Renal principal cell-specific expression of green fluorescent protein in transgenic mice

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Departments of 1Pediatrics and 2Medicine, University of Utah, Salt Lake City, Utah 84132; and 3Program in Membrane Biology and Renal Unit, Massachusetts General Hospital, and 4Department of Medicine, Harvard Medical School, Boston, Massachusetts 02114

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Zharkikh, Ludmilla, Xiaohong Zhu, Peter K. Stricklett, Donald E. Kohan, Greg Chipman, Sylvie Breton, Dennis Brown, and Raoul D. Nelson. Renal principal cell-specific expression of green fluorescent protein in transgenic mice. Am J Physiol Renal Physiol 283: F1351–F1364, 2002.—The purpose of this study is to develop transgenic mice with principal cell-specific expression of green fluorescent protein (GFP). After the cloning and sequencing of the mouse aquaporin-2 (AQP2) gene, 9.5 kb of the promoter were used to drive expression of GFP in transgenic mice. In transgenic mice, GFP was selectively expressed in principal cells of the renal collecting duct and not in intercalated cells. Expression was increased by dehydration of mice. AQP2 and GFP expression was maintained in primary cultures of renal medulla that were stimulated with cAMP or vasopressin analogs. GFP-expressing cells were then isolated by fluorescence-activated cell sorting. RT-PCR analysis showed expression of AQP2, AQP3, AQP4, vasopressin type 2 receptor, and cAMP response element binding protein but not H⁺-ATPase B1 subunit or anion exchanger 1. After expansion of these cells in culture, RT-PCR analysis showed continued expression of the same genes. This pattern of gene expression is that of principal cells rather than intercalated cells. This transgenic mouse model can be used in future studies of gene expression during the development, differentiation, and maturation of renal principal cells.

renal collecting duct; aquaporin-2; gene expression regulation

THE RENAL COLLECTING DUCT is responsible for regulation of extracellular volume, osmolality, and pH and is involved in disorders of acid-base and salt and water balance, including hypertension. The collecting duct is composed of intercalated and principal cells. Intercalated cells make up ~30–40% of the cortical and outer medullary collecting duct cells and 10% of inner medullary collecting duct cells (10, 50). These cells are characterized by the expression of specific genes, such as the vacuolar H⁺-ATPase B1 subunit (37), anion exchanger 1 (AE1) (1), and carbonic anhydrase type II (45). These genes are essential to the specialized function of intercalated cells, which includes regulated secretion and/or reabsorption of hydrogen and bicarbonate ions. Principal cells make up ~60–70% of the cortical and outer medullary collecting duct cells and 90% of inner medullary collecting duct cells (10, 50). They are characterized by the expression of specific genes such as aquaporin-2 (AQP2) (19, 20, 39, 44), AQP3 (15, 16), AQP4 (18, 51, 53), and vasopressin type 2 receptors (V₂R) (41). AQP2 is expressed in the apical plasma membrane and subapical vesicles of the entire collecting duct. AQP3 is expressed in the basolateral plasma membrane of the entire collecting duct. AQP4 is expressed throughout the entire collecting duct but is expressed at higher levels in the inner medullary collecting duct. AQP4 is also expressed in the S3 segment of the mouse proximal tubule (34, 53). Ultimately, AQP2, AQP3, AQP4, and the vasopressin receptor are involved in vasopressin-regulated water reabsorption by the principal cells within the collecting duct (35, 40).

The development and differentiation of the renal collecting duct have been studied in some detail (47, 49), but the regulation of intercalated and principal cell development and differentiation is less well understood. This process begins as the ureteric bud, a derivative of the metanephric mesenchyme, and primitive mesenchyme, a derivative of the intermediate mesoderm, coinduce each other to differentiate into the metanephric kidney. The ureteric bud repeatedly branches and elongates. Each branch of the ureteric bud induces the metanephric mesenchyme to form individual nephrons. Ultimately, the ureteric bud and mesenchyme collectively give rise to the glomerulus and tubule. The terminal portion of the tubule is the collecting duct. In the final stage, the collecting duct further differentiates into intercalated and principal cells (3, 13, 33). The intercalated and principal cells likely mature further after their initial formation (3, 6, 55). The factors that control development, differentiation, and maturation of principal cell-specific gene expression are incompletely understood.

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One of the limiting factors in the study of principal cell development and differentiation is the lack of model systems. The most powerful approach to study regulation of gene expression in vivo is to use a transgenic mouse model. Such a model would preserve the complex cellular relationships found within kidney that are required for principal cell-specific gene expression. However, the expense and time required for analysis must be minimized by initial in vitro experiments using a cell culture system, from the same species, that retains differentiated features. The cell culture system that would most likely retain differentiated features is a primary culture or cultured cells that have only been passaged in vitro a limited number of times. The use of such a system would then require one to be able to isolate principal cells from the primary culture in a specific and efficient manner. The development of model systems is essential to make further progress in the study of collecting duct development, differentiation, and maturation.

The purpose of this paper is to describe such a new model system for the in vivo and in vitro study of the development, differentiation, and maturation of principal cells within the renal collecting duct. Because AQP2 is a specific marker of the principal cell phenotype, these studies have focused on the principal cell-specific regulation of AQP2 gene expression. The mouse AQP2 gene was cloned and sequenced, and the mouse AQP2 promoter was used to drive the expression of enhanced green fluorescent protein (EGFP) in principal cells of transgenic mice. Primary cultures of the medulla from these transgenic mice were then optimized for the maintenance of AQP2 and EGFP transgene expression. EGFP-expressing cells were isolated by fluorescence-activated cell sorting (FACS). The sorted cells expressed principal cell-specific marker genes, such as AQP2 and AQP3, but not intercalated cell-specific marker genes, such as H^+–ATPase and AE1. These sorted cells were propagated and continued to express principal cell-specific genes such as AQP2, AQP3, AQP4, and V_2R. This transgenic mouse model should prove useful in further studies of principal cell biology both in vivo and in vitro.

**MATERIALS AND METHODS**

**PCR.** Mouse DNA or cDNA were amplified by PCR using 0.05 units/μl Taq DNA polymerase (Life Technologies, Rockville, MD), 200 μM each of dATP, dCTP, dGTP, and dTTP, 400 nM each of forward and reverse primers (Table 1), 1.5 mM MgCl_2, 50 mM KCl, and 10 mM Tris-HCl, pH 8.4, in 25 μl. The thermocycling program consisted of a 94°C hot start followed by 20–35 cycles of 94°C for 20 s, 58–72°C for 20 s, and 72°C for 60 s and a final extension at 72°C for 5 min (Table 2). The cycle number and annealing temperature were varied depending on the primer set. The cycle number was limited to maintain nonsaturating conditions of amplification.

**DNA sequencing.** Plasmids or PCR products were sequenced by the University of Utah DNA Sequencing Facility. It utilizes the dye primer system for universal primers and dye terminator system for other primers. The products were analyzed by using the ABI PRISM 377 or 3700 DNA analyzers (Applied Biosystems, Foster City, CA). The results were analyzed by using Sequencer (Gene Codes, Ann Arbor, MI). The results have been submitted to GenBank.

**A Cloning and sequencing of the mouse AQP2 gene.** A PCR method for library screening was developed. Mouse genomic DNA was prepared by standard methods (26) and 100 ng were amplified by PCR using AQP2GF and AQP2GR2 (Tables 1 and 2). The PCR product was isolated by the Wizard PCR Preps DNA purification system (Promega, Madison, WI) and sequenced. These PCR primers were used to screen an arrayed 129/OLA mouse genomic library that was packaged and sequenced. The sequence was compared by primer walking. The sequence was compared with a partial sequence for the mouse promoter and exon 1 using primers HAQP2F1 and AQP2G5PER and exon 4 by PCR using primers AQP2GF and AQP2G5PER and AQP2G5PER (Tables 1 and 2). Clone 13430 was digested with EcoRI and a 21-kb fragment subcloned into pKSII (Stratagene, La Jolla, CA). Both strands of this plasmid were sequenced by primer walking. The sequence was compared with a partial sequence for the mouse promoter (accession no. D87129) and cDNA (accession no. AF020519).

**Construction of the mAQP2 promoter-EGFP transgene.** A 9.5-kb EcoRI–Eco47III fragment was ligated into EcoRI and Smal digested, calf intestinal phosphatase-treated pEGFP-1 (Clontech, Palo Alto, CA). The product was restriction mapped and the ligation junctions were sequenced. The transgene was excised from the vector by digestion with EcoRI and AllI and purified away from the vector by electrophoresis through low-melting-point agarose gel. The linear transgene was isolated by digestion of the gel slice with β-agarase I, phenol/chloroform extraction, ethanol precipitation, and purification with an Elutip-D column (Schleicher &

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<th>Name</th>
<th>Sequence</th>
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Schuell, Keene, NH). The DNA was quantitated by OD260 and agarose gel electrophoresis with a quantitative standard.

**Generation and breeding of transgenic mice.** Transgenic founder mice were created by the Transgenic and Gene Targeting Mouse Facility at the University of Utah according to standard methods (25). The transgene was injected into the male pronucleus of single-cell embryos derived from C57BL6 × CBA F1 mice, and the embryos were implanted into pseudopregnant females. Candidate transgenic mice were weaned, ears punched, and a tail clip was obtained just before 21 days of age. After genotyping, the founder mice were mated with C57BL6 × CBA F1 animals. F1–F4 transgenic mice were analyzed for transgene expression. Line 14 was also mated with the Immortomouse, which contains the H2Kb-SV40Tag transgene (32), to obtain double transgenic mice. Transgenic mice were euthanized by exanguination after methoxyflurane or halothane anesthesia, and the kidneys or other organs were dissected for the various studies described below.

**Genotyping of transgenic mice.** Tail DNA was isolated by standard methods (26). Tail DNA (100 ng) was amplified by PCR using the stated primers (Tables 1 and 2). The MAQP2F1 and EGFPN primers were used to detect the mAQP2 promoter-EGFP transgene, the SV40F2 and PCH110R3 primers were used to amplify the EGFP. The GAPDHF and GAPDHHR primers were used to amplify GAPDH from organ cDNA, and EF1αF and EF1αR were used to amplify elongation factor 1α (EF1α) from cellular cDNA. All PCR reactions were carried out in the presence and absence of RT to demonstrate that there was no contaminating DNA.

**Dehydration of mice.** Water was withheld from transgenic mice for 48 h. Age- and sex-matched mice served as controls. Mice were euthanized and RNA was prepared from organs as stated above.

**Fluorescence microscopy of kidney.** Transgenic mice were anesthetized by inhalation of methoxyflurane or halothane and fixed by cardiac perfusion with PBS (10 mM sodium phosphate buffer containing 0.9% NaCl, pH 7.4) and 2% paraformaldehyde in PBS at room temperature. The kidneys were then dissected and fixed by immersion for 4–6 h at room temperature.

Transgenic mice were initially screened for EGFP expression by examining thick kidney sections. Kidneys were embedded in 3% agarose in PBS and cut into 200- to 300-μm sections with an oscillating vibratome. The sections were viewed by epifluorescence microscopy using a Nikon E800 microscope equipped with the PCM2000 confocal system having filters specific for EGFP. The images were captured digitally by using the SimplePCI imaging software package (Compix, Cranberry, PA). A Z-series was collected at 10-μm intervals and a montage was created.

Thick kidney sections from transgenic mice expressing EGFP were then immunostained for AQP2. The 200- to 300-μm sections were permeabilized by incubation with PBS-0.5 g/l saponin, 2 g/l BSA, 2 g/l gelatin in PBS (SBG) for 30 min, and nonspecific binding was blocked by incubation with 10% goat serum in PBS-SBG for 60 min. The sections were incubated overnight with a 1:5,000 dilution of a purified rabbit antibody to AQP2 (14) (provided by Dr. Mark Knepper, National Institutes of Health) in PBS-SBG at 4°C and then washed four times with PBS-SBG for 30 min. The sections were then incubated overnight with a 1:200 dilution of CY5-conjugated goat anti-rabbit antibody (Amersham Pharmacia Biotech, Piscataway, NJ) in PBS-SBG at 4°C and washed four times with PBS-SBG for 30 min. The sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and viewed with the same confocal microscope previously described with laser excitation and filters specific for EGFP and CY5. The pseudocolor images were generated by

### Table 2. PCR and RT-PCR conditions

<table>
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<tr>
<th>Name</th>
<th>Purpose</th>
<th>PCR Product</th>
<th>Oligonucleotide Primer Pairs</th>
<th>Product Size, bp</th>
<th>Thermocycling Program</th>
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<td>MAQP2R</td>
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<td>1,100</td>
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<td>H2Kb-SV40Tag</td>
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<td>V2RR</td>
<td>371</td>
<td>72</td>
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AQP2, AQP3, AQP4, aquaporin-2, -3, -4, respectively; EGFP, enhanced green fluorescent protein; EF1α, elongation factor 1α; CREB, cAMP response element binding protein.
using SimplePCI (Compix, Cranberry, PA). EGFP appears as green and Cy5 appears as red.

Thin kidney sections from transgenic mice expressing EGFP were immunostained with antibodies to AQP2 and H\(^{-}\)-ATPase. Kidneys were fixed as previously described. The kidneys were then cryoprotected by immersion in 30% sucrose in PBS for 4 h, mounted in Tissue-Tek (Miles, Elkhart, IN), and frozen at -29\(^\circ\)C in a Reichert Frigocut cryostat (Reichert Jung, Derry, NH). Sections were cut at 4 \(\mu\)m and picked up onto Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA).

Sections were hydrated 5 min in PBS and treated for 4 min with 1% SDS in PBS, an antigen retrieval technique previously described (11). After three washes of 5 min each in PBS, nonspecific staining was blocked by using a solution of 1% BSA in PBS for 15 min. An affinity-purified chicken antibody raised against the COOH-terminal 14 amino acids of the 31-kDa subunit (E subunit) of the H\(^{-}\)-ATPase was applied at a concentration of 10 \(\mu\)g/ml for 1.5 h at room temperature. This antibody has been characterized previously (9). Similarly, an affinity-purified rabbit antibody raised against the COOH-terminal 14 amino acids of AQP2 was used in other experiments (8). Sections were then washed two times for 5 min each time in PBS containing 2.7% NaCl to reduce nonspecific binding, followed by one wash in normal PBS. Secondary donkey anti-chicken IgG conjugated with Cy3 (Jackson Immunologicals) was applied at a dilution of 1:800 for 1 h at room temperature, and washes were performed as for the primary antibody. Slides were mounted in Vectashield (Vector Laboratories) diluted 1:2 in Tris-HCl buffer (1.5 M, pH 8.9). The images were captured with a Hamamatsu Orca digital camera. Pseudocolor images were merged by using IP Lab Spectrum software (Scanalytics). Cy3 appears as red and EGFP appears as green.

For quantitative immunofluorescence studies, the pseudocolored images were merged into a single file and a grid was overlaid by using Adobe Photoshop. The EGFP- and AQP2-positive cells were counted in the cortex, outer medulla, and inner medulla of kidneys from three control mice and three dehydrated mice. The results were expressed as means \pm SE. Significance was determined by using two-sided Student’s t-test.

Cells cultured on coverslips were visualized by phase contrast and fluorescent microscopy using a Nikon Eclipse E800 microscope equipped with a CoolSNAP digital camera (Roper Scientific, Trenton, NJ) and by fluorescence microscopy using a Nikon Eclipse and PCM2000 confocal system.

**Cell culture.** Primary cultures of medulla were obtained according to modifications of published protocols (22, 30). The kidneys were dissected from mice that were heterozygous for the mAQP2-EGFP and H2Kb-SV40Tag transgenes. The medulla was dissected and minced into 1-mm fragments with a razor blade. The fragments were digested in a bicarbonate-free Krebs solution (in mM) 145 NaCl, 10 HEPES, 5 KCl, 1 Na\(_2\)HPO\(_4\), 2.5 CaCl\(_2\), 1.8 MgSO\(_4\), and 5 glucose, pH 7.3] with 1 mg/ml class IV collagenase ( Worthington, Lakewood, NJ) and 0.1 mg/ml DNase I ( Sigma, St. Louis, MO) at 37\(^\circ\)C for 15–20 min until tubules were dispersed into cylindrical fragments. The tubular fragments were centrifuged at 400 g for 5 min in 10% albumin in PBS. The pellet was resuspended in medium and cultured in six-well Primaria plates or standard six-well plates with 22-mm-diameter polyethylene terephthalate membrane inserts coated with mouse collagen IV (BD Biosciences, San Jose, CA). The cells were cultured in DMEM/F-12 (1:1) (Life Technologies) with 15 mM HEPES, 100 U/ml penicillin, 10 U/ml streptomycin, 2 mM glutamine, 10 \(\mu\)g/ml insulin, 5.5 \(\mu\)g/ml transferrin, 6.7 ng/ml selenium, 10\(^{-7}\) M dexamethasone, 10\(^{-7}\) M aldosterone, 5 nM T3, 10 ng/ml mouse EGF, 10 U/ml mouse interferon-\(\gamma\), and 10% fetal bovine serum at 33\(^\circ\)C until confluent. The cells were then shifted to medium without serum or interferon-\(\gamma\) at 37\(^\circ\)C for 24–48 h. The cells were then stimulated with 500 \(\mu\)M 8-(4-chlorophenylthio) cAMP (CPT-cAMP) or 400 \(\mu\)M 3-isobutyl-1-methylxanthine (IBMX) and 10\(^{-7}\) M DDAVP for 72 h. FACS-derived cells were cultured with the same medium and stimulated as stated. The cells were typically passaged by treatment with trypsin/EDTA before confluence.

**FACS.** After stimulation with CPT-cAMP or DDAVP and IBMX, primary cultured cells were dispersed into a single-cell suspension by scraping and treatment with 0.05% trypsin and 0.5 mM EDTA. The trypsin was quenched with serum-containing medium. The cells were resuspended in ice-cold medium and pushed through a 40-\(\mu\)m filter. The cells were sorted in ice-cold medium with a FACS Vantage Cell Sorter (BD Biosciences) equipped to sort EGFP. Nontransgenic cells were used to determine the level or autofluorescence. RNA was prepared from the cells directly or after the cells were expanded by cell culture.

**RESULTS**

**Cloning, sequencing, and structural analysis of the mouse AQP2 gene.** To perform further experiments to understand the basis for principal cell-specific expression of AQP2, the mouse AQP2 gene was isolated from a PAC library created from the SVJ129 mouse strain. Oligonucleotide primers were designed to amplify regions of the mouse AQP2 cDNA and gene that were homologous to exons 2 and 3 from the human AQP2 gene (accession no. Z29491). These primers were then used for PCR screening of an arrayed mouse genomic library in the PAC vector pAD10SacBII (42). The four clones contained inserts with common patterns of restriction fragments (data not shown). Using the mouse cDNA (accession no. AF020519), the human promoter (accession no. U40369), and the human intron-exon boundaries (accession no. D31846, Z29491), additional PCR primers were designed to amplify other regions of the AQP2 gene. PCR analysis with these primers demonstrated that all four clones contained sequences homologous to the human proximal promoter exon 1 and exon 4 (Fig. 1). A 21-kb EcoRI fragment was subcloned from the PAC clone into pBluescript KS II, and both strands were sequenced by primer walking to establish the overall gene structure (Fig. 2). The results were submitted to GenBank (accession no. AJ055468).

The identity of the mouse AQP2 gene was confirmed by comparison of the DNA sequence to the human AQP2 gene, the proximal mouse AQP2 promoter, and the mouse cDNA. The proximal 1.2 kb of promoter sequence is identical to the published mouse AQP2 promoter (accession no. D87129), and the coding exon sequences are identical to the published mouse AQP2 cDNA (accession no. AF020519), with the exception of several single-nucleotide polymorphisms. The coding exon and intron boundaries were conserved relative to the human gene (52) (accession no. D31846). This 21-kb subclone of the mouse AQP2 gene was used for further experiments designed to understand the basis for principal cell-specific expression of the AQP2 gene.
Design of an EGFP transgene using the mouse AQP2 promoter. Although we previously demonstrated that 14 kb of the human AQP2 promoter was sufficient to confer principal cell-specific expression of a transgene, the expression was incomplete (38). We therefore used the mouse AQP2 promoter in hopes of achieving more complete expression of GFP. Mouse AQP2 promoter (9.5 kb) was fused to an EGFP cassette that included the EGFP coding region with its own translational initiation site and an SV40 early region polyadenylation signal (Fig. 2). The mouse AQP2 promoter contained the TATA-box and the predicted downstream transcription initiation site but did not contain the translation initiation site for the mouse AQP2 gene. This transgene was designed to achieve principal cell-specific transcription via the AQP2 promoter and efficient translation utilizing the EGFP cassette. It will be referred to as mAQP2 promoter-EGFP.

Creation, breeding, and genotyping of transgenic mice. Linearized mAQP2 promoter-EGFP was used to create transgenic mice by standard pronuclear microinjections (27). Founders were identified by PCR analysis of tail DNA using oligonucleotide primers that anneal within the promoter and EGFP cassette (Figs. 2 and 1). PCR analysis showed that all 4 P1 artificial chromosome clones contain all 4 coding exons and the promoter of the mouse aquaporin-2 (AQP2) gene. Four P1 artificial chromosome clones were analyzed by PCR using primers that amplify the AQP2 genomic regions, including the promoter and coding exon 1, coding exon 4 only, and coding exons 3 and 4. Mouse tail DNA and kidney cDNA are shown as positive controls.

Fig. 1. PCR analysis showed that all 4 P1 artificial chromosome clones contain all 4 coding exons and the promoter of the mouse aquaporin-2 (AQP2) gene. Four P1 artificial chromosome clones were analyzed by PCR using primers that amplify the AQP2 genomic regions, including the promoter and coding exon 1, coding exon 4 only, and coding exons 3 and 4. Mouse tail DNA and kidney cDNA are shown as positive controls.

Fig. 2. Structure of the mouse AQP2 gene and the mAQP2-enhanced green fluorescent protein (EGFP) transgene. Top: 21-kb AQP2 gene. Sizes of the 5'- and 3'-flanking regions, 4 coding exons, and 3 introns are shown. Bottom: structure of the mAQP2-EGFP transgene. AQP2 promoter (9.5 kb) was fused to the EGFP coding region with the SV40 early region polyadenylation signal. Regions of the transgene that were amplified by PCR for genotyping and by RT-PCR for mRNA expression analysis are also shown.
and 3). Primers specific for the endogenous AQP2 gene were used to confirm integrity of the tail DNA (Fig. 3). Single copy detection was demonstrated by analysis of transgene added to normal mouse DNA to simulate 1–100 copies/cell equivalent (Fig. 3). At least three F1 animals derived from each of the four founders were analyzed for expression of the transgene. Only one founder ultimately was found to express the transgene. This particular founder transmitted the transgene to >50% of offspring, suggesting more than one integration site. Several F1 animals were used to generate F2 animals. An F1 animal that transmitted the transgene to 50% of offspring and whose offspring always expressed the transgene was used to propagate the transgenic line of mice for further experiments. The PCR genotyping of the tail from the original founder and the F1 animal are shown in Fig. 3. The line of mice derived from this F1 animal contains 1–3 copies/cell equivalent of the transgene. This copy number has remained stable for at least four generations.

**RT-PCR analysis for transgene expression.** Each transgenic line was screened by RT-PCR for expression of the transgene in kidney. One of the four lines consistently expressed the transgene in kidney. Organ panels were analyzed by RT-PCR for expression of EGFP and AQP2 in two male and two female mice. A representative organ panel for each sex is shown in Figs. 2 and 3. EGFP was expressed in kidney but not other organs, including the male reproductive system (Fig. 4). AQP2 not only was expressed in kidney but also was expressed in the vas deferens of the male reproductive system. The integrity of the RNA and similar loading was confirmed by RT-PCR analysis for GAPDH (accession no. M32599). Parallel reactions carried out in the absence of RT verified that the kidney-specific products did not result from amplification of genomic DNA contaminating the RNA. The identity of the RT-PCR products was verified by direct sequencing. These results show kidney-specific expression of the mAQP2-EGFP transgene that parallels that of endogenous AQP2 in the kidney but not in the male reproductive system.

Transgenic mice were then dehydrated for 48 h, and kidney RNA was analyzed by RT-PCR for expression of AQP2. The results are shown for three control and three dehydrated mice (Fig. 5). The results were replicated in an additional experiment. Thirsting resulted in an increase in EGFP expression in parallel with AQP2. There was no change in expression of the housekeeping gene GAPDH. This is consistent with vasopressin induction of transgene and AQP2 expression. This maneuver will be potentially useful in future studies on gene regulation.

**Fluorescence microscopic analysis of kidney for EGFP expression.** All transgenic mouse lines were screened for expression of EGFP by fluorescence microscopy of thick sections of mouse kidney. Mouse kidneys were fixed and sectioned to 200-μm thickness with an oscillating microtome. These thick sections were then examined by fluorescence microscopy. This is a rapid and easy method to screen for EGFP expression in mAQP2 promoter-EGFP transgenic mice. The transgenic line that expressed EGFP in kidney by

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**Fig. 3.** PCR genotyping showed that the mAQP2-EGFP transgene was present in founder tail DNA. Purified transgene DNA plus normal mouse DNA or transgenic tail DNA were amplified by PCR using primers specific for the mAQP2-EGFP transgene (top) and the endogenous mAQP2 gene (bottom). Left: results of purified transgene DNA added to normal mouse DNA to simulate 1, 3, 10, 30, and 100 copies/cell equivalent. Right: results from PCR analysis of tail DNA from the founder and the F1 animal that carried the active transgene integration site.

**Fig. 4.** RT-PCR analysis showed that EGFP and AQP2 were expressed in the kidney of male (A) and female (B) mAQP2-EGFP transgenic mice and that GAPDH was expressed in all organs. Parallel analyses performed in the absence of RT did not reveal any DNA contamination of the RNA.
RT-PCR also expressed EGFP in kidney by fluorescence microscopy. At least three animals from the F1–F5 generations showed expression of EGFP in an identical pattern. Representative images demonstrate that EGFP is expressed in a radial pattern from the cortex through the outer medulla into the inner medulla (Fig. 6). Close examination reveals variegated expression, which is what one would expect if the transgene were expressed only in principal cells but not in intercalated cells. This pattern of expression suggests that the mAQP2 promoter-EGFP transgene is expressed within the collecting duct and perhaps only in AQP2-expressing principal cells.

The pattern of expression was confirmed by immuno-fluorescence studies performed on these thick sections of transgenic kidney to determine whether EGFP was found only in AQP2-expressing collecting ducts rather than other tubules that do not express AQP2. Transgenic kidney slices were permeabilized with saponin and immunostained for AQP2 by indirect immunofluorescence using a CY5-conjugated secondary antibody (Fig. 7). The pattern of EGFP expression was very similar to the pattern of endogenous AQP2 in the cortex, outer medulla, and inner medulla. However, there were consistently fewer EGFP-expressing tubules than AQP2-expressing tubules. These results were seen in three different animals. Breeding the transgene to homozygosity did not significantly change the pattern of expression (data not shown). This pattern of expression suggests that mAQP2 promoter-EGFP transgene was expressed in AQP2-expressing collecting ducts but that expression was incomplete.

Immunofluorescence studies were performed on thin cryosections of kidney to determine whether EGFP was expressed only in AQP2-expressing principal cells rather than in H+-ATPase-expressing intercalated cells within the collecting duct (Fig. 8). EGFP retained fluorescence despite formaldehyde fixation, cryosectioning, and immunostaining. EGFP colocalized with
AQP2 (Fig. 8, A–C) but did not colocalize with H\(^+\)-ATPase E subunit (Fig. 8, C–E). However, EGFP was not expressed in all AQP2-expressing cells (Fig. 8, A–C). These studies demonstrate that EGFP is only expressed in AQP2-expressing principal cells and not in the H\(^+\)-ATPase-expressing intercalated cells.

Further quantitative immunofluorescence studies of AQP2 and EGFP expression were performed in control and dehydrated mice to determine the extent of expression of EGFP in principal cells and the effects of dehydration on this expression. Mice were dehydrated for 48 h. Then, the kidneys from three control and three dehydrated mice were fixed, cryosectioned, and immunostained for AQP2. Digital images of AQP2 and EGFP were merged and displayed with a grid overlay. There were no EGFP-positive cells that were AQP2 negative. Therefore, 500–1,500 AQP2-positive cells were counted in each region of the kidney. The percentage of AQP2-positive cells that were also EGFP-positive was 10, 12, 18, and 34% in the cortex, outer stripe of outer medulla, inner stripe of outer medulla, and inner medulla, respectively, in control animals and 24, 36, 44, and 44% in the cortex, outer stripe of outer medulla, inner stripe of outer medulla, and inner medulla, respectively, of dehydrated animals (Fig. 9). Dehydration caused a statistically significant increase in the per-
percentage of EGFP-positive cells in the cortex and outer medulla (P < 0.05, Student’s t-test) but not in the inner medulla (P = 0.06). These results indicate that EGFP expression in principal cells was incomplete but could be increased significantly by dehydration.

Expression of EGFP in primary cultures of renal medulla. The mAQP2 promoter-EGFP transgenic mice were bred with H2Kb-SV40Tag transgenic mice to generate mice with both transgenes. Double transgenic kidneys were then used for the culture of renal medulla. When induced in culture with interferon-γ, the H2Kb-SV40Tag transgene produces a temperature-sensitive version of the SV40 virus T antigen in all cell types (32). The SV40 T antigen should facilitate the expression of active SV40 T antigen (32) (33°C) (32). The SV40 T antigen should facilitate the passage of principal cells expressing EGFP.

Therefore the renal medulla was cultured from mice that were transgenic for both mAQP2 promoter-EGFP and H2Kb-SV40Tag by a modification of established methods (29). The tubule fragments were cultured on standard tissue cultureware or type IV collagen-coated polyethylene terephthalate filters. The cells were cultured under conditions that result in high-level expression of active SV40 T antigen (32) (33°C with interferon-γ in the medium). When the cells approached confluence, they were shifted to conditions that would reduce the level of SV40 T antigen expression. After 24–48 h, the cells were stimulated for 48–72 h with 10−7 M DDAVP plus 400 μM IBMX or with 500 μM CPT-cAMP. RT-PCR analysis demonstrated that AQP2 and EGFP mRNA were variably present in primary culture without stimulation but were consistently present after stimulation with DDAVP and IBMX or with CPT-cAMP (Fig. 10). The expression of EF1α, a housekeeping gene, was detected with or without stimulation (Fig. 10). In addition, islands of green fluorescence were observed in these cells after stimulation with DDAVP and IBMX or with CPT-cAMP (Fig. 11). These results have been reproduced in at least three experiments. These results demonstrate that EGFP- and AQP2-expressing principal cells could be maintained in culture to carry out FACS.

FACS. Cells were cultured on collagen IV-coated filters and stimulated with DDAVP and IBMX or cultured in flasks and stimulated with CPT-cAMP, as previously stated, dispersed into single cells, and subjected to FACS. The data are shown for DDAVP- and IBMX-treated cells (Fig. 12). The results were identical for stimulation with CPT-cAMP. The nontransgenic and transgenic cells are similar in terms of the forward scattering and side scattering shown in the scatter plots (Fig. 12, top left). However, there is a difference between transgenic and nontransgenic cells on the basis of forward scattering of light and fluorescence (GFP; Fig. 12, top right). There is also a marked diff-

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**Fig. 8.** Fluorescence microscopy of thin cryosections of transgenic kidney showing that EGFP (green) was present in AQP2-positive (red) cells (A–C) but was not present in H+-ATPase-positive (red) intercalated cells (D–E). Cryosections (4 μm) of transgenic kidney were immunostained with a primary rabbit antibody to AQP2 (A–C) or H+-ATPase 31-kDa subunit (D and E) and a secondary CY3-conjugated antibody. A: AQP2 (red); C: EGFP (green); B: both AQP2 and EGFP; D and E: both H+-ATPase E subunit (green) and EGFP (red).

**Fig. 9.** Quantitation of EGFP-positive principal cells (PC) in control and dehydrated animals. Immunostaining and fluorescence microscopy were performed as stated in Fig. 8. EGFP- and AQP2-positive cells were counted. Values are means ± SE of the percentage of AQP2-positive principal cells that were EGFP positive for the cortex, outer stripe of outer medulla (OS), inner stripe of outer medulla (IS), and inner medulla (IM). There is a significant difference between the control (n = 3) and dehydrated (n = 3) animals in the cortex, OS, and IS (P < 0.05, Student’s t-test) but not in the IM (P = 0.06).
ference between transgenic and nontransgenic cells on the basis of the EGFP fluorescence shown in the histogram (Fig. 12, bottom). The cells with fluorescence >10^2 units and forward scattering >20 units (regions M1 and R2) were collected under sterile conditions. Typically, this was 1–3% of the total cells. When a sample of these cells was immediately reanalyzed, >95% of cells exhibited a high level of fluorescence. Either RNA was prepared directly from these cells or the cells were expanded by cell culture for further experiments.

Characterization of cells obtained by FACS. EGFP-positive cells were initially analyzed directly. RNA was directly prepared from 10^5-positive cells, and gene expression was determined by RT-PCR. The cells expressed AQP2, AQP3, AQP4, V_2R, and cAMP response element binding protein (CREB) but not the H^+/ATPase B1 subunit or AE1, indicating they retained the principal cell phenotype rather than the intercalated cell phenotype (Fig. 13). Next, cells prepared in the same way were passaged five times after reaching 80% confluence. After the final passage, the cells were cultured on collagen IV-coated polyethylene terephthalate filters until confluent and then stimulated with DDAVP and IBMX for 72 h. Endogenous gene expression was determined by RT-PCR analysis (Fig. 14). The cells clearly continued to express AQP2, AQP3, AQP4, V_2R, and CREB. AQP2 and AQP3 expression was increased by stimulation, whereas AQP4, V_2R, and CREB were not increased by stimulation. The housekeeping gene EF1α was unchanged by stimulation. The cell population obtained by FACS retains the ability to express AQP2, AQP3, AQP4, V_2R, and CREB for five passages.

DISCUSSION

The goal of these studies was to develop a new transgenic mouse model for parallel in vivo and in vitro studies of gene expression regulation in principal cells within the renal collecting duct. Transgenic mice were created with 9.5 kb of the mouse AQP2 promoter driving expression of an EGFP. The mouse AQP2 promoter was sufficient to drive EGFP expression in vivo in a kidney and principal cell-specific manner. In addition, the mouse AQP2 promoter was also sufficient to confer increased expression of EGFP in response to dehydration, as has been demonstrated for AQP2 in rats (23, 39). This increase in expression is manifested as an increase in the level of EGFP mRNA and abundance of principal cells expressing EGFP. Finally, the mouse AQP2 promoter confers increased expression of EGFP in response to stimulation with a vasopressin analog, DDAVP, in the presence of a phosphodiesterase inhibitor, IBMX, or a cAMP analog, CPT-cAMP. These results agree with the previous demonstration that vasopressin and cAMP stimulate expression in AQP2 reporter constructs in vitro in transient transfection experiments using renal cell lines (28, 56). These transgenic mouse experiments demonstrate that the stimulation of AQP2 mRNA expression in vivo by dehydration and by vasopressin or cAMP in vitro results from the stimulation of transcription of the AQP2 gene. The cell-specific and temporal regulation of EGFP expression in the kidney of this transgenic mouse model suggest that the mouse model could be very useful in the study of AQP2 gene expression and principal cell biology.
A potential limitation of this transgenic model is the incomplete expression of EGFP in principal cells that is observed in intact kidney. This incomplete expression likely explains the expression level observed in primary cultures. Incomplete expression has been observed with other transgenic models, including those that have achieved kidney-specific gene expression (5, 31, 38). This type of incomplete expression is often referred to as position effect variegation. It may be due to the tandem arrays of the transgene at the site of integration (21). In this case, Cre/lox technology has been used to reduce the transgene copy number and enhance expression of the transgene (21). Incomplete expression may also be due to the lack of chromatin insulator sequences or boundary elements in the transgene. Improved expression could be accomplished by the flanking of the transgenes with insulator sequences (43) or creating transgenic mice using BAC clones containing gene loci with intact insulator sequences (24, 54). These approaches are technically more difficult but will be considered in the future design of transgenes in attempts to improve the expression level of EGFP.

Incomplete expression of EGFP in this transgenic model can be partially overcome. In vivo expression of EGFP can be increased by thirsting animals. Expression in primary culture may be enhanced by stimulating with DDAVP and IBMX or with CPT-cAMP for longer periods of time or more selective dissection of inner medulla, where there is an increased abundance of collecting ducts. Despite the incomplete expression of EGFP, the expression of EGFP is very specific for principal cells. This is the essential feature required for the use of this transgenic model for future studies.

It is interesting to note that the EGFP transgene was not expressed in the male reproductive system where AQP2 is expressed. In the male reproductive system, AQP2 is expressed in the principal cells of the distal vas deferens in a vasopressin-independent manner (38, 48). It is possible that the lack of expression of the...
transgene was due to an unfavorable transgene integration site in this particular line of mice. Alternatively, it is also possible that 9.5 kb of the mouse AQP2 promoter did not contain the required vas deferens-specific enhancer sequences. Further studies are in progress to determine the mechanism of vas deferens-specific expression of AQP2. Such investigations may contribute to a better understanding of the mechanism of vasopressin-independent expression of AQP2 in the vas deferens (48) and therefore will provide unique insight into the development and differentiation of the principal cells of the vas deferens.

This transgenic model was used to establish conditions that maintain expression of EGFP in principal cells in culture. In culture, viable EGFP-expressing principal cells can be identified by fluorescence microscopy and isolated by FACS. Whether principal cells are analyzed directly or after passage, the cells express marker genes typical of principal cells after stimulation with CPT-cAMP or with DDAVP plus IBMX. The marker genes include AQP2, AQP3, AQP4, V2R, and CREB. EGFP-positive cells that were isolated by FACS were expanded in culture, plated on collagen-coated filters, and stimulated with DDAVP and IBMX. The expression of the stated genes was determined by RT-PCR analysis. Parallel reactions without RT show that there was no DNA contamination of the RNA. Kidney RNA was used as a positive control. EF1α was used as a control housekeeping gene.

In summary, we have shown that the mouse AQP2 promoter is sufficient to drive renal principal cell-specific expression of EGFP and that the expression is temporally modulated in vivo by dehydration and in vitro by vasopressin and cAMP analogs. Thus we have created a transgenic mouse model that should prove to be useful for a variety of in vivo and in vitro studies of AQP2 gene expression and principal cell biology.

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**Fig. 14.** RT-PCR analysis of EGFP-positive cells that were passaged, showing continued expression of principal cell markers including AQP2, AQP3, AQP4, V2R, and CREB. EGFP-positive cells that were isolated by FACS were expanded in culture, plated on collagen-coated filters, and stimulated with DDAVP and IBMX. The expression of the stated genes was determined by RT-PCR analysis. Parallel reactions without RT show that there was no DNA contamination of the RNA. Kidney RNA was used as a positive control. EF1α was used as a control housekeeping gene.
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