Expression of an AQP2 Cre recombinase transgene in kidney and male reproductive system of transgenic mice

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[1]*University of Utah Medical School, Salt Lake City 84132;* [2]*Veterans Affairs Medical Center, Salt Lake City, Utah 84148; and* [3]*Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129*

Nelson, Raoul D., Peter Stricklett, Corrine Gustafson, Anna Stevens, Dennis Ausiello, Dennis Brown, and Donald E. Kohan. Expression of an AQP2 Cre recombinase transgene in kidney and male reproductive system of transgenic mice. *Am. J. Physiol.* 275 (*Cell Physiol.* 44): C216–C226, 1998.—A transgenic mouse approach was used to examine the mechanism of principal cell-specific expression of aquaporin-2 (AQP2) within the renal collecting duct. RT-PCR and immunocytochemistry revealed that murine AQP2 was expressed in principal cells in the renal collecting duct, epithelial cells of the vas deferens, and seminiferous tubules within testis. The vas deferens expression was confirmed in rats. RT-PCR and immunocytochemistry showed that 14 kb of the human 5′-flanking region confers specific expression of a nucleus-targeted and epitope-tagged Cre recombinase in the principal cells within the renal collecting duct, in the epithelial cells of the vas deferens, and within the testis of transgenic mice. These results suggest that cell-specific expression of AQP2 is mediated at the transcriptional level and that 14 kb of the human AQP2 5′-flanking region contain *cis* elements that are sufficient for cell-specific expression of AQP2. Finally, renal principal cell expression of Cre recombinase is the first step in achieving cell-specific gene knockouts, thereby allowing focused examination of gene function in this cell type.

water-electrolyte balance; kidney collecting tubule; ion channel physiology; gene expression regulation; promoter region; aquaporin

AQUAPORIN-2 (AQP2) belongs to a family of water channel proteins and is fundamentally important in regulation of renal water excretion (1, 28). The renal site of AQP2 expression is crucial to its function. AQP2 is expressed exclusively in collecting duct principal cells (12, 13, 35). These cells are located in the distal and terminal nephron, the region of the kidney where final adjustments to urine concentration are made. In addition, principal cells are concentrated in the renal medulla, where water reabsorption is facilitated by high interstitial tonicity. It is evident, therefore, that principal cell-specific expression of AQP2 is of primary importance to the regulation of renal water excretion.

AQP2 is regulated in two ways. Long-term regulation occurs by arginine vasopressin (AVP) augmentation of AQP2 mRNA and protein levels (6, 20, 21, 29, 35), resulting in increased numbers of water channels in the kidney. Short-term regulation occurs by vasopressin-induced movement of AQP2 protein from subapical-cytosolic vesicles to the apical plasma membrane by exocytic insertion of AQP2-containing vesicles into the plasma membrane (35, 39). These processes result in enhanced water permeability of the collecting duct, thereby allowing water to be osmotically reabsorbed by the kidney across collecting duct epithelium as a result of the interstitial hypertonicity that is generated in the medulla. To date, AQP2 is the only known mechanism by which AVP directly controls renal water excretion.

Although factors modulating AQP2 expression are beginning to be identified, very little is known about the mechanism of principal cell-specific expression of this AVP-sensitive water channel. One potential mechanism of cell-specific expression involves cell-specific gene transcription mediated by *cis*-acting sequences in the human AQP2 gene and transcription factors expressed in principal cells. In this context, regulatory regions of the AQP2 gene have been examined. The human AQP2 5′-flanking region contains a TATA box with transcription initiation sites 92 and 93 bp upstream from the translational initiation site (44). Further sequence analysis reveals putative transcriptional regulatory sequences, including several cAMP response elements (CREs), several GATA consensus sites, E-boxes, and AP-1, AP-2, and SP1 sites (24, 44). Studies have demonstrated a role for the CREs and GATA sites in altered levels of AQP2 gene transcription by cAMP-responsive element binding protein and GATA-3 (24, 33, 43), respectively. It is unclear whether these sequences play a role in kidney- and principal cell-specific expression of AQP2.

To begin to examine how principal cell-specific expression of AQP2 occurs, a transgenic mouse approach was utilized. A 14-kb section of the human AQP2 gene 5′-flanking region was used to determine whether transcriptional elements contained within this region were responsible for cell-specific expression. We report that this reporter region does indeed confer principal cell-specific expression within the kidney. An unanticipated result was transgene expression in the vas deferens and testis, where AQP2 is also expressed. Finally, an added feature of the transgene was the inclusion of Cre recombinase and an epitope tag as the reporter (CreTag). As described, this system will ultimately be used to attempt principal cell-specific gene targeting within the kidney.

METHODS

Construction of the AQP2-CreTag transgene. All cloning and DNA manipulations were done using conventional meth-

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ods (32). pMC-Cre (gift from Kirk Thomas, Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT) includes a Cre recombinase gene that was modified at the amino-terminal end to include the nuclear localization signal from the simian virus 40 (SV40) large T antigen (18). pMC-Cre was further modified at the carboxy-terminal end to include an 11-amino acid herpes simplex virus (HSV) glycoprotein D epitope tag (QPELAPEDPED) (27). To do this, the Cre gene was cut from pMC-Cre with *Eco*R I/*Xho* I and subcloned into *Eco*R I/*Xho* I-digested pBluescript KS II (Stratagene, La Jolla, CA) without the *Bsp*H I sites in the vector, resulting in pBS-Cre. Overlapping and complementary 80-mer oligonucleotides were synthesized (Bob Schackman, DNA/Peptide Facility, University of Utah), annealed, and extended with Klenow enzyme. The resulting double-stranded DNA contains sequence encoding the carboxy-terminal Cre extending from a *Bsp*H I site, the carboxy-terminal 11-amino acid epitope tag, an added stop codon, and *Xba* I and *Xho* I sites. This DNA was digested with *Bsp*H I/*Xho* I and ligated into *Bsp*H I/*Xho* I-digested pBS-Cre, resulting in pBS-CreTag. An *Xba* I linker was ligated into pBS-CreTag that was digested with *Mlu* I and blunted with Klenow enzyme. This modified pBS-CreTag was digested from the vector with *Xba* I and ligated into the *Xba* I site of pUHD 10-3 (16), becoming pUHD-CreTag. pUHD provided a SV40 late region polyadenylation signal downstream from CreTag. CreTag with the polyadenylation signal was digested from pUHD-CreTag with *Hin*D III, blunted with Klenow enzyme, and cut with *Sma* I. This fragment was ligated into the *Sma* I site of pBluescript KS II that resides downstream from the 14-kb human AQP2 5′-flanking region, resulting in pAQP2-CreTag. Cloning of the human AQP2 5′-flanking region was previously described (24). pAQP2-CreTag was digested with *Xho* I/*Not* I, and the AQP2-CreTag transgene was separated from the vector sequences by electrophoresis through low-melting-point agarose. The transgene was purified with an Elutip-D column (Schleicher & Schuell, Keene, NH) after digestion of the agarose with β-agarase I (New England Biolabs) and suspended in injection buffer. All ligation junctions, the entire CreTag sequence and the 5′ and 3′ 300 bp of the AQP2 5′-flanking region were sequenced to verify the structural integrity of the transgene. Finally, to express CreTag in cultured cells, CreTag was subcloned downstream from a cytomegalovirus (CMV) promoter.

Generation and breeding of transgenic mice. Transgenic mice were created by the Transgenic Mouse Core Facility at the University of Utah. The linearized transgene was injected into pronuclei of C57BL/CBA single cell embryos, and the injected embryos were transferred into pseudopregnant mice according to standard techniques (22). Pups were analyzed for the presence of the transgene by PCR amplification of tail DNA. Three founders were each bred to nontransgenic C57BL/CBA mice. F_1 and F_2 animals from each founder line were identified by PCR of tail DNA and used for analysis.

Identification of transgenic animals. Tail DNA was prepared by standard methods (23). The transgene was detected by PCR amplification of tail DNA using oligonucleotide primers AQP2+310 (5′-GGA CGT CAG TCC TTA TCT GGA G-3′) and CreDown (5′-GCG AAC ATC TTC AGG TTC TGC GG-3′), which span 625 bp of the junction between the AQP2 promoter and CreTag reporter. Normal mouse DNA with 1–100 copies/cell equivalent of the transgene DNA was always run as a control to estimate copy number. Equal loading and amplification efficiency of genomic DNA was controlled for by amplification with RAPSYN(+) (5′-AGG ACT GGG TGG CTT CCA ACT CCC AGA CAC-3′) and RAPSYN(−) (5′-AGC TTC TCA TTG CTG CGC GCC AGG TTC AGG-3′), which amplify 590 bp of the endogenous gene RAPSYN (19). The products were electrophoresed through 2% agarose and were visualized by ethidium bromide staining and ultraviolet (UV) transillumination.

Expression of CreTag by transient transfection into cultured cells. Two micrograms of the plasmid containing CreTag and a CMV promoter (CMV-CreTag) were transiently transfected into a 35-mm dish of 3T3 cells using Lipofectamine (GIBCO BRL, Gaithersburg, CA) according to the manufacturer's protocol. RNA was prepared with the acid phenol method (5) 48 h posttransfection.

RT-PCR determination of AQP2, CreTag, and glyceraldehyde-3-phosphate dehydrogenase mRNA expression. RNA was prepared from transgenic mouse organs with the acid phenol method (5); 2.5 μg total RNA from organs or cells was reverse transcribed using oligo$(dT)_{12–18}$ and Superscript II according to the manufacturer's procedure (GIBCO BRL). The following oligonucleotide primers were used to PCR amplify AQP2, CreTag, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from one-tenth of the reverse transcription reaction: AQP2F (5′-GTG GCT GCC CAG CTG CTG GG-3′) and AQP2R2 (5′-AGC TCC ACC GAC TGC CGC CG-3′) were used to amplify 500 bp of the mouse AQP2 cDNA; CreTagUP (5′-GGC TCT AGC GTT CGA ACG CAC TGA TTT CGA-3′) and SV40LateR (5′-T_{24} GTT GTT AA-3′) were used to amplify 844 bp of CreTag cDNA; CreTagUP and CreTagDown2 (5′-GGC TAT CGC CAT CTT CCA GCA-3′) amplify the 635 bp of Cre recombinase cDNA or gene, and GAPDHF (5′-CCT TCA TTG ACC TCA ACT ACA TGG-3′) and GAPDHR (5′-GCA GTG ATG GCA TGG ACT GTG GT-3′) were used to amplify 442 bp of the mouse GAPDH cDNA. All RT-PCR reactions were carried out with and without RT to establish whether cDNA or genomic DNA was being amplified. A negative control without template was also run with each set of reactions to demonstrate that there was no contamination of the PCR reaction. In selected experiments, 10 ng of the CMV-CreTag plasmid DNA was used as a template for PCR amplification of CreTag. The products were electrophoresed through 2% agarose and were visualized by ethidium bromide staining and UV transillumination. PCR products were directly sequenced using a dye terminator cycle sequencing system with Amplitaq DNA polymerase FS (Perkin Elmer, Norwalk, CT) in conjunction with an ABI fluorescent sequencing system (DNA Sequencing Core Facility, University of Utah).

Single- and double-label immunohistochemistry. Kidneys and male reproductive organs were fixed by cardiac perfusion and immersion in 4% paraformaldehyde in Dulbecco's PBS at 4°C, dehydrated with graded ethanol, paraffin embedded, and sectioned to 5 μm thickness. The sections were deparaffinized and microwaved in an antigen retrieval solution (Biogenex, San Ramon, CA) (41).

Single-label immunohistochemical visualization of AQP2 and CreTag was accomplished by the following protocol. Sections were blocked with 10% goat serum in PBS (blocking solution) for 2 h and incubated overnight at 4°C with 1:2,000 dilution of an affinity-purified polyclonal rabbit antiserum to AQP2 (35) (gift from Mark Knepper, National Institutes of Health, Bethesda, MD) or a 1:1,000 dilution of a mouse monoclonal antibody to the HSV epitope tag (Novagen, Madison, WI) in blocking solution. Sections were washed with 0.1% Triton X-100 in PBS and incubated with a 1:100 dilution of a biotinylated goat anti-mouse or goat anti-rabbit antibody in blocking solution for 2 h at 4°C. Sections were washed, incubated with preformed complexes of streptavidin and biotinylated-peroxidase conjugate (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, washed again, and incubated with 3′,3′-diaminobenzidine and H_2O_2 with

metal enhancement (Vector Laboratories). Selected sections were counterstained with hematoxylin.

Double-label immunofluorescent visualization was accomplished using the following protocol. Sections were blocked with 5% goat serum and 5% horse serum in PBS (blocking solution) and incubated with a 1:2,000 dilution of an affinity-purified rabbit polyclonal antibody to AQP2 and a 1:1,000 dilution of a mouse monoclonal antibody to the HSV epitope tag in blocking solution overnight at 4°C. Sections were washed and incubated with a 1:200 dilution of fluorescein-conjugated goat anti-rabbit IgG antibody (Vector Laboratories) and a 1:200 dilution of a Texas red-conjugated horse anti-mouse IgG antibody (Vector Laboratories) in blocking solution for 2 h at 4°C. Sections were washed, mounted in Vectashield (Vector Laboratories) and viewed with an Olympus BX50 fluorescent microscope equipped with a CH250 Photometrics charge-coupled device (CCD) camera. Images were digitally acquired with Visis Smartcapture version 2.4.

Immunofluorescent visualization in the rat vas deferens was accomplished as previously described (3). The tissue was fixed in paraformaldehyde lysine periodate. After cryoprotection in 30% sucrose, 5-μm sections were cut and incubated with a polyclonal anti-AQP2 carboxy-terminal peptide antibody that had been previously characterized (39). After they were washed, the sections were incubated with goat anti-rabbit IgG-FITC and counterstained with Evan's blue. Sections were examined on a Nikon FXA photomicroscope equipped with an Optronics three-bit color CCD camera. Images were acquired digitally with IP Lab Spectrum software.

Immunoblotting analysis for CreTag in kidney and testis. Kidney and testis from AQP2-CreTag transgenic and non-transgenic male mice were minced and nuclei isolated by a modification of a previously described method (8). The tissues were homogenized using a Dounce homogenizer and pushed through a nylon mesh (Small Parts, Miami, FL). Nuclei were pelleted and nuclear proteins isolated using previously described methods (7, 36). In brief, the nuclear pellet was resuspended in 20 mM HEPES (pH 7.9), 420 mM NaCl, 0.2 mM EDTA (pH 8), 1.5 mM $MgCl_2$, 25% (vol/vol) glycerol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5 mM dithiothreitol (DTT), incubated on ice for 15 min, and centrifuged at 15,000 *g* for 15 min at 4°C. The supernatant was diluted 1:5 in 20 mM HEPES (pH 7.9), 0.2 mM EDTA (pH 8), 50 mM KCl, 20% (vol/vol) glycerol, 0.5 mM PMSF, and 0.5 mM DTT, and aliquots were removed for protein determination using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). Nuclear proteins were electrophoresed through a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride-plus membrane (Micron Separations, Westboro, MA) using standard protocols (14). Immunoblotting was performed using the enhanced chemiluminescence protocol (Amersham, Arlington Heights, IL). A mouse monoclonal antibody to the HSV epitope tag (Novagen, Madison, WI) was used as primary antibody at a dilution of 1:20,000, and a horseradish peroxidase-conjugated sheep anti-mouse antibody was used as a secondary antibody at a dilution of 1:5,000 (Amersham). Detection was accomplished by enhanced chemiluminescence, using Hyperfilm-MP (Amersham). The relative molecular mass was calculated for CreTag by comparison to the standards.

RESULTS

Transgene design. To achieve principal cell-specific gene expression in the kidney and to begin to achieve principal cell-specific gene targeting, we designed a transgene with the AQP2 5′-flanking region driving expression of a modified Cre cassette that was designated CreTag (Fig. 1). The original Cre cassette included an SV40 nuclear localization signal on the amino terminus (18). An 11-amino acid epitope tag, derived from HSV glycoprotein D (27), was added to the carboxy-terminal end to facilitate detection of Cre by immunoblotting and immunohistochemistry using a mouse monoclonal antibody (Novagen). The 362-amino acid CreTag has a predicted molecular mass of 41 kDa. The polyadenylation signal from the SV40 late region, without an intron, was also added for efficient expression (accession no. V01380). The CreTag cassette was inserted into a CMV expression vector, resulting in CMV-CreTag, and was transiently transfected into 3T3 cells. CreTag mRNA was detectable by RT-PCR, and CreTag protein was detectable in the nucleus of cells by immunocytochemistry and immunoblotting analysis (Stricklett, Nelson, and Kohan, unpublished results). In addition, nuclear extracts from the cells expressing CreTag exhibit loxP site-specific recombinase activity in vitro (Stricklett and Kohan, unpublished results). A transgene was then constructed with 14 kb of the human AQP2 5′-flanking region (24) driving expression of the CreTag cassette (Fig. 1). The AQP2 5′-flanking

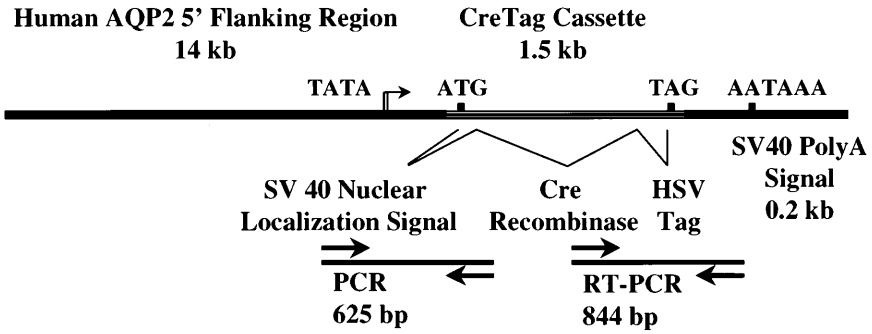

Fig. 1. Aquaporin-2 (AQP2)-CreTag transgene: 14 kb of human AQP2 5′-flanking region includes TATA box and transcription initiation sites from human AQP2 gene. Cre recombinase cassette (CreTag cassette) includes an amino-terminal simian virus 40 (SV40) nuclear localization signal, Cre recombinase coding region, carboxy-terminal herpes simplex virus (HSV) epitope tag, and SV40 virus late region polyadenylation signal. ATG is translational initiation site, and TAG is translational stop site. Transgenic animals were genotyped by PCR amplification of junction between AQP2 5′-flanking region and CreTag cassette (625 bp). AQP2-CreTag mRNA expression was determined by RT-PCR analysis for 3′ end of mRNA produced by CreTag cassette (850 bp).

region included the transcription initiation sites from the human AQP2 gene but excluded the translational initiation site. This transgene is referred to as AQP2-CreTag.

Generation of AQP2-CreTag transgenic mice. Three independent founders were obtained following pronuclear injections of the AQP2-CreTag transgene. PCR analysis of founder tail DNA demonstrated that the CreTag transgene varied between 2 and 100 copies/cell equivalent among the different founder lines compared with standards of transgene mixed with normal mouse genomic DNA (Fig. 2). The transgene was transmitted according to Mendelian genetics. Transgenic F_1 and F_2 animals from each founder line and nontransgenic animals were analyzed for expression of CreTag and AQP2.

AQP2 expression in mice. Mouse AQP2 cDNA and amino acid sequences had not been reported when these studies were initiated, but rat and human AQP2 cDNA and amino acid sequences were known to be highly conserved (13, 40). Comparison of rat and human AQP2 cDNA sequences (accession nos. L28112 and Z29491) identified regions that were 100% conserved but differed from rat AQP1 (accession nos. X67948, X70257), rat AQP3 (accession no. D17695), rat AQP4 (accession no. U14077), rat AQP5 (accession no. U16245), human AQP6 (accession no. AB006190), rat AQP7 (accession no. AB000507), and rat AQP8 (accession no. AB005547). PCR primers were designed to anneal to these regions and selectively amplify 510 bp of mouse AQP2 cDNA from kidney. The internal 450 bp of the PCR products were directly sequenced, and the sequence was translated. The products predict an amino acid sequence that is 98% identical to rat AQP2, but only 42, 20, 44, 60, 33, 40, and 40% identical to rat AQP1, AQP3, AQP4, AQP5, AQP7, and AQP8, and human AQP6, respectively, for 150 amino acids that were compared. Furthermore, the sequence was 100% identical to a sequence that was recently entered into GenBank (accession no. AF020159). The PCR products generated by these primers, therefore, were derived from mouse AQP2 cDNA.

The pattern of mouse AQP2 mRNA expression was next determined by RT-PCR analysis of RNA derived from a panel of mouse organs (Fig. 3). The correct sized products were observed in kidney, vas deferens, and testis. No products were present in other organs, such as the brain, liver, heart, intestine, stomach, spleen, ovary, fallopian tube, or uterus. The cDNA sequence of the PCR products was identical in kidney, vas deferens, and testis. These results indicated that AQP2 was selectively expressed in kidney, vas deferens, and testis.

Immunohistochemistry was used to confirm the cellular pattern of expression of AQP2 in kidney and to determine the cellular pattern of expression in the male reproductive system. An affinity-purified antibody to rat AQP2 was used (35). Immunoperoxidase (Fig. 4) and immunofluorescent staining (Fig. 5) showed mouse AQP2 in the cytoplasm and apical pole of 75–100% of collecting duct cells in the cortex, outer medulla, and inner medulla of mouse kidney. The appearance of less predominant apical staining in the immunofluorescence studies, compared with prior published studies of rat, may be due to the image capture techniques or redistribution during fixation. Otherwise, the pattern of staining is identical to that observed in rat (35) and mouse (2), suggesting that the observed staining is in principal cells. The collecting duct cells that do not stain for AQP2 are intercalated cells, as previously reported in the mouse kidney (2). In the male reproductive system, immunoperoxidase staining showed mouse AQP2 in apical and subapical regions of the principal cells in the vas deferens and the central regions of seminiferous tubules but not in the mature sperm in the epididymis or in the epididymis itself (Fig. 6). The pattern of staining suggests that AQP2 is expressed in postmeiotic sperm. However, further investigations will be required to define the exact stage of spermatogenesis at which AQP2 is expressed. This is the first

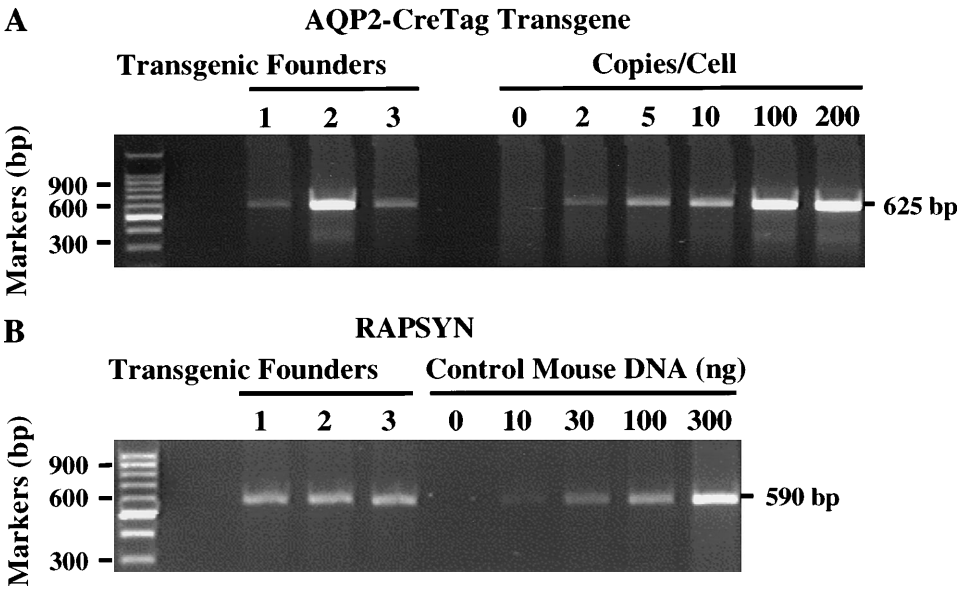

Fig. 2. PCR determination of AQP2-CreTag transgene copy number in 3 founder animals. *A*: results of PCR amplification of tail DNA from transgenic founder *lines 1–3* and 0–200 copies/cell equivalent of transgene DNA added to normal mouse DNA. Product size is 625 bp. *B*: results of PCR amplification of endogenous RAPSYN gene from transgenic founder line and control mouse DNA. Product size is 590 bp. Equal intensity of RAPSYN amplification products indicates equal amplification efficiency of tail DNA preparations. Relative intensity of AQP2-CreTag transgene indicates that transgene is present at 2, 100, and 2 copies/cell in *lines 1–3*, respectively.

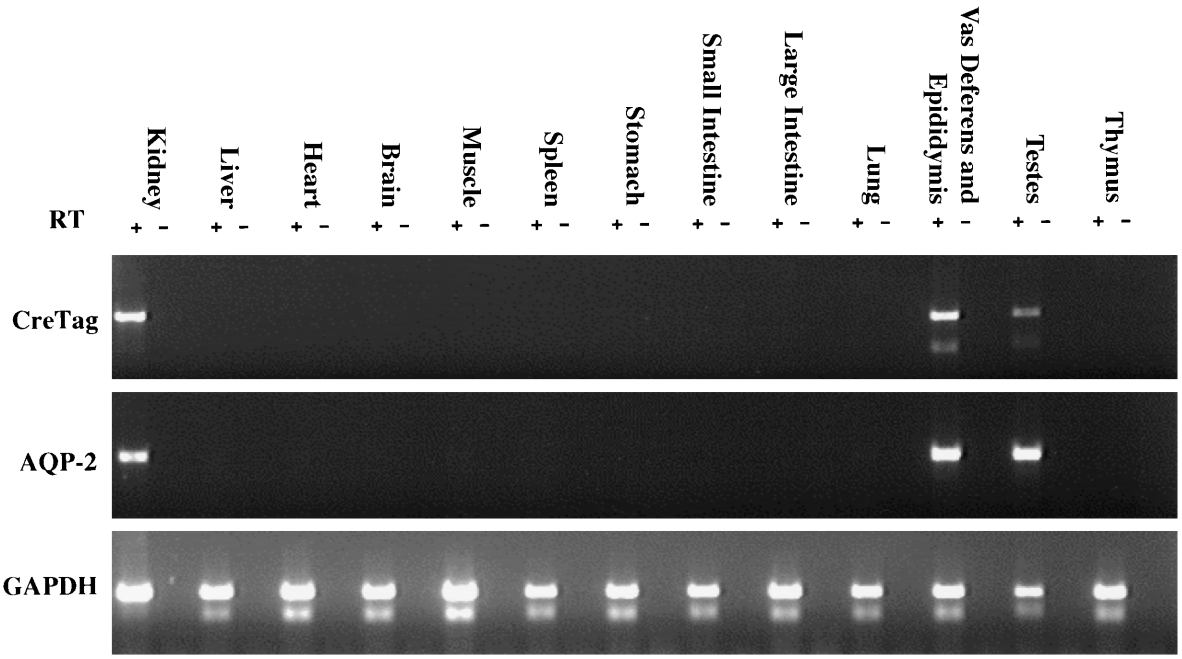

Fig. 3. RT-PCR determination of AQP2, CreTag, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in transgenic mouse *line 2*. AQP2 and CreTag are expressed in kidney, testes, and vas deferens and epididymis. GAPDH is expressed in all organs. No product is observed in absence of RT (−).

reported immunostaining for AQP2 in the male reproductive system.

AQP2 expression in the rat vas deferens. To determine whether the unexpected presence of AQP2 in the vas deferens was restricted to the mouse, we also examined sections of the rat vas deferens. As shown in Fig. 7, the apical membrane domain and microvilli of principal cells of this species were also strongly labeled with anti-AQP2 antibodies. Such staining was not observed with preimmune or normal rabbit serum. The presence of AQP2 in rat vas deferens was confirmed by PCR analysis. A total of 750 bp were obtained, and the sequence was identical to that reported for rat AQP2 (results not shown) (13)

RT-PCR method for detection of CreTag mRNA. Initial RT-PCR studies of transgene expression were complicated by contaminating genomic DNA amplification products derived from the intronless AQP2-CreTag transgene, which were indistinguishable in size from cDNA amplification products. An alternative RT-PCR method was used to amplify cDNA without amplifying contaminating genomic DNA. This was accomplished with a novel antisense PCR primer. This primer included an oligo(dT) sequence at the 5′ end that anneals to the poly(A) tail of mRNA and a 6-bp anchor sequence at the 3′ end that anneals to the site upstream of the SV40 late region polyadenylation site (11) in the CreTag reporter cassette. This antisense primer was used in combination with a sense primer that annealed upstream in the CreTag coding region. At the optimized annealing temperature, these primers amplify CreTag cDNA without amplifying the CreTag transgene or genomic DNA.

To demonstrate that this method works, a CMV expression vector containing CreTag was transiently transfected into 3T3 cells. RT-PCR analysis was performed using a standard and a novel antisense primer in combination with a common sense primer (Fig. 8). The standard antisense primer amplified product from a cDNA reaction carried out in the absence of RT as well as from a plasmid template (Fig. 8, *middle*). In contrast, the novel antisense primer only amplified product in the presence of RT and did not amplify plasmid template (Fig. 8, *top*). This validates this RT-PCR method for detection of authentic CreTag cDNA.

CreTag expression in transgenic mice. CreTag transgene mRNA expression was compared with endogenous AQP2 mRNA expression in multiple mouse organs. RT-PCR analysis was used to detect mRNA expression by the CreTag transgene and AQP2 in F_1 transgenic animals derived from each of the three founders.

CreTag and AQP2 mRNA expression was determined by RT-PCR of total RNA from F_1 transgenic mouse organs derived from each of the three transgenic founder lines. CreTag was expressed in kidney of all three lines of transgenic mice but was not expressed in nontransgenic mice. *Lines 1* and *2* exhibited expression of CreTag mRNA in kidney, vas deferens, and testis, which paralleled the expression of the AQP2. The RT-PCR results for *line 2* are shown (Fig. 3). *Line 2* showed much stronger CreTag expression than *line 1* in kidney as well as vas deferens and testis, perhaps reflecting the relative copy number of the transgene. However, despite a relatively lower transgene copy number, *line 3* showed high-level CreTag expression in kidney and many other organs. This is likely due to transgene integration near strong enhancer elements.

The cellular patterns of CreTag and AQP2 expression within kidney were determined with immunohistochemistry. Single-label peroxidase immunohistochemistry

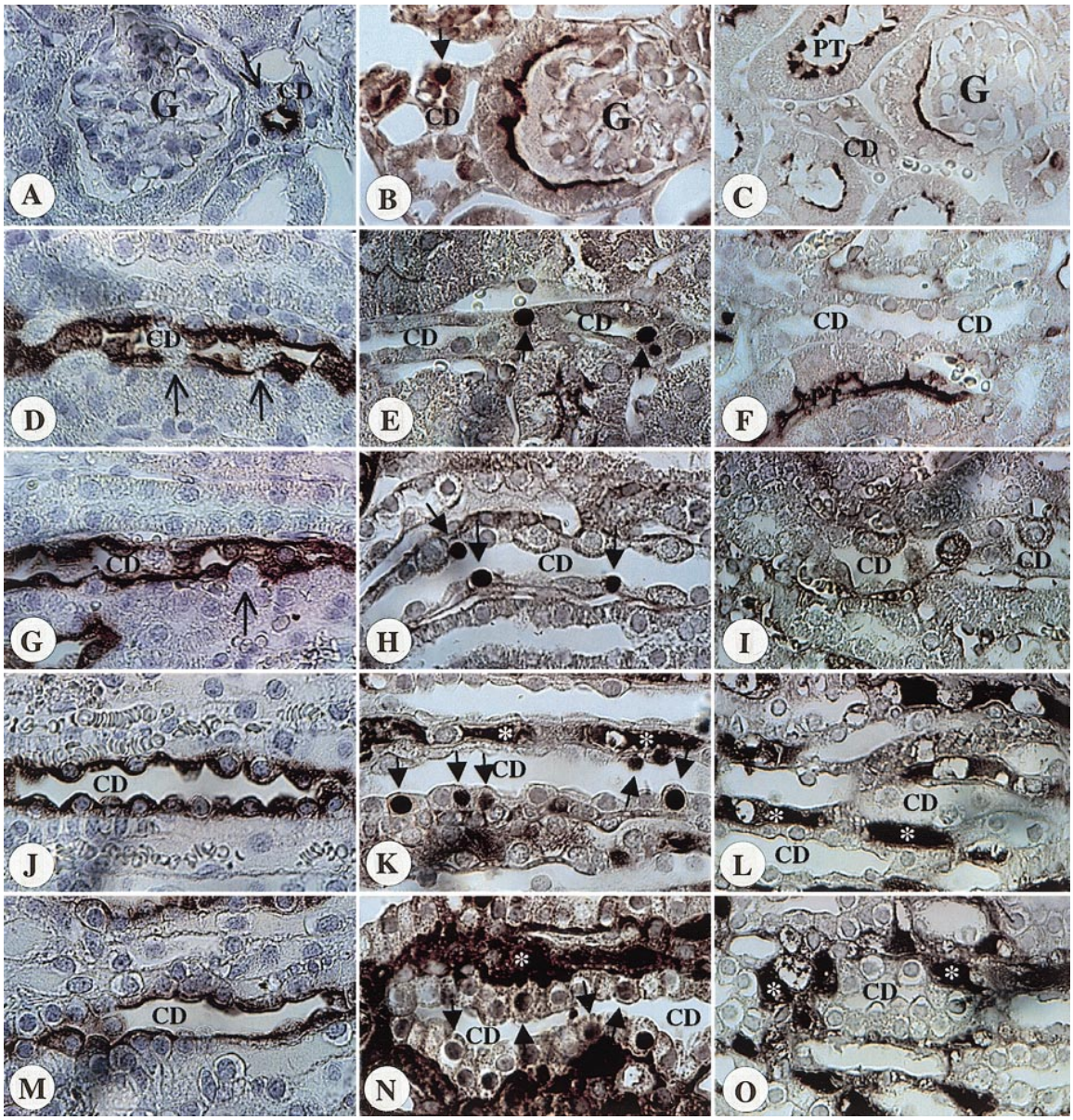

Fig. 4. Immunohistochemical localization of AQP2 and CreTag in transgenic and nontransgenic mouse kidney. *A*, *D*, *G*, *J*, and *M*: immunostaining for AQP2 in transgenic renal collecting duct within cortex, outer stripe of outer medulla, inner stripe of outer medulla, outer half of inner medulla, and papilla, respectively. Arrows, AQP2-negative intercalated cells within collecting ducts. *B*, *E*, *H*, *K*, and *N*: immunostaining for CreTag in renal collecting duct within cortex, outer stripe of outer medulla, inner stripe of outer medulla, outer half of inner medulla, and papilla, respectively, of a kidney from transgenic mouse *line 2*. Arrows with triangular heads denote nuclear CreTag immunostaining in collecting duct of transgenic but not nontransgenic animals. *C*, *F*, *I*, *L*, and *O*: immunostaining for CreTag in renal collecting duct within cortex, outer stripe of outer medulla, inner stripe of outer medulla, outer half of inner medulla, and papilla, respectively, of a kidney from a nontransgenic mouse. Asterisks, nonspecific immunostaining for CreTag in nonnuclear regions in and around tubules and glomeruli; CD, collecting ducts; G, glomeruli; PT, proximal tubule. *A*–*O*: ×360 magnification.

for CreTag and AQP2 was performed on nontransgenic and transgenic mouse kidneys from *line 2* (Fig. 4). Nuclear CreTag immunostaining was observed in collecting duct nuclei in the cortex, outer medulla, and inner medulla of transgenic kidney but not in nontransgenic kidney. Extranuclear staining was observed in both transgenic and nontransgenic kidneys when the monoclonal antibody to the HSV epitope tag or an irrelevant mouse monoclonal primary antibody was used, suggesting that nonnuclear staining is due to staining of endogenous mouse immunoglobulin. Nuclear immunostaining for CreTag was observed in fewer cells than would be expected if CreTag were expressed in all AQP2-expressing principal cells. This indicated that expression of the AQP2-CreTag transgene was variegated.

Double-label immunofluorescent staining was performed on transgenic kidney from *line 2* to demonstrate that CreTag was expressed in the nucleus of AQP2-

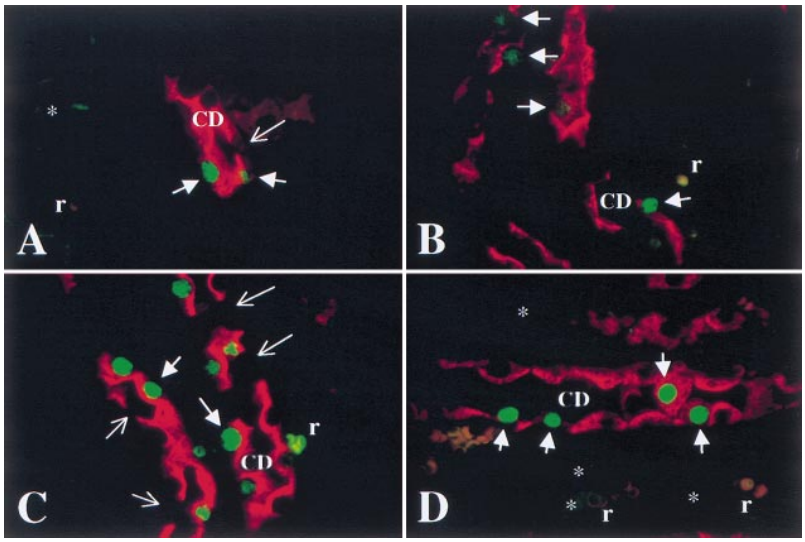

Fig. 5. Immunofluorescent colocalization of AQP2 and CreTag in kidney. *A–D*: collecting duct in cortex, outer stripe of outer medulla, inner stripe of medulla, and inner medulla, respectively. AQP2 was immunostained with a rabbit polyclonal antibody to AQP2 and a Texas red-conjugated goat anti-rabbit antibody. CreTag was immunostained with a mouse monoclonal antibody to HSV epitope tag and a fluorescein-conjugated horse anti-mouse antibody. Note that nuclear CreTag immunostaining (green) was always associated with AQP2 immunostaining (red). Arrows with triangular heads, CreTag-positive cells. Regular arrows, AQP2-negative intercalated cells. No nuclear CreTag staining was associated with AQP2-negative intercalated cells. Asterisks, nonspecific immunostaining for CreTag in nonnuclear regions around tubules and glomeruli; r, nonspecific staining of red blood cells. *A–D*: ×540 magnification.

expressing principal cells within the collecting duct rather than in intercalated cells (Fig. 5). Nonnuclear CreTag immunofluorescent staining was present even in nontransgenic animals, again suggesting that it represented nonspecific background immunostaining due to endogenous mouse immunoglobulin. However, it was easy to distinguish this background staining from specific nuclear staining. Nuclear CreTag immunofluorescent staining always colocalized with nonnuclear AQP2 immunofluorescent staining. No cells lacking AQP2 immunofluorescent staining, including intercalated cells, exhibited nuclear CreTag immunofluorescent staining. In addition, not all renal principal cells displaying AQP2 immunofluorescent staining also stained for CreTag. This again indicates that the transgene expression is variegated.

Next, single-label peroxidase immunohistochemistry was performed for CreTag and AQP2 in nontransgenic and transgenic mouse vas deferens, epididymis, and testis from *line 2* (Fig. 6). Nuclear CreTag immunostaining was observed in principal cells of the vas deferens but not the epididymis. Immunostaining for CreTag could not be demonstrated in the testis because the monoclonal antibody to the HSV epitope tag exhibited nonspecific immunostaining of all nontransgenic germ cell nuclei (Fig. 6).

Finally, immunoblotting analysis was performed to determine whether CreTag was expressed in the testis of AQP2-CreTag transgenic mice. Nuclear proteins from the kidney and testis of male transgenic and nontransgenic mice were fractionated by SDS-PAGE, transferred to membranes, and immunoblotted for CreTag using the murine monoclonal antibody to the HSV epitope tag. The relative molecular mass was estimated on the basis of comparison to molecular mass standards. In kidney and testis of transgenic AQP2-

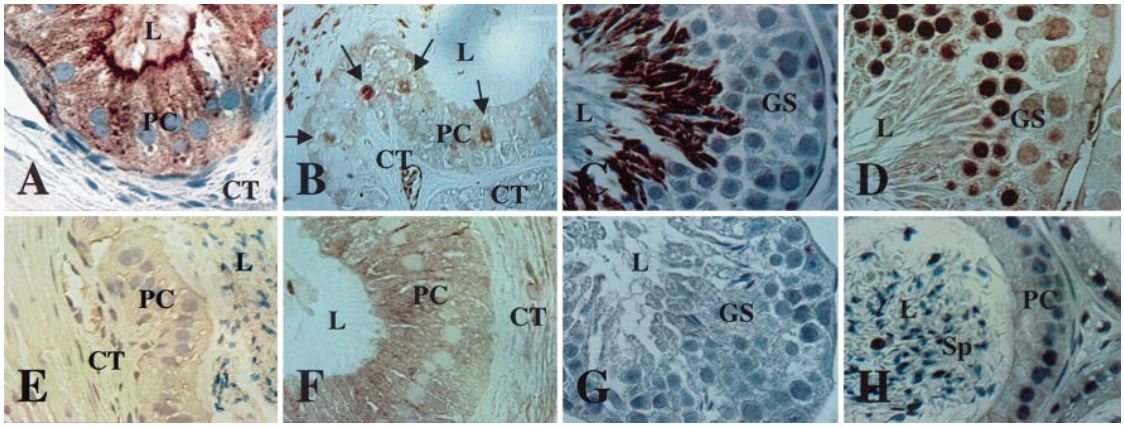

Fig. 6. Immunocytochemical localization of AQP2 and CreTag in male reproductive system. *A* and *C*: immunostaining with a rabbit polyclonal antibody for AQP2 in principal cells of vas deferens and in seminiferous tubules of testis, respectively. *E* and *G*: minimal background immunostaining with an irrelevant rabbit polyclonal antibody in vas deferens and seminiferous tubules of testis, respectively. *H*: there is no immunostaining for AQP2 in epididymis. *B*: CreTag immunostaining with a mouse monoclonal antibody to HSV epitope tag is localized to some of principal cells of vas deferens. Arrows, nuclear Cre immunostaining. *F*: minimal background immunostaining with an irrelevant mouse monoclonal antibody in vas deferens. *D*: strong background immunostaining in nontransgenic testis with HSV epitope tag antibody. CreTag could not be localized in testis for this reason. PC, principal cells in vas deferens; Sp, mature sperm; GS, germ cells and Sertoli's cells; L, lumen; CT, connective tissue. *A–H*: ×360 magnification.

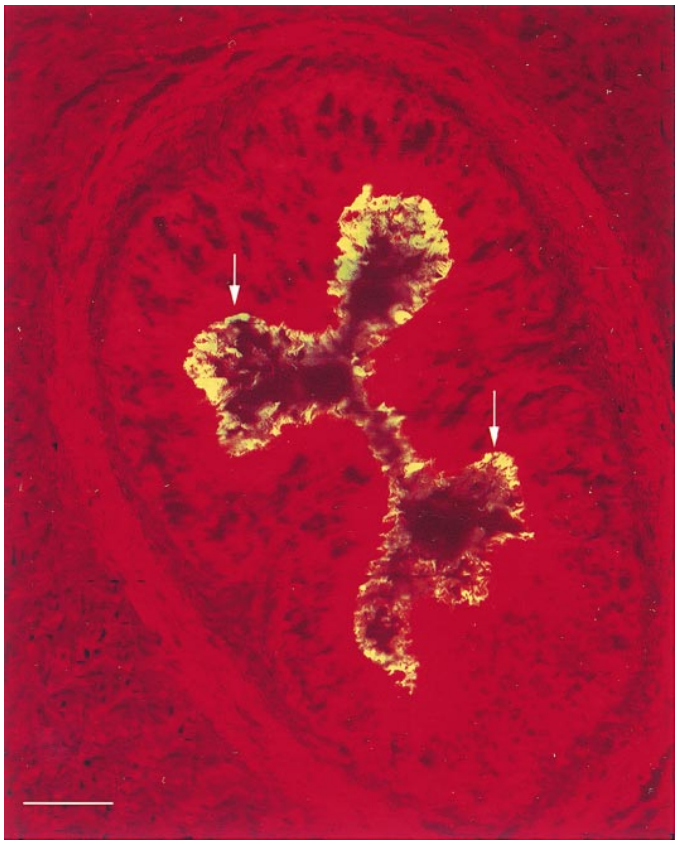

Fig. 7. Immunocytochemical localization of AQP2 in rat vas deferens. A cross section of rat vas deferens was immunostained with anti-AQP2 antibodies and counterstained with Evan's blue (red staining). Specific yellow/green AQP2 staining is restricted to apical pole of principal cells of vas deferens epithelium. Apical stereocilia that project from these cells into lumen appear to be heavily stained. No basolateral or extensive intracellular vesicular staining was detectable in these cells. Arrows, apical AGP2 immunostaining of vas deferens epithelium. Bar, 30 μm.

CreTag mice, but not of nontransgenic mice, a polypeptide with relative molecular mass of 43 kDa was detected (Fig. 9). The size of this polypeptide was in agreement with the mass predicted on the basis of the primary structure. Other polypeptides of different relative molecular mass were observed in transgenic and nontransgenic testis, which were likely the nonspecific polypeptides found in transgenic and nontransgenic seminiferous tubules by immunocytochemistry.

DISCUSSION

Before the current study, AQP2 expression had been demonstrated in principal cells of mouse kidney by immunocytochemistry, but neither the cDNA sequence nor the pattern of mRNA expression had been determined. Hence, before commencing mouse transgenic studies, it was first necessary to confirm that mice express AQP2 and to determine the tissue pattern of such expression. Partial cDNA sequence analysis confirmed that AQP2 mRNA was expressed in mice. RT-PCR analysis of whole-organ RNA demonstrated that AQP2 mRNA was selectively expressed in kidney and male reproductive organs. Immunocytochemical analysis revealed that AQP2 protein was only expressed in principal cells of the collecting ducts within the kidney, principal cells in the vas deferens, and seminiferous tubules in the testis. The kidney- and renal principal cell-specific expression of AQP2 was identical to that reported in the past for the mouse (2) and paralleled rat (13, 35) and human (40). These findings suggest that AQP2 is involved in water reabsorption in the collecting duct of the mouse, as in other species.

AQP2 expression in testis and vas deferens has not previously been reported. The functional significance of AQP2 in seminiferous tubules of the mouse testis remains unclear, as does the reason that studies of rat testis did not reveal AQP2 (13). Interestingly, AQP7 and AQP8 are expressed in postmeiotic sperm (25, 26). These aquaporins could play a role in sperm maturation. The presence of AQP2 in the vas deferens may reflect a function of this tubule segment in modifying the luminal fluid content in a hormone-sensitive manner. The potential physiological regulation of AQP2 in the vas deferens is the subject of ongoing studies in our laboratory. In previous studies, AQP1 was most abundant in the efferent ducts of the testis, in which much of the fluid secreted by the seminiferous tubules is reabsorbed. It was not present in epithelial cells of the epididymis or most of the vas deferens, but it was present in cells of the terminal ampulla of the vas deferens, seminal vesicle, and prostate (4). The proposed role of AQP1 was to modulate the luminal fluid content in the male reproductive system. Studies are clearly needed to determine the physiological role of aquaporins in the male reproductive system.

The mechanism of kidney- and principal cell-specific expression of the AQP2 gene was investigated in transgenic mice. Fourteen kilobases of the human AQP2 gene 5′-flanking region conferred kidney-, vas deferens-, and testis-specific expression of a CreTag reporter cassette in transgenic mice. Furthermore, CreTag was localized to AQP2-expressing principal cells in the kidney and vas deferens. Finally, CreTag was targeted to the nucleus. These results indicate that organ- and cell-specific expression of AQP2 results from regulated AQP2 gene transcription. In addition, the AQP2 gene 5′-flanking region contains the *cis*-acting sequences that are sufficient to confer organ- and cell-specific expression.

Not all AQP2-expressing cells contained detectable CreTag, indicating a variegated pattern of transgene activity. Such variegated cellular expression patterns have been observed with many transgenes (10, 37, 38). Several explanations have been proposed. First, the promoter may not be expressed efficiently in mice because of species differences within the AQP2 promoter. Second, the 14-kb AQP2 5′-flanking region may be missing upstream or downstream regulatory elements. Such elements may include enhancers (45), chromatin boundary elements, insulator DNA sequences (9, 15), or locus-controlling regions (10). Third, bacterial reporter sequences may be inactivated by some as yet unknown mechanism. Inactivation by methylation of hemizygous loci may be one such mecha-

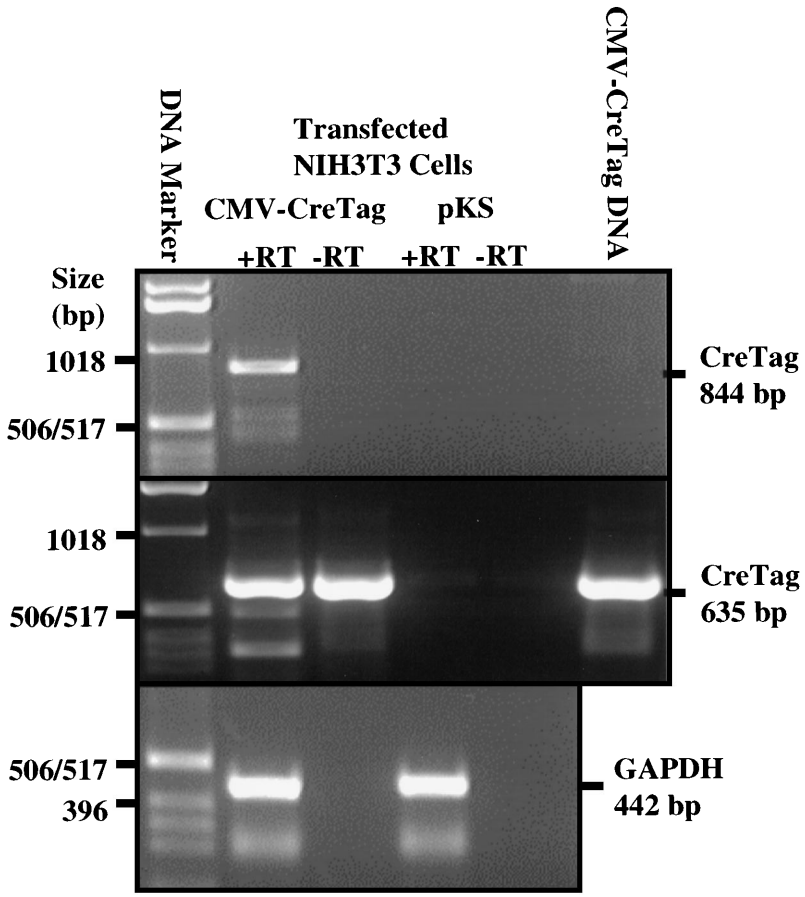

Fig. 8. Testing of method for RT-PCR analysis to determine CreTag mRNA. Murine 3T3 cells were transiently transfected with pCMV-CreTag, which has a cytomegalovirus (CMV) promoter and enhancer driving expression of CreTag cassette, or pKS, which contains no promoter or expression cassette. RNA was reverse transcribed. PCR analysis was performed on cDNA or plasmid DNA using oligonucleotide primer pairs. *Top*: PCR analysis using specialized primers CreTagUP and SV40LateR. Products are only observed in presence of RT. *Middle*: PCR analysis using conventional primers CreTagUP and CreTagDown. Products are observed in presence and absence of RT and in presence of pCMV-CreTag plasmid DNA. *Bottom*: PCR analysis using primers that amplify GAPDH. Molecular mass markers are shown at *left*.

nism (34). Further studies are needed to examine these possibilities.

Sequence analysis of the human AQP2 gene proximal promoter reveals several consensus sites for transcription factors that may be involved in cell-specific transcription (24, 44). These elements include CREs and several GATA sites. cAMP and AVP, which acts through cAMP, have been implicated in the regulation of AQP2 gene expression. For example, cAMP and AVP increase AQP2 promoter-reporter activity and AQP2 mRNA levels in cell lines (24, 33). Similarly, AVP treatment increases APQ2 levels in kidneys of Brattleboro rats with congenital diabetes insipidus (6). Furthermore, mutation or deletion of the CRE sites in the AQP2 promoter ablates cAMP and AVP-induced promoter activity (24, 33). There is also evidence to suggest a role for GATA sites in transcriptional regulation of AQP2. GATA-3 is the only GATA transcription factor that is known to be expressed in the renal collecting duct (43). The finding that overexpression of GATA-3 increases AQP2 promoter-reporter activity in kidney cell lines suggests a role for GATA-3 in principal cell-specific expression of AQP2 (43). Studies in transgenic mice with reporter genes containing 14 kb of the AQP2 5′-flanking region with GATA or CRE site mutations will be required to demonstrate their role in mediating principal cell-specific expression of AQP2.

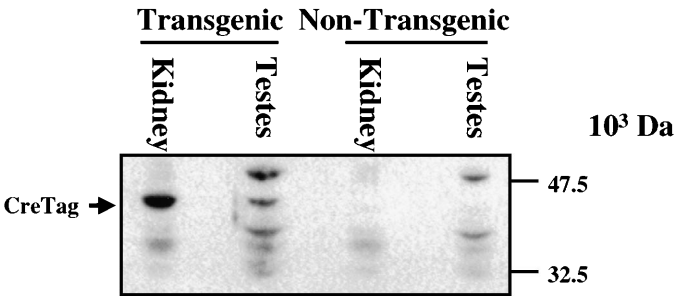

Fig. 9. Immunoblotting analysis of nuclear proteins from transgenic and nontransgenic mouse kidney medulla and testis. Nuclear proteins from testis and kidney medulla were fractionated by SDS-PAGE, transferred to a membrane, and probed for CreTag by immunoblotting using a murine monoclonal antibody to HSV epitope tag using enhanced chemiluminescence. Relative molecular mass of markers is shown at *right*. Arrow, CreTag protein. CreTag is only found in transgenic kidney and testis.

A potential application for transgenic mice expressing AQP2-CreTag is in principal cell-specific gene targeting. The AQP2-CreTag-expressing mouse could be mated with a mouse containing loxP sites flanking the gene of interest. The progeny having both Cre recombinase expressed in principal cells and a loxP-flanked gene would theoretically contain deletion of the gene of interest in principal cells (17, 42). These animals could then be used to establish the principal cell function of a given gene. In addition, this system avoids lethal mutations that result from loss of function of the gene of interest in other cell types. For example, deletion of the endothelin-1 gene by conventional gene targeting is

lethal in the newborn period due to pharyngeal arch malformation (31). Principal cell-specific gene targeting would allow one to examine the role of endothelin-1 production in the collecting duct, a region where this peptide is known to be produced in large amounts (30). Clearly, before such physiological studies are successful, it will first be necessary to optimize expression of Cre recombinase. As alluded to earlier, this requires efforts to reduce the variegation of Cre expression in principal cells.

Finally, it should be emphasized that variegated Cre expression within the collecting duct may prove to be useful. In this scenario, a subset of principals would contain deletions of the gene of interest. One could compare principal cells with and without expression of the gene of interest using histological, biochemical, or physiological techniques that can discriminate between cells.

In summary, we confirm that AQP2 is expressed in principal cells in the collecting duct of mouse kidney. Unexpectedly, AQP2 is also expressed in principal cells of the vas deferens and seminiferous tubules of the testis. The function of AQP2 in the male reproductive system is unknown. Fourteen kilobases of the human AQP2 5′-flanking region confers principal cell-specific expression in the collecting duct of the kidney, principal cell-specific expression in the vas deferens, and seminiferous tubule-specific expression in the testis of a Cre-Tag reporter gene in transgenic mice. These results suggest that cell-specific expression of AQP2 is mediated at the transcriptional level and that 14 kb of the human AQP2 gene contain *cis* elements that are sufficient for expression of AQP2 in principal cells of the kidney and vas deferens and in seminiferous tubules of the testis.

We acknowledge Steve Johnson for excellent technical assistance. We thank Mark Knepper for kindly providing the rabbit antibody to AQP2. We thank Peggy Moody and Brad Preston in the University of Utah Transgenic Mouse Core Facility for helping in the creation of the transgenic mice.

Portions of this work were funded by the Primary Children's Research Foundation (R. D. Nelson), National Institutes of Health Grants K08-DK-02132-06 (R. D. Nelson), RO1-DK-52043 (D. E. Kohan), RO1-HL-256857 (D. E. Kohan), DK-19406 (D. Ausiello), and DK-38452 (D. Brown), and National Cancer Institute Grant 5-P30-CA42014 (R. D. Nelson and D. E. Kohan).

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Received 7 January 1998; accepted in final form 10 April 1998.

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