°C.

Reverse Transcription

Three micrograms of amplified aRNA were converted into first-strand cDNA for 1 h at 37°C in a final volume of 25 μl with 1× first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol, 6 ng/μl random hexamers, 500 μM each dNTP, 50 U RNase inhibitor (Promega, Madison, WI), and 400 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Second-strand synthesis was performed for 2 h at 16°C in a final volume of 150 μl with 1× second-strand buffer (20 mM Tris-HCl, pH 6.9, 90 mM KCl, 4.6 mM $MgCl_2$, 0.15 mM β-NAD^+, 10 mM $(NH_4)_2SO_4$), 250 μM each dNTP, 40 U DNA polymerase I, 2 U *Escherichia coli* RNase H, and 10 U *E. coli* DNA ligase (all reagents from Invitrogen). Double-stranded DNA was purified with the Qiaquick PCR purification kit (Qiagen) and quantified with the PicoGreen dsDNA quantification reagent (Molecular Probes, Eugene, OR) by using a spectrofluorometer.

Polymerase Chain Reaction

Reverse transcription products were used as templates for traditional end point polymerase chain reaction (PCR) and real-time quantitative PCR. For end-point PCR, reaction mixtures consisted of a 20-μl final volume containing 2 ng template, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 2.0 mM $MgCl_2$, 1.0 mM each dNTP and 0.5 μM forward and reverse oligonucleotide primers. PCR was performed in a Flexigene thermal cycler (Techne, Princeton, NJ) with the following parameters: 8 min at 95°C to activate the polymerase, followed by 30–40 cycles of melting for 1 min at 95°C, annealing for 30 sec at 60°C, extension for 45 sec at 72°C, and a final extension for 10 min at 72°C. PCR products were resolved in a 2.5% agarose gel containing GelStar stain (Cambrex, Rockland, ME). The 746-bp *Aqp2* PCR product was purified with the Qiaquick PCR purification kit and submitted for direct sequencing at the Massachusetts General Hospital sequencing core facility.

Quantitative PCR analysis of AQP expression was performed with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Each reaction was performed in triplicate by using 5 ng of cDNA from each sample and the iTaq SYBR Green Supermix with ROX reagent (Bio-Rad, Hercules, CA). A no-template control (NTC) was included in each PCR plate to check for any contamination in the reaction mix or formation of primer dimer. A dissociation curve was generated after each SYBR Green PCR run to further confirm the specificity of the amplification. For *Aqp9*, a 5′ nuclease assay was performed using the TaqMan Universal PCR master Mix (Applied Biosystems) and a specifically designed fluorogenic probe (see Table 1). The relative standard curve method was used for linear regression analysis of unknown samples. Data are presented as fold change between samples calculated from at least three experiments. Oligonucleotide primer pairs were designed using the Primer Express software (Applied Biosystems) to amplify a short sequence in the 3' end of the rat AQP cDNAs. Primers were synthesized by Sigma-Genosys (The Woodlands, TX). The sequences are as indicated in Table 1.

Antibodies

Primary antibodies. Peptides corresponding to the C-terminus of rat AQPs 1, 2, 3, 4, 5, 7, 8, and 9 were coupled to keyhole limpet hemocyanin (KLH) and used to immunize rabbits using standard protocols. The specificity of the

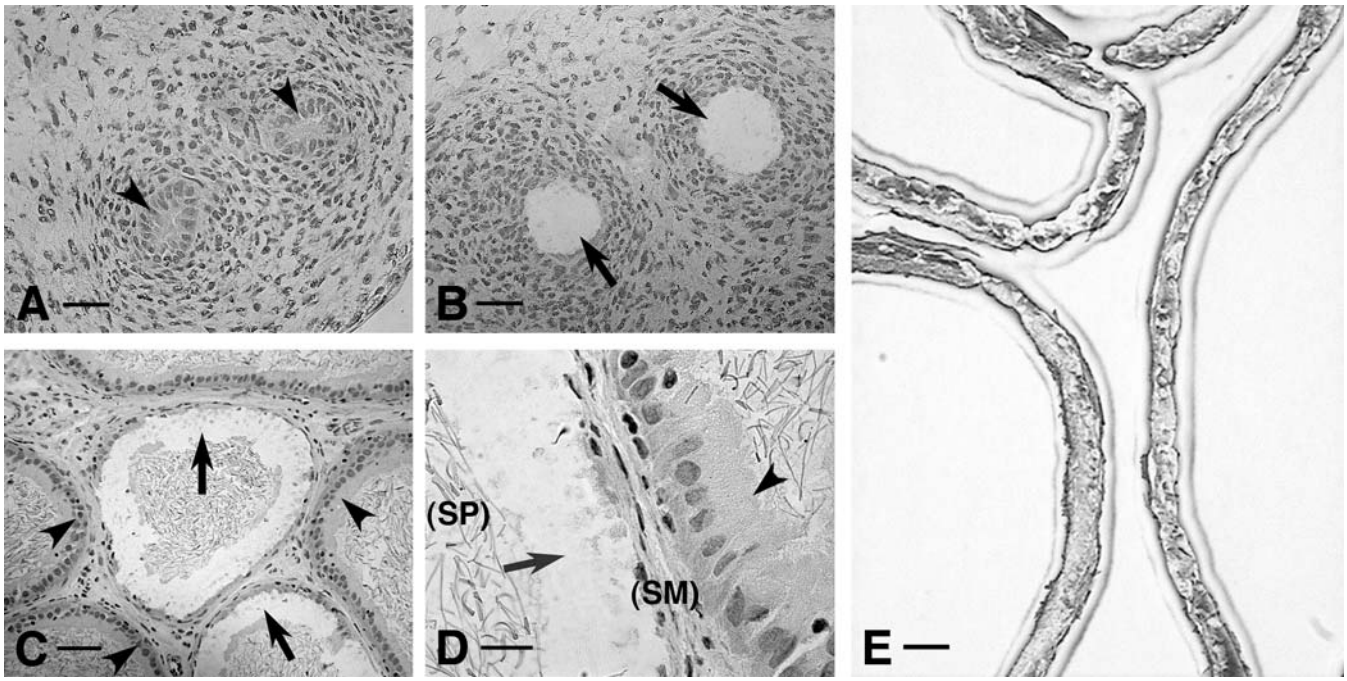

FIG. 1. LCM-mediated isolation of epithelial cells from 6-μm-thick sections of rat epididymis. Intact epithelial cells are indicated by arrowheads, microdissected areas are indicated by arrows. **A**) Cross section of a newborn rat cauda epididymidis before microdissection. **B**) A similar epididymis section after immature epithelial cells microdissection. **C**) Section of an adult rat cauda epididymidis with intact epithelial tubules (arrowheads) and two tubules where epithelial cells have been microdissected (arrows). **D**) Higher magnification of the same section showing that epithelial cells can be isolated with limited contamination by smooth muscle cells (SM) and spermatozoa (SP). **E**) Epithelial cells from an adult epididymis captured on the LCM cap. Bars = 25 μm for A, B, C, E; 10 μm for D.

antibodies against AQP1, 2, 4, and 9 has been reported previously [23, 29, 33, 39]. All the antibodies were affinity purified against the immunizing peptide using an affinity purification column kit (Pierce, Rockford, IL) according to the manufacturer's instructions. A polyclonal antibody against the C-terminal tail of the E2 subunit (previously called subunit E, or 31-kDa subunit) of the bovine kidney medulla vacuolar H^+-ATPase (V-ATPase), was raised in chicken and affinity purified. This antibody has been previously characterized [40, 41]. A previously characterized antibody against the second extracellular loop of AQP2 was also used [42]. Rabbit anti-rat IgG, affinity purified antibodies against the C-terminal of AQP0, 4, 5, 8, and 10, and against the N-terminal of AQP7, were purchased from Alpha Diagnostic Intl. (San Antonio, TX). A goat anti-rat IgG affinity-purified antibody against an internal region of rat AQP6 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). A mouse antiactin monoclonal antibody (clone C4) was purchased from Chemicon International (Temecula, CA).

The secondary antibodies were donkey anti-chicken IgG conjugated to fluorescein isothiocyanate (FITC), and goat anti-rabbit IgG conjugated to indocarbocyanine (Cy3) (Jackson Immunologicals, West Grove, PA) for immunofluorescence, and goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma, St Louis, MO) for Western blotting.

Experimental Animals and Tissue Preparation

For immunofluorescence studies, organs were perfused via the left cardiac ventricle with 1× phosphate-buffered saline (PBS), pH 7.4, followed by paraformaldehyde-lysine-periodate (PLP) [33]. The epididymis, vas deferens, kidney, and testis were dissected and placed in PLP buffer overnight at 4°C. Tissues from young rats (1 day, 1–4 postnatal wk) were fixed in PLP by immersion. Tissues were then washed three times in PBS and stored at 4°C in PBS containing 0.02% Na-azide.

Immunofluorescence

Fixed tissues were cryoprotected in PBS with 30% sucrose for 2 h at room temperature, embedded in Tissue-Tek OCT compound (Sakura Finetek U.S.A., Torrance, CA), and mounted and frozen on a cutting block. Tissues were cut in a Reichert Frigocut microtome at 4–5 μm thickness and sections were placed

TABLE 1. Primers used for PCR analysis.

Protein	Gene	GenBank accession no.	Forward primer	Reverse primer	Amplicon
MIP	*Mip*	X53052	CGCCGCTGGGTTTCAG	AAGTTAAAAAATAAAGAAGTACCGATTGATC	95 bp
AQP1	*Aqp1*	NM_012778	GCTCAGCAGTCAAAGCCATGT	AAACGGAGAAGCTGGAAATGA	76 bp
AQP2	*Aqp2*	D13906	GACTGTGCTTAGTGCATCTCATTTTAT	AAACTCTGTTCGTGAGGAAAAGAAA	81 bp
AQP2	*Aqp2*	NM_012909	CTCAGATCCATAGCCTTCTCCCGAG	CGCACTTCACGTTCCTCCCA	746 bp
AQP3	*Aqp3*	NM_031703	TTGCTTCAGGGACTCCAGTGT	CCCGCTCCCTAAGCCTAGAA	103 bp
AQP4	*Aqp4*	NM_012825	GGCCCCAAAACTAGATGTTT	AGCATGCATCACATATACCAAATTC	89 bp
AQP5	*Aqp5*	NM_012779	GCTTTGGAATCAGGCAGAATG	AGCAAGCTGTCGTCCCTTTC	112 bp
AQP6	*Aqp6*	NM_022181	AGTTGTTCCCTTTCCATTTTTCC	GCTAGTCCCAGCCATTTCACA	121 bp
AQP7	*Aqp7*	BC072695	GGCATGAACACAGGATATGCA	CCCAGCCAGCAATGAAAGTG	76 bp
AQP8	*Aqp8*	NM_019158	GCAGGTGGAGCATGCAGTT	GGAGGCTCCGTAGCAAAGTG	123 bp
AQP9	*Aqp9*	NM_022960	CCTCCCCAGAATAGCACTGACT	AGCCAAGCACATGGTCAAGTT	101 bp
			fluorogenic probe: VIC-CCTAGGAGTAAGGACTTCCATCAAATACCCATCATA-TAMRA		
AQP11	*Aqp11*	NM_173105	GGTTTGGGCACCTTTCAAAC	ATACAGGCTGCCTCATTTTCACA	111 bp
GAPD	*Gapd*	BC059110	AGAGAGAGGCCCTCAGTTGCT	TGGAATTGTGAGGGAGATGCT	77 bp

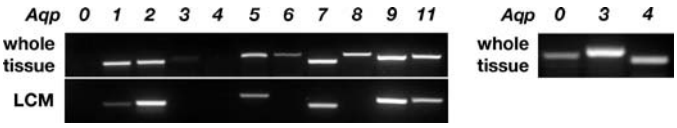

FIG. 2. Expression of AQPs in the adult rat epididymis by RT-PCR. Total RNA from the entire epididymis was probed for *Mip* (*Aqp0*), *Aqp1, 2, 3, 4, 5, 6, 7, 8, 9,* and *11*. Strong signals for *Aqp1, 2, 5, 6, 7, 8, 9,* and *11* were detected, while *Aqp3* showed a weak signal, and *Mip* and *Aqp4* mRNAs were not detected (left panel, whole tissue). Control RT-PCR showed that *Mip, Aqp3,* and *4* are detected in RNA samples extracted from the eye (*Aqp0*), kidney (*Aqp3*), and brain (*Aqp4*) (right panel, whole tissue). Strong signals were revealed for *Aqp2, 5, 7, 9,* and *11* in amplified RNA from epithelial cells isolated by LCM; a weak band for *Aqp1* and no signal for *Aqp3, 4, 6,* and *8* are shown (left panel, LCM).

onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were rehydrated in PBS for 10 min and pretreated with 1% (w/v) SDS, an antigen-retrieval technique previously described [43]. After three washes in PBS, slides were preincubated in 1% (w/v) BSA in PBS with 0.02% Na-azide for 30 min to block nonspecific staining, after which they were incubated with the different primary antibodies for 1–2 h at room temperature. For detection of AQP0, 1, 2, 3, 4, and 5, the antibodies were diluted 1:200 in PBS. The antibodies against AQP6, 7, 8, and 10 were diluted 1:100 in PBS. Antibody against AQP9 was diluted 1:3000 in PBS. After two washes in high-salt PBS (containing 2.7% NaCl) and one wash in PBS, slides were incubated for 1 h at room temperature with secondary antibody Cy3-conjugated goat anti-rabbit IgG (2 μg/ml final concentration), and washed again. Double labeling was performed by subsequent incubation of some sections with chicken anti-V-ATPase antibody (subunit E2) and FITC-conjugated donkey anti-chicken IgG. After washes, slides were mounted in Vectashield (Vector Labs, Burlingame, CA) diluted 1:1 in Tris buffer, pH 8.5, and examined using a Nikon E800 epifluorescence microscope. Sections were digitally imaged using a Hamamatsu Orca CCD camera and IP Lab Spectrum software (Scanalytics, Fairfax, VA). Final images were imported into Adobe Photoshop and printed. To determine the specificity of the AQP primary antibodies, control incubations using antibodies preabsorbed with their respective immunizing peptides were performed as described previously [33, 44].

Immunoblot Analysis

The epididymis, vas deferens, kidney, and lung were dissected, snap frozen in liquid nitrogen, and stored at −80°C. Tissues were placed in cold buffer containing 250 mM sucrose, 18 mM Tris-Hepes, pH 7.4, 1 mM EDTA, Complete protease inhibitor (Roche Applied Science, Indianapolis, IN) and were disrupted using a PRO 200 homogenizer (PRO Scientific Inc., Monroe, CT). Differential centrifugations were performed at 500 × *g* for 10 min, 10 000 × *g* for 10 min, and 100,000 × *g* for 1 h. The last centrifugation pellet, which contains vesicles and plasma membranes, was resuspended in buffer containing 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.2 mM Na-orthovanadate, 0.5 mM IGEPAL, 1% Triton X-100, and protease inhibitors. Some homogenates were used to prepare an enriched preparation of epididymis brush-border membranes (BBMs) using the Mg^{2+} precipitation technique, as described previously [33]. BBMs were prepared from total epididymis homogenates as well as from homogenates of cauda epididymidis. Protein concentration was determined by the bicinchoninic acid assay (Pierce). SDS-PAGE was performed with an XCell Mini-Cell electrophoresis system (Invitrogen) and 10% acrylamide/Tris-glycine/SDS precast gels. Samples were prepared at room temperature in Laemmli sample buffer containing 0.5% β-mercaptoethanol, as previously described [44]. For Western blot analysis, proteins were transferred to a polyvinylidene fluoride membrane by electro-transfer at 5–7 V for 18 h in 1 mM Na_2CO_3, 4 mM $NaHCO_3$, and 0.01% SDS (pH 9.9) [44]. The membrane was blocked for 1 h in PBS with 0.05% Tween 20, containing 5% nonfat milk. The membrane was then incubated for 2 h at room temperature with anti-AQP antibodies (diluted 1:2000), washed with PBS containing 0.05% Tween 20, and incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase (Sigma, St Louis, MO). After three additional washes, proteins were detected using Western Lightning Chemiluminescence reagent (Perkin Elmer Life Science, Boston, MA). To confirm the specificity of the antibodies, some membranes were incubated with primary antibodies that had been preincubated with the immunizing peptide.

RESULTS

Aquaporin mRNA Expression in Total Epididymis

Sequence-specific primer sets were designed to amplify a short region of the 3′ end of the rat *Aqp0–9* and *Aqp11* mRNAs (Table 1). Although *Aqp10* and *Aqp12* mRNA have been reported in human and mouse [45, 46], their rat sequences remain unknown and we failed to obtain specific PCR products with our rat epididymal samples using primers that recognize both human and mouse sequences. Of the 11 AQPs examined, *Aqp1, 2, 5, 6, 7, 8, 9,* and *11* were clearly detected in RNA samples extracted from the entire adult rat epididymis (Fig. 2, left panel, whole tissue). A very weak signal for *Aqp3* was obtained and no signals for *Aqp0* and *4* were detected. Control amplifications were, therefore, performed for *Aqp0, 3,* and *4* to assess the specificity of their corresponding primer sets (Fig. 2, right panel, whole tissue). *Mip* mRNA, which codes for a major protein of the fiber cells of the lens, also known as AQP0, was detected in the eye (lane 0). *Aqp3* and *Aqp4* were detected in kidney (*Aqp3*, lane 3) and brain (*Aqp4*, lane 4) samples, as well as in several other tissues (small intestine, pancreas, spleen, testis, and tongue for *Aqp3*; kidney and pancreas for *Aqp4*; data not shown). A more precise characterization of AQP gene expression was next performed using microdissected epithelial cell samples.

Aquaporin mRNA Expression in Microdissected Epithelial Cells

Figure 2 (left panel, LCM) shows strongly amplified *Aqp2* and *Aqp9* fragments and less intense bands for *Aqp1*, *5*, *7,* and *11* in epithelial cells isolated by LCM. Thus, of the nine AQP mRNAs detected in total epididymis, *Aqp1*, *2*, *5*, *7*, *9,* and *11* were also detected in microdissected epithelial cell samples. This end-point RT-PCR analysis indicates that *Aqp9* and *Aqp2* mRNAs are expressed at a relatively high level in the adult epididymal epithelium. *Aqp1*, *5*, *7,* and *11* were also detected, but at a lower level in these samples. To determine whether the absence of detectable levels of *Aqp3*, *6,* and *8* in the aRNA samples was a consequence of using an RNA amplification procedure, the RNA extracted from two total epididymis samples was amplified using the same protocol. Strong signals were obtained for these three genes after one and two rounds of amplification (not shown), demonstrating the ability of their specific primer sets to detect them, even after amplification. Thus, whereas *Aqp3*, *6,* and *8* mRNAs are present in the adult rat epididymis, they are not specifically expressed in epithelial cells.

Aquaporin mRNA Expression During Postnatal Development

Rat epididymal epithelial cells were carefully isolated by LCM at different times postnatally, from the day of birth to 4 wk. Total RNA was extracted and linearly amplified. The amplified aRNA was then reverse transcribed and subjected to traditional end-point PCR analysis and real-time quantitative PCR (qRT-PCR). AQP gene expression was normalized for glyceraldehyde-3-phosphate dehydrogenase (Gapd), which showed no visible variation during postnatal development. RT-PCR indicated significant *Aqp9* and *Aqp11* mRNA levels at birth (D0), whereas *Aqp2*, *5,* and *7* were detected only after the first (W1) or second week (W2) of postnatal development (Fig. 3). *Aqp2* expression dramatically increased between the second (W2) and the fourth week (W4), and abundant *Aqp2* mRNA was detected in the adult epididymis. *Aqp9* and *11*

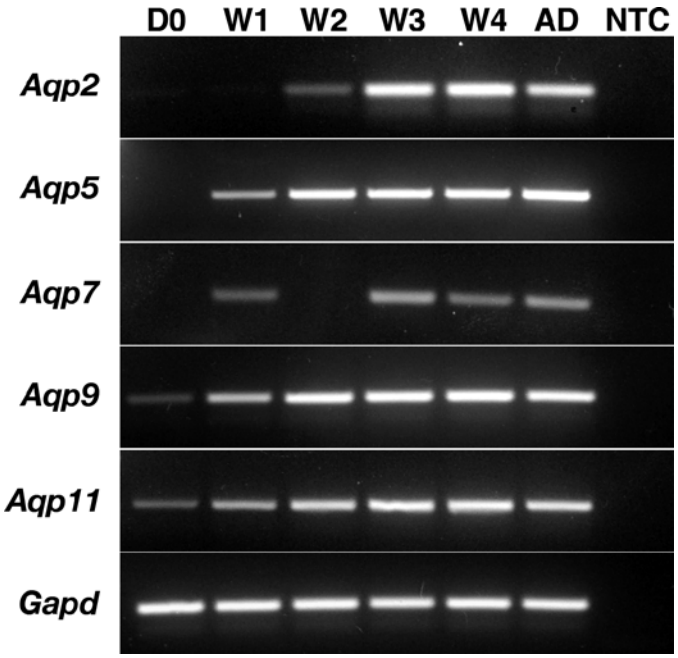

FIG. 3. RT-PCR analysis of AQP expression during rat postnatal development. Expression of *Aqp2, 5, 7, 9,* and *11* in epithelial cells isolated by LCM was monitored at birth (D0) and during the first 4 wk of postnatal development (W1, W2, W3, and W4), and in adulthood (AD), by traditional end-point RT-PCR. Gapd amplification was used as control. NTC: no-template control.

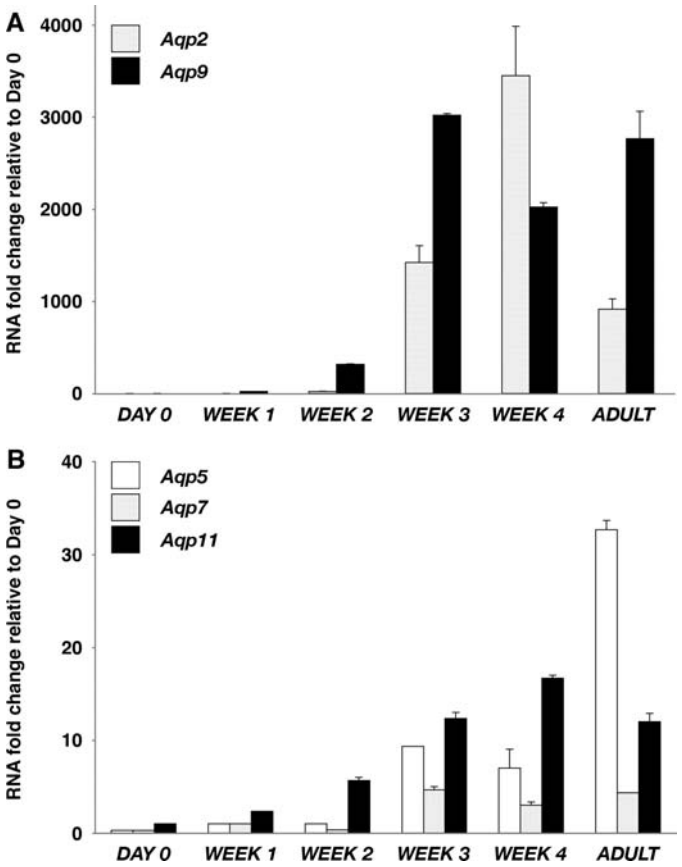

FIG. 4. The qRT-PCR analysis of AQP expression during rat postnatal development. Real-time quantitative RT-PCR was performed using epithelial cells isolated by LCM at birth (*DAY 0*), during the first 4 postnatal wk (*WEEK 1–4*) and in adulthood (*ADULT*). **A**) A tremendous increase in *Aqp2* and *Aqp9* mRNA expression is shown, leading to mRNA levels that are over 3000-fold higher at Weeks 3 and 4 compared with Week 1. No significant signal is detected for *Aqp2* and *9* at birth. In adult rats, *Aqp9* mRNA remains abundant, while *Aqp2* mRNA expression is significantly reduced compared with week 4. **B**) *Aqp5, 7,* and *11* mRNA expression is modulated to a lesser extent, the levels reaching values that are 30 (*Aqp5*), 2 (*Aqp7*), and 15 (*Aqp11*) fold higher compared with the day of birth. In contrast with *Aqp2* and *9,* significant levels of mRNAs were detected at birth. Error bars : ± SEM.

signals increased gradually between birth and adulthood. The qRT-PCR analysis (Fig. 4) showed that the level of expression of *Aqp2* and *Aqp9* varies considerably between birth and adulthood (Fig. 4A). A 3000- to 4000-fold increase of the signal was observed between birth and Week 3 for *Aqp9* and between birth and Week 4 for *Aqp2*. In addition, this analysis showed that *Aqp2* mRNA is less abundant in the adult than after 3 and 4 wk of postnatal development. As seen in Figure 4B, the magnitude of mRNA increase during postnatal development is considerably smaller for *Aqp5* (33-fold), *Aqp7* (5-fold), and *Aqp11* (17-fold).

Immunolocalization of Aquaporins in Adult Rats

No significant staining was detected by indirect immunofluorescence in adult rat epididymal epithelial cells for AQP0, 1, 2, 3, and 4 (Fig. 5, left column). As previously shown [26, 27], a strong labeling was detected for AQP1 in endothelial cells from epididymal blood vessels and a weaker staining in the muscle cells surrounding the epididymal tubules (Fig. 5B). Positive controls were performed to confirm the validity of our antibodies (Fig. 5, right column). AQP0 is strongly expressed in the lens (F), AQP1 in kidney proximal tubules (G), and AQP2, 3, and 4 in the kidney collecting duct (H–J). AQP5 was detected in the apical membrane of a subpopulation of epithelial cells of the corpus (Fig. 6B) and cauda epididymidis (Fig. 6C). No AQP5 staining was detected in initial segments (Fig. 6A) and caput (not shown). Double labeling for the E2 subunit of the V-ATPase was performed to identify clear cells [47, 48]. Cells positive for AQP5 are negative for the V-ATPase E2 subunit, demonstrating that they represent a subpopulation of principal cells (Fig. 6D).

No specific staining was detected in epithelial cells using either our antibodies or commercial antibodies against AQP6 and 8 (data not shown), consistent with the absence of their respective mRNA in epithelial cell samples. Surprisingly, no labeling was obtained for AQP7 using two antibodies against the C- and N-termini of this protein (data not shown). However, these antibodies also did not recognize AQP7 in tissues known to express this protein, and at present, we cannot conclude whether or not AQP7 protein is expressed in epithelial cells. Regarding AQP11, for which a strong mRNA signal was detected in the LCM samples, no antibody is currently available to determine whether AQP11 protein is expressed in the epididymis.

In view of the strong *Aqp2* mRNA signal that was detected in the LCM samples, the absence of AQP2 staining by immunofluorescence in adult tissue was surprising. The absence of AQP2 from the adult epididymis was confirmed by Western blotting (Fig. 7A), while strong signals were detected in the vas deferens and kidney. No AQP2-specific band was detected in epididymal BBMs, indicating that the absence of signal in the total homogenates was not attributed to a low level of expression. In addition, whereas a strong signal for AQP5 was detected in the lung (Fig. 7B, middle panel), where this protein was previously described [49], no signal could be detected in total epididymis homogenates (data not

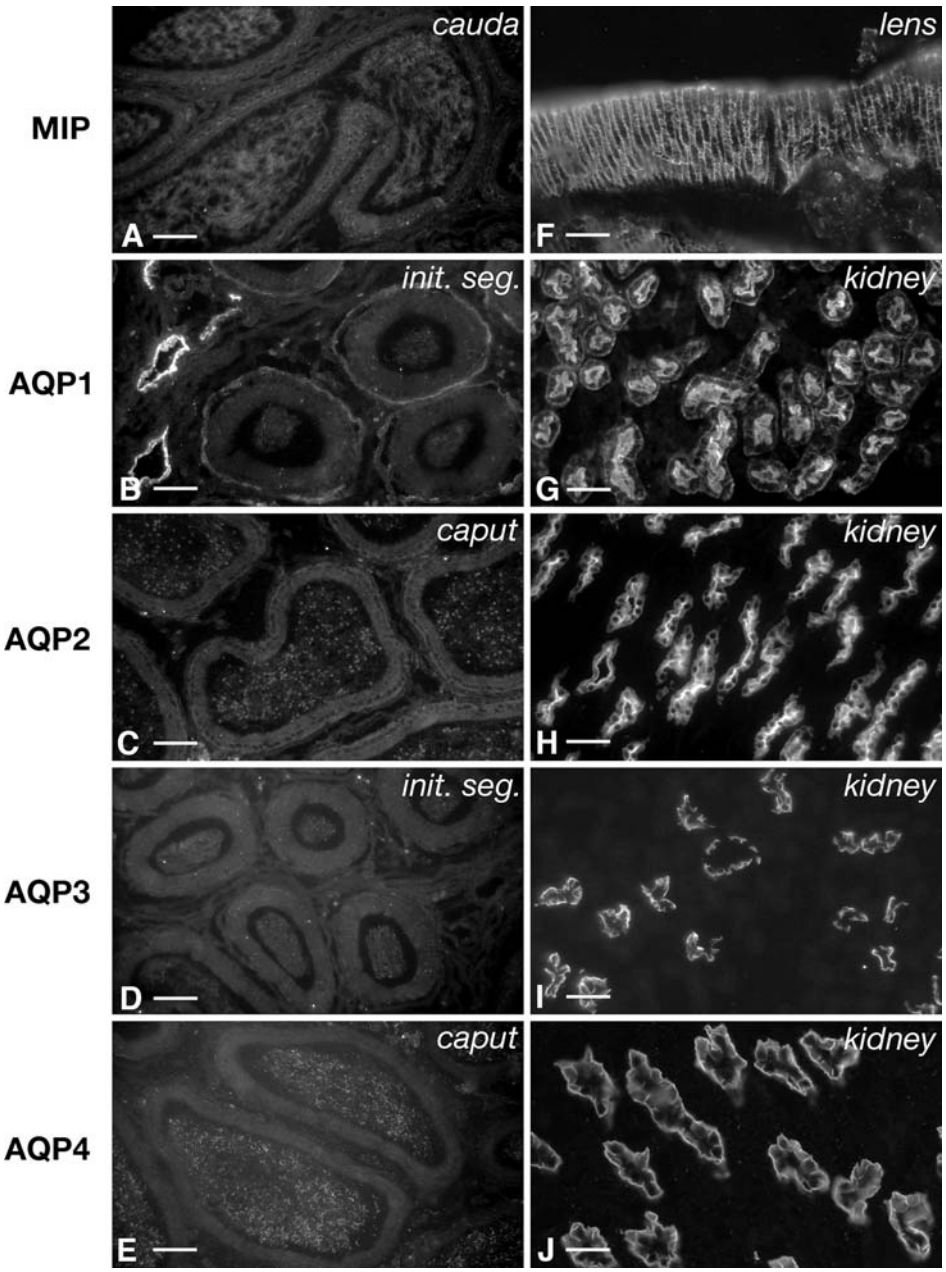

FIG. 5. Immunofluorescence detection of AQP0 (MIP), AQP1, 2, 3, and 4 in adult epididymis. Negative staining is shown in epithelial cells (**A**, MIP; **B,**: AQP1; **C**, AQP2; **D**, AQP3; **E**, AQP4). Strong AQP1 staining is shown in endothelial cells and weaker staining is seen in the smooth muscle cells surrounding the tubules. AQP4 (**E**) is detected in epididymal spermatozoa, which are visible in the lumen. The panels included in the right column show positive staining for AQP0 in the lens (**F**), AQP1 in kidney proximal tubules (**G**), AQP2 in the apical membrane of kidney collecting duct principal cells (**H**), and of AQP3 and 4 in the basolateral membrane of kidney collecting duct principal cells (**I** and **J**, respectively). *Caput,* caput epididymidis; *init. seg.,* initial segments; *cauda,* cauda epididymidis. Bars = 20 μm.

shown) by Western blotting. However, a weak signal was detected in BBM isolated from the entire epididymis, and a stronger signal was seen in BBM isolated from the cauda epididymidis (Fig. 7B, left panel), consistent with the immunofluorescence detection of AQP5 in a few principal cells of the distal epididymis only. Because the AQP5 antibody has not been characterized previously, we tested its specificity. No signal was obtained in lung samples after preincubation of the antibody with the AQP5 peptide, showing specificity of our AQP5 antibody (Fig. 7B, right panel). Interestingly, while one single band at the molecular weight of 25 kDa was detected in lung, two additional bands at higher molecular weights were detected in epididymis BBM, indicating the presence of different glycosylation states. To confirm the validity of the epididymal samples, positive controls were performed using our AQP9 antibody (Fig. 7C). As we have previously shown [33], a strong AQP9 signal was detected in BBM isolated from the entire epididymis and from the cauda. No difference in the intensity of the signal was observed between these samples, consistent with the uniformly high expression of AQP9 in principal cells of all regions of rat epididymis.

Expression of Aquaporin Protein in Rat Epididymis During Postnatal Development

To determine the pattern of AQP protein expression in the postnatal epididymis, tissues were immunostained at different times from the day of birth to adulthood. AQP2 staining was not detected at birth (not shown) and during the first and second postnatal weeks (Fig. 8, A and B). In contrast, significant apical AQP2 staining was seen in a subpopulation of epithelial cells in the cauda epididymidis at Weeks 3 and 4 (Fig. 8, B and C). No staining was detected in the more proximal regions of the epididymis at any time during postnatal development (data not shown). Surprisingly, as described above, no AQP2 immunofluorescence staining was detectable in the adult epididymis. Thus, AQP2 protein is expressed

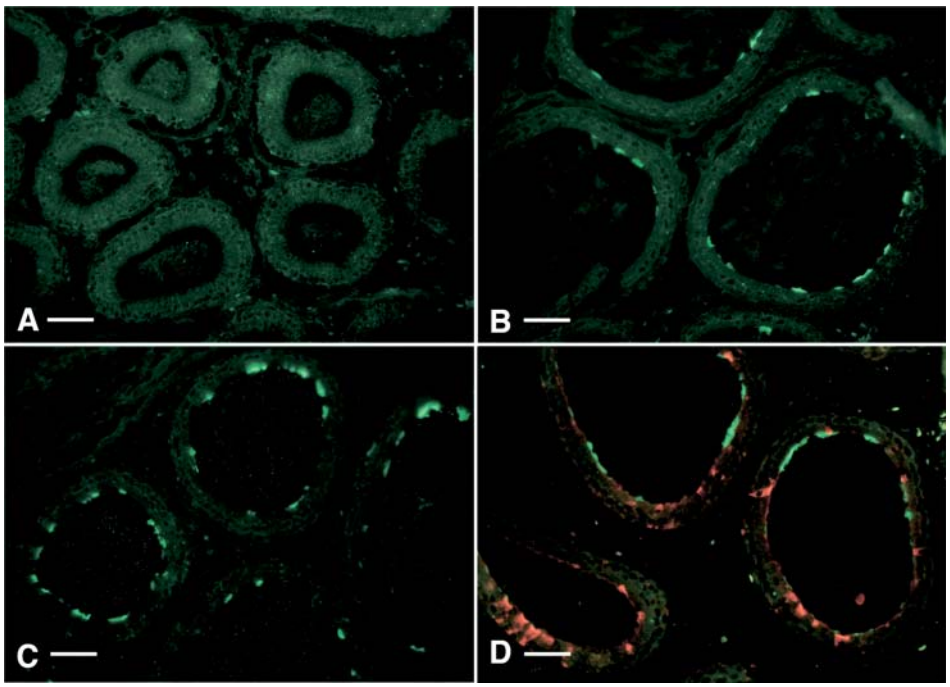

FIG. 6. Immunofluorescence detection of AQP5 in the adult epididymis. No staining for AQP5 (green) is detectable in the initial segments (**A**). AQP5 is present in the apical membrane of a subpopulation of epithelial cells in the corpus (**B**) and cauda (**C**) epididymidis. **D**) Cauda epididymidis was double labeled for AQP5 (green) and the E2 subunit of the V-ATPase (red), to identify clear cells. AQP5-positive cells do not express the V-ATPase, indicating that they are principal cells. Bars = 25 μm.

transiently in the distal region of the epididymis during postnatal development. Downstream from the epididymis, AQP2 immunostaining began to appear in the vas deferens during the first postnatal week (Fig. 9). An intense staining was detected in the apical pole of epithelial cells during Weeks 1, 2, 3, and 4 (Fig. 9, B–E). As we have previously shown [29], in the adult vas deferens, a weaker AQP2 staining was detected in a subpopulation of principal cells in the middle region (Fig. 9F). Interestingly, intensely stained immunoreactive material was detected in the lumen of the vas deferens at Postnatal Weeks 3 and 4. By Western blot, in agreement with the immunofluorescence data, no AQP2 protein was detected in the caput epididymidis at any postnatal time point (Fig. 10, caput). In the cauda epididymidis, whereas no AQP2 band was

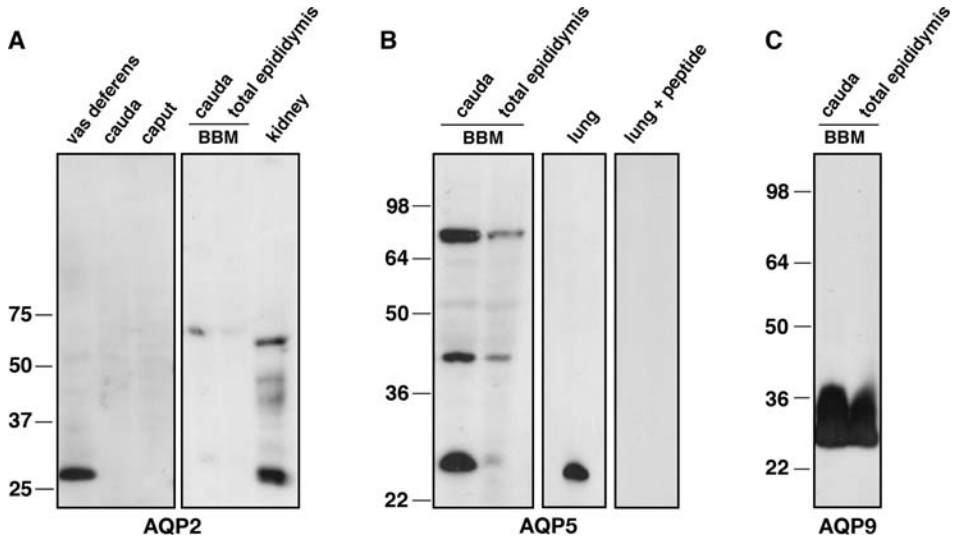

FIG. 7. Western blot analysis of AQP2, 5, and 9 expression in the adult rat epididymis. (**A**: left panel) Twenty-five micrograms of total homogenates from vas deferens, and cauda and caput epididymidis were loaded onto the gel. (**A**: right panel) Seventy-five micrograms of a BBM preparation isolated from the cauda or the entire epididymis was loaded. A positive control was performed using total kidney homogenates (25 μg protein). In total homogenates from vas deferens and kidney, AQP2 shows a strong band at around 30 kDa. In contrast, no AQP2 is detected in the caput or cauda epididymides. In addition, no signal is shown in BBM isolated either from the whole epididymis or from the cauda epididymidis. Higher molecular-weight bands are detected in the kidney sample, indicating the presence of glycosylated AQP2 in this tissue. (**B**: left panel) AQP5 shows a weak band at around 30 kDa in total epididymal BBM (75 μg protein). A much stronger signal is seen in BBM isolated from the cauda epididymidis compared with BBM from the entire epididymis. Additional higher molecular-weight bands are detected, indicating the presence of glycosylated AQP5 protein in the epididymis. (**B**: middle panel) Specificity of the anti-AQP5 antibody was assessed in the lung (0.75 μg). A strong band at around 30 kDa was detected. (**B**: right panel) This band was completely abolished after preincubation of the antibody with the immunizing AQP5 peptide. (**C**) Detection of AQP9 in BBM isolated from the whole epididymis and from the cauda epididymidis (75 μg protein). A strong band at around 30 kDa and higher molecular-weight bands for AQP9 are detected. The intensity of the bands is similar in both BBM preparations.

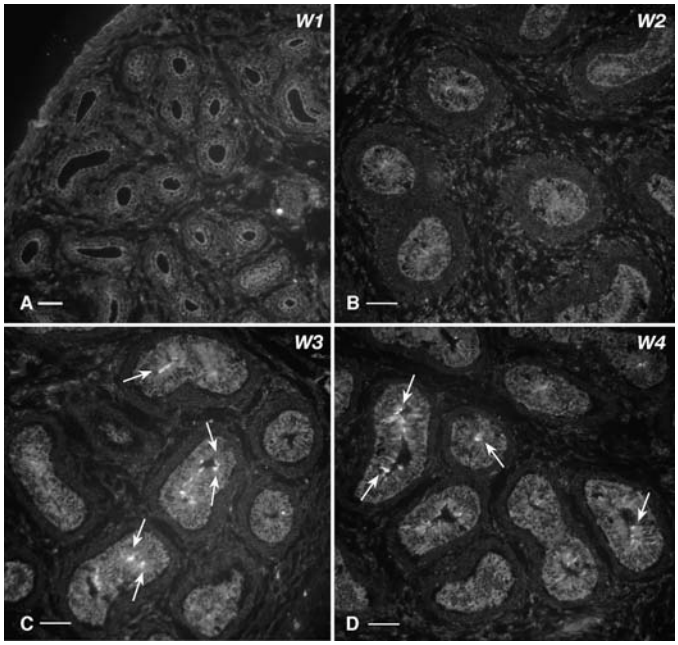

FIG. 8. Immunolocalization of AQP2 in the epididymis during postnatal development. AQP2 is not detected above background levels in cauda epididymidis after Postnatal Week 1 (**A**: *W1*) and Week 2 (**B**: *W2*). After 3 wk (**C**: *W3*) and 4 wk (**D**: *W4*), AQP2 staining is visible in the apical pole of some epithelial cells (arrows). Bars = 25 μm.

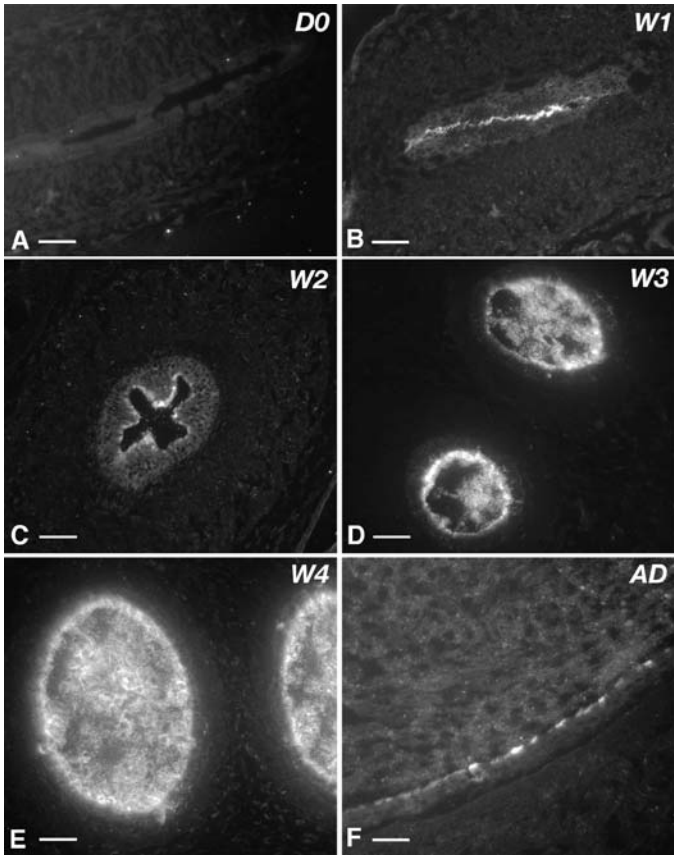

FIG. 9. Immunolocalization of AQP2 in the vas deferens during postnatal development. AQP2 is not visible at birth (**A**: *DO*). After the first (**B**: *W1*) and second (**C:** *W2*) postnatal weeks, AQP2 is detected in the apical pole of epithelial cells. At Weeks 3 (**D**: *W3*) and 4 (**E**: *W4*), intensively labeled luminal material is seen in addition to the apical staining in epithelial cells. In the adult animal, AQP2 is located in a subpopulation of principal cells in the midportion of the vas deferens and positive luminal material is not detected (**F**: *AD*). Bars = 25 μm

detected at the early time points, a faint band was seen at Postnatal Week 3 and a strong signal was obtained at Week 4 (Fig. 10, cauda). In the vas deferens, AQP2 was detected from the first week of development to adulthood, and the signal reached a maximum after 4 wk (Fig. 10, vas deferens). Loading controls were performed by blotting the same membrane for actin (Fig. 10, lower panels).

Figure 11 shows that AQP5 was detected in the cauda epididymidis only after the fourth week of postnatal development. At this time point, a subpopulation of epithelial cells exhibited a strong apical AQP5 staining (Fig. 11B). No staining was observed at birth and after Weeks 1, 2 (not shown), and 3 (Fig. 11A). The initial segments and caput epididymidis remained negative throughout development.

Aquaporin 2 Coding Sequence Expression in Adult Rat Epididymis

The primer sets described above were specifically designed to amplify short fragments that are located in the 3′ end of the cDNA. Thus, the amplicons obtained with these primers contain a short fragment of the coding sequence or even no coding sequence at all. As described above, *Aqp2* mRNA is abundant in adult epididymis epithelial cells, but the protein is not detectable, either by immunofluorescence or Western blotting. To determine whether the coding sequence of *Aqp2* is present in the epididymis, we performed an additional RT-PCR study with a second set of primers (see Table 1), which allows the amplification of a 746-bp fragment of the *Aqp2* mRNA, including approximately 80% of the published coding sequence. This long PCR product was detected in the adult caput, corpus, and cauda epididymidis (Fig. 12) as well as in the kidney (not shown), and its specificity was confirmed by direct sequencing (not shown). This semiquantitative analysis demonstrates that the coding sequence of *Aqp2* is present in epididymal epithelial cells and also shows that the *Aqp2* mRNA expression level is significantly higher in the cauda epididymidis compared with the caput and corpus.

DISCUSSION

In actively transporting epithelia, including kidney, lung, efferent ducts, and eye, water flow involves the coordinated activity of apical and basolateral AQPs [9]. In the epididymis, water transport is essential to establish the environment in which sperm mature and are stored. However, to date, very few AQPs have been described in the epididymal epithelium. The purpose of this study was, therefore, to conduct an exhaustive evaluation of the expression of all known AQPs in this tissue. Our results are consistent with previous data showing expression of *Aqp1* in endothelial cells and muscle cells [26, 27] and *Aqp9* in principal cells of the epididymis [26, 32, 33]. In addition, we show that the mRNAs coding for other AQPs (*Aqp2*, *3*, *5*, *6*, *7*, *8,* and *11*) are present in this organ. Out of all AQP mRNAs detected in extracts from the entire epididymis, only *Aqp1*, *2*, *5*, *7*, *9,* and *11* were detected in epithelial cells isolated by laser capture microdissection (LCM). Thus, LCM combined with in vitro RNA amplification allowed for an epithelial cell-specific analysis of AQP gene expression in the epididymis.

In the total epididymis sample, the RT-PCR signal obtained for *Aqp3*, whose protein has been recently reported in basal

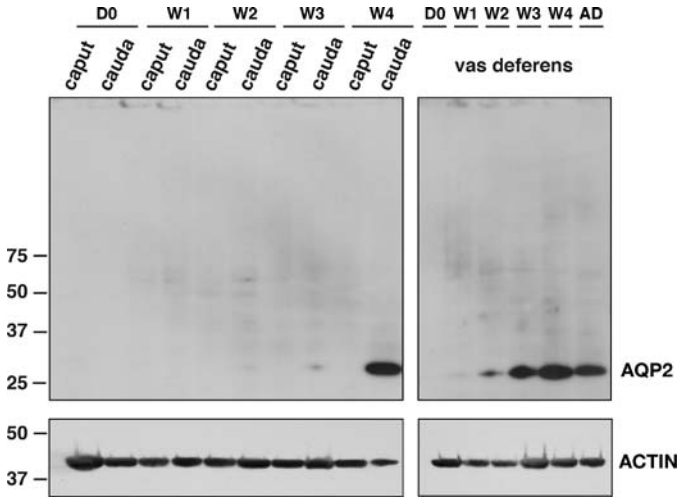

FIG. 10. Western blot analysis of AQP2 expression in the epididymis and vas deferens during postnatal development. Vesicle and plasma membrane preparations (25 μg protein) isolated from the caput or cauda epididymidis (top left panel) or from the vas deferens (top right panel) were loaded onto the gel. At all time points during postnatal development, no signal for AQP2 was detected in the caput epididymidis samples (caput). In the cauda epididymis (cauda), while no signal was detected at the early time points (D0 and W1), a faint band at around 30 kDa (AQP2) starts to appear at Week 2 (W2) and a slightly stronger band is detected at Week 3 (W3). At Week 4 (W4), a strong AQP2 band is seen. In the vas deferens, no AQP2 band is visible at birth (D0), and AQP2 signal progressively increases in intensity between Week 1 and Week 4 (W1, 2, 3, 4). The signal reaches maximal intensity at Week 4 and remains strong in the adult (AD). Bottom panels) Western blot loading controls using a β-actin antibody.

cells [35], was surprisingly weak. Moreover, no significant *Aqp3* signal was detected in the amplified RNA generated from the LCM samples, despite the fact that basal cells are probably represented in these samples. In addition, the anti-AQP3 antibody used in this study did not detect AQP3 in any cell type of the epididymis by immunofluorescence. This antibody could, however, reveal a strong staining in principal cells of the kidney collecting duct, showing its ability to detect AQP3. In addition, we showed that, whereas mRNA transcripts specific for *Aqp6* and *8* are present in the whole organ, they are not detectable in the LCM samples, indicating that the genes coding for those AQPs are not specifically expressed in epithelial cells. These results are in agreement with previous studies showing the absence of AQP8 from the rat epididymis [26, 50]. However, expression of AQP8 in basal cells was reported by another group [36].

We describe here for the first time the expression of AQP5 in the epididymis. The mRNA is present in microdissected epithelial cells, and the protein is located in the apical membrane of a subpopulation of principal cells in the corpus and cauda epididymidis. Isolation of BBM from the distal epididymis was necessary to detect a significant signal for AQP5 by Western blotting, corroborating its immunofluorescence localization. AQP5, which is selectively permeant to water, has been previously described in the salivary, lachrymal, and sweat glands, as well as in the lung, the ear, and the cornea [51–53]. In the epididymis, AQP5 is located in the apical membrane of principal cells, where AQP9 is also expressed [26, 32, 33]. AQP5 may be involved in the final regulation of water transport in the distal epididymis, or it could represent a potential backup water-transport mechanism in situations where the function of AQP9 is impaired. The expression of two AQPs in the same membrane domain of one given cell type has also been reported in other epithelia actively involved in water transport, including the efferent ducts, the distal vas deferens, and kidney collecting ducts. In efferent ducts, both AQP9 and AQP1 are coexpressed in the apical membrane of nonciliated cells [26, 27, 33]. In the ampulla of the vas deferens, AQP1, AQP2, and AQP9 are all expressed in the apical membrane of principal cells [27, 29, 33]. In the kidney collecting duct, principal cells contain both AQP3 and AQP4 in their basolateral membrane, as well as AQP2 in some kidney regions [20, 54, 55] (see also Fig. 4). The functional importance of the coexpression of selective water channels (AQP1, AQP2, AQP4, or AQP5) together with aquaglyceroporins (AQP3 or AQP9) in kidney, efferent ducts, vas deferens, and epididymis still remains to be determined. AQP1, 2, and 9 in the vas deferens and AQP5 and AQP9 in the epididymis probably contribute to the establishment and maintenance of an appropriate luminal environment for sperm maturation, concentration, and storage. In agreement with this notion, previous studies have shown the presence of a significant AQP9-dependent water transport across the epididymal epithelium [56, 57].

We also detected significant mRNA levels of the recently described *Aqp11* in epididymal epithelial cells. However, no antibody is available to date to determine whether AQP11 protein is expressed and to characterize its cellular distribution in the epididymis. This will be the subject of future work. Surprisingly, the mRNAs coding for *Aqp1*, *Aqp2,* and *Aqp7* were also detected in epithelial cell samples of the adult

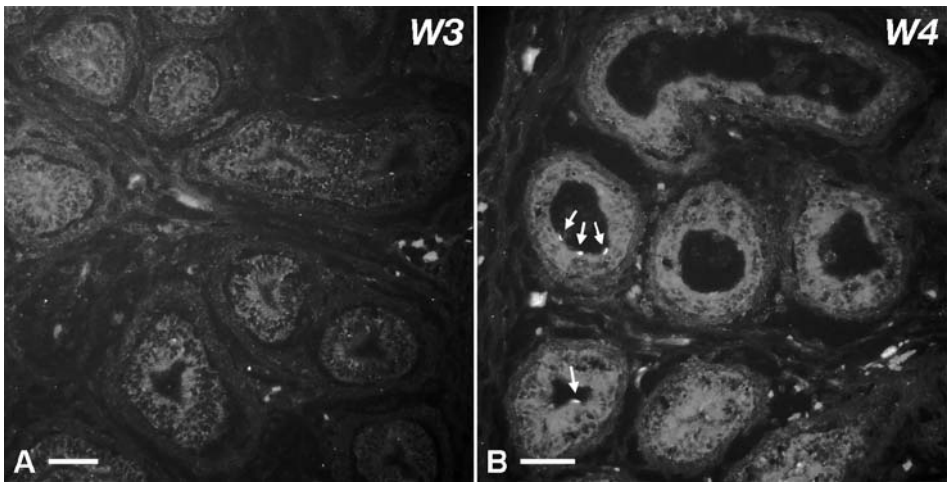

FIG. 11. Immunofluorescence detection of AQP5 in the cauda epididymidis during postnatal development. **A**) AQP5 is not detected in epithelial cells after 3 wk (*W3*). **B**) Apical AQP5 staining is visible in a subpopulation of epithelial cells (arrows) after 4 wk of development (*W4*). Bars = 20 μm.

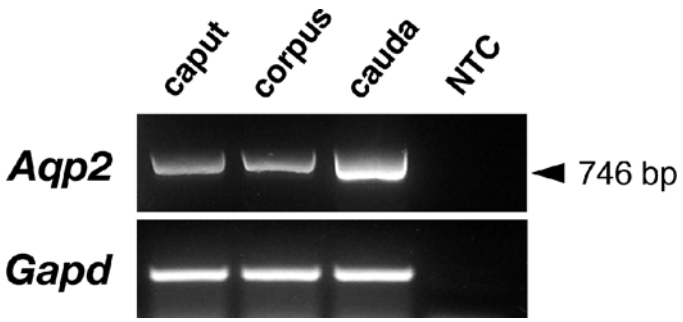

FIG. 12. Expression of *Aqp2* coding sequence in the adult rat epididymis. A 746-bp fragment, representing 80% of the *Aqp2* coding sequence, is amplified from RNA extracted from the caput, corpus, and cauda epididymidis. NTC, No template control.

epididymis, while our laboratory and others have constantly failed to immunolocalize these proteins in these cells [26, 27, 29, 50]. It is likely that the faint *Aqp1* mRNA signal that was obtained by RT-PCR might be attributed to a minor contamination by the underlying smooth muscle cells, as these cells do express AQP1. Improvement of the resolution of the laser microdissection technologies, allowing for the isolation of single cells, will undoubtedly help to resolve this issue. In the present study, AQP7 was not detected by immunofluorescence or Western blotting, despite the utilization of two antibodies directed against different domains of the protein (our antibody against the C-terminus and a commercial antibody against the N-terminus of AQP7). In agreement with our results, a previous study also showed the absence of AQP7 from epithelial cells of the epididymis [50]. However, with these two antibodies, we also did not detect any specific staining in the kidney and testis, which have been shown to express AQP7 [58–60], and the present study does not, therefore, allow us to conclude whether or not AQP7 is expressed in the epididymis. Further studies using novel AQP7 antibodies will be required to demonstrate the absence (or presence) of AQP7 in epididymal epithelial cells and to compare it with the expression of *Aqp7* mRNA in these cells.

As the rats matured toward adulthood, a marked increase in *Aqp2* mRNA levels was detected by qRT-PCR, with a maximum expression level observed at Postnatal Week 4. During this period, AQP2 protein exhibited a complex pattern of spatial expression, with a transient increase in the vas deferens and cauda epididymidis during the first 4 wk, followed by a disappearance of the protein from the cauda epididymidis and the proximal vas deferens ([29] and the present study) in adult animals. Interestingly, significant *Aqp2* mRNA levels were detected in LCM samples from adult rats and the coding region of the *Aqp2* gene was expressed in whole adult epididymis extracts. Thus, these results suggest that AQP2 expression is regulated by posttranscriptional or translational mechanisms. We have previously shown that dehydration of adult rats increases *Aqp2* mRNA levels in the kidney and vas deferens, but that an increase in AQP2 protein occurs only in the kidney [29]. This indicates that AQP2 expression is modulated differently in the kidney and vas deferens. Whether this occurs at the level of translational regulation, protein degradation, or some other posttranslational processes remains unknown. Another major difference between AQP2 in the kidney and the vas deferens is the presence of glycosylated AQP2 in the kidney and its absence from the vas deferens ([29] and the present study). Also, while AQP2 is recycling between the apical membrane and subapical vesicles in kidney principal cells, it is constitutively expressed in the apical membrane of vas deferens principal cells ([29] and the present study). It was proposed that the glycosylation state of AQP2 might modulate its intracellular targeting. AQP2 glycosylation could also contribute to the stability of the protein, which would explain the absence of AQP2 in the adult epididymis and its abundance in the kidney. In addition, while AQP2 was detected exclusively in epithelial cells of the vas deferens at early time points during postnatal development, at Weeks 3 and 4, an intensely stained luminal material was observed in addition to epithelial cell staining. Interestingly, in the kidney, significant levels of AQP2 are detected in the urine, secondary to the shedding of AQP2 into the lumen of the collecting duct [61]. Similarly, AQP2 disappearance from the adult epididymal epithelium may be partially attributed to the shedding of AQP2 into the lumen of the epididymal and vas deferens tubule.

Aqp9 mRNA levels also markedly increased during the first 4 postnatal wk, but the transcript remained abundant in adulthood. These results are in agreement with our previously published study showing a progressive increase in AQP9 protein expression during postnatal development [33]. In addition, both AQP2 (the present study) and AQP9 [33] proteins start to be expressed in the vas deferens at earlier postnatal time points compared with the epididymis. Thus, the very large increase in both *Aqp2* and *Aqp9* mRNAs together with their protein expression patterns during postnatal development indicate that *Aqp2* and *Aqp9* genes may share common mechanisms of expression regulation, at least during the first weeks of postnatal development. Because these major variations occurred before puberty, the arrival of luminal sperm does not seem to participate in this process. The *Aqp9* promoter contains a putative steroid receptor binding site [62], and we have previously shown that flutamide treatment or castration significantly reduce AQP9 protein expression in the adult epididymis [34]. Interestingly, previous studies have shown that estrogens play a critical role in the development of the male reproductive tract and in fluid reabsorption via regulation of AQP1 expression in the efferent duct [24, 28, 63, 64]. In addition, whereas both androgens and estrogens are involved in the regulation of AQP9 expression in the efferent duct, expression of AQP9 in the initial segment seems to be dependent on androgen stimulation only [25].

Further studies will be required to determine whether the dramatic hormonal changes, including increase in androgen levels, that occur during puberty are responsible for the disappearance of AQP2 protein in the adult epididymis. The role of estrogens will also have to be investigated. Interestingly, the mRNAs coding for *Aqp5*, *7,* and *11* varied to a much lesser extent, compared with *Aqp2* and *9*, showing a moderate and progressive increase from birth to adulthood. These results indicate that the expression of *Aqp5*, *7,* and *11* mRNAs is modulated by factors that are distinct from those that induce the dramatic increase observed for *Aqp2* and *9* mRNAs.

In conclusion, this study confirms that AQP9 is the main AQP of the epididymis, where it may contribute to the apical membrane permeability to water and neutral solutes. While the mRNAs coding for numerous AQPs were detected in the epididymis, only AQP5 protein was clearly detectable in the adult epididymis, where it was located in the apical membrane of a few principal cells. The identity of the AQP or water exit pathway that may participate in water transport across the basolateral membrane leading to transepithelial flow remains unknown. Characterization of the posttranscriptional processes responsible for the regulation of *Aqp2*, for which the mRNA was detected in the absence of protein, will be important to

understand better the mechanisms underlying the regulation of water permeability in the male reproductive tract as well as in other systems in which this AQP is expressed.

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