Abstract. Gene therapy has the potential to provide a therapeutic strategy for numerous renal diseases such as diabetic nephropathy, chronic rejection, Alport syndrome, polycystic kidney disease, and inherited tubular disorders. In previous studies using cationic liposomes or adenoviral or retroviral vectors to deliver genes into the kidney, transgene expression has been transient and often associated with adverse host immune responses, particularly with the use of adenoviral vectors. The unique properties of recombinant adeno-associated viral (rAAV) vectors permit long-term stable transgene expression with a relatively low host immune response. The purpose of the present study was to evaluate gene expression in the rat kidney after intrarenal arterial infusion of a rAAV (serotype 2) vector encoding green fluorescence protein (GFP) induced by a cytomegalovirus-chicken beta-actin hybrid promoter. The left kidney of experimental animals was treated with either saline or transduced with rAAV2-GFP (0.125 ml/100 g body wt, 1 × 10^{10} /ml infectious units) through the renal artery. A time-dependent expression of GFP was observed in all kidneys injected with rAAV2-GFP, with maximal expression observed at 6 wk posttransduction. The expression of GFP was restricted to cells in the S3 segment of the proximal tubule and intercalated cells in the collecting duct, the latter identified by colocalization with H^+/ATPase. No transduction was observed in the glomeruli or the intrarenal vasculature. These studies demonstrate successful transgene expression in tubular epithelial cells, specifically in the S3 segment of the proximal tubule and intercalated cells, after intrarenal administration of a rAAV vector and provide the impetus for further studies to exploit its use as a tool for gene therapy in the kidney.

Gene therapy represents a promising therapeutic modality for several diseases. Gene therapy-related research has been directed toward the development of an ideal vector that shows high transduction efficacy, no cytotoxicity, long-term expression, and lack of a host immune response. Many delivery systems have been reported, including both viral (e.g., adenovirus, retrovirus, lentivirus, adeno-associated virus) and nonviral (e.g., naked plasmid DNA and liposomal-DNA complexes) modalities (1–6). Unfortunately, only limited success has been achieved with most of these modalities owing to either short duration of transgene expression or adverse host immune responses (7). Recombinant adeno-associated viral vectors (rAAV) have several distinct advantages over other gene delivery vectors because rAAV results in long-term transgene expression and infects cells with no significant side effects, particularly with respect to immune responses (reviewed in reference 4). rAAV has been used in several animal and human clinical trials in diseases such as cystic fibrosis (8), alpha-1-antitrypsin deficiency (9), and hemophilia B (10). rAAV has a broad host range and is capable of transducing both dividing and non-dividing cells (4). In addition, genes introduced by rAAV can provide continuous production of the recombinant transgene after a single application (9–11). Infection with wild-type AAV alone leads to the establishment of a long-term latency, which is due primarily to site-specific integration in the AAVS1 site on human chromosome 19 (12), although some forms persist as episomes or are integrated at other sites (13).

The adeno-associated viruses are members of the parvovirus family. There are six known serotypes of rAAV that infect primate cells, designated AAV1 through AAV6. Two additional serotypes, AAV7 and AAV8, have recently been described (14). Thusfar, AAV serotype 2 (AAV2) has been the most extensively studied serotype. The AAV2 virion is a...
non-enveloped, icosahedral particle approximately 25 nm in diameter (15). The AAV2 genome consists of a single strand of DNA of 4680 nucleotides in length (16). There are two open reading frames, the rep and cap genes, flanked by two palindromic inverted terminal repeats (ITR), which contain the cis-acting sequences that serve as the origins for DNA replication and the packaging signal for encapsidation (16). The left open reading frame encodes the nonstructural replication initiator polypeptides (rep), which regulate AAV DNA replication, transcription, and the accumulation of single-stranded progeny genomes. The rep proteins are important regulators of transcription both from the endogenous AAV promoter and from promoters within the helper virus (16).

rAAV-mediated gene delivery resulting in long-term expression has been reported in a wide variety of tissues, including retina (17), lung (8), muscle (9–11), liver (18), brain (19), spinal cord (20), and pancreatic islets (21). However, to the best of our knowledge, only one previous study has reported results with the use of rAAV in the kidney. In this study, Lipkowitz et al. (22) injected rAAV carrying green fluorescence protein (GFP) and/or ß-galactosidase directly into the renal parenchyma of mouse kidneys and demonstrated transduction of renal tubular cells only along the needle track. While these results were encouraging, the disadvantage of the direct intra-parenchymal approach, as well as the limited transduction observed, prompted us to evaluate other delivery strat-

Figure 1. Vector map of rAAV2-GFP (A). The elements of the cassette are as follows: ITR, the inverted terminal repeat of AAV serotype 2 (AAV2) virus; CBAp, the cytomegalovirus immediate early enhancer linked to chicken ß-actin promoter and derived from pCacMam-1 (Novagen, Madison, WI); a chimeric intron from rabbit ß-globin; GFP, a “humanized” green fluorescent protein cDNA (716 bp) from pTR-UF2 (23); poly(A), the bovine growth hormone polyadenylation site from pTR-UF2. Technique for intrarenal arterial administration of vector for transduction of rat kidneys (B through E). The infrarenal segment of the aorta was isolated and cannulated just below the origin of the renal arteries after clamping the suprarenal aorta, the right renal artery, and aortic branches near the left renal artery (B). An incision was made on the left renal vein. The left kidney was flushed with 1.0 ml of cold normal saline through the renal artery via the infrarenal aorta (C). The left renal vein was clamped thereafter and saline (0.125 ml/100 g body wt) or rAAV2-GFP (0.125 ml/100gm body weight, titer: $1 \times 10^{10}$/ml infectious units) was injected through the left renal artery into the kidney. The artery and vein were clamped, and the vector was allowed to dwell in the kidney for 45 min (D). The cannula was removed, the incisions in the aorta and left renal vein were sutured (E), and the blood supply to the kidney was restored.
egies in the kidney. The purpose of the present study was to evaluate the pattern of gene expression in the rat kidney after intrarenal arterial infusion of a rAAV (serotype 2) vector encoding GFP as a reporter gene. This approach would be clinically feasible in renal disorders in which gene therapy is contemplated and would be particularly applicable to renal transplantation, where the kidney is available \textit{ex vivo} for transduction before transplantation.

**Materials and Methods**

**Preparation of rAAV2-GFP Vector**

The structure of the rAAV2-GFP vector (UF11) plasmid is shown in Figure 1A. The plasmid contains a “humanized” GFP as the transgene and is driven by a cytomegalovirus-chicken \( \beta \)-actin hybrid promoter (CBAp), as described previously (23). The rAAV2 production was performed according to the previously described method of double transfection of a permissive human cell line (HEK293) (24). Plasmid pDG containing both the AAV2rep and cap genes as well as a subset of adenovirus (Ad) 5 genes (E2a, E4, and VA-RNA) were co-transfected along with rAAV2-GFP vector plasmid into HEK293 cells (Ela\(^{+}\), Elb\(^{+}\)) grown in cell factories (Nalge Nunc, Rochester, NY). Cells were harvested and lysed by a freeze-thaw method to release virions. The virus containing supernatant was subsequently purified by iodixanol gradient ultracentrifugation followed by heparin sepharose affinity chromatography. The purity of the preparations was determined by silver-stained SDS acrylamide gel electrophoresis. Infectious center assays were used to determine rAAV virus titer, with dot blot analysis performed to quantify the titer of the rAAV physical particles and determine the particle to infectivity ratio. The particle-to-infectivity ratio averaged approximately 10 in all the rAAV preparations.

**Intrarenal Artery Administration of Vector**

Male Lewis rats weighing 80 to 100 g were anesthetized intraperitoneally with pentobarbital sodium (30 to 40 mg/kg body wt). A

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Figure 2. Histologic evaluation of transduced kidneys. Hematoxylin and eosin–stained sections of the left kidney transduced with either saline (control; A and B) or rAAV2-GFP \( 1 \times 10^{10} \) IU/ml (C and D). Representative low magnification \((\times 25)\) images of cortex and medulla are shown in A and C. Higher magnifications \((\times 100)\) of the outer medullary region are shown in B and D. Arrowheads indicate areas of mononuclear cell infiltration, tubular atrophy, and scar formation.
Figure 3. Immunohistochemical staining of rat kidneys 6 wk after transduction with either saline (control; A) or rAAV2-GFP $1 \times 10^{10}$ IU/ml (B through G). Staining for GFP (arrows, black color) was performed using a rabbit anti-GFP antibody (1:15 dilution; Chemicon, Temecula, CA) detected by a vector Elite ABC kit using DAB nickel as the chromagen (Vector Labs, Burlingame, CA). Sections were counterstained with Fast Green. As shown in C through G, significant positive staining for GFP was present in renal tubular epithelial cells in animals that received...
midline laparotomy incision was made, and administration of the vector was performed through the left renal artery as described in Figures 1B through 1E. Briefly, the left kidney was flushed with saline after clamping of the left renal vein, and saline (0.125 ml/100 g body wt; n = 7) or rAAV2-GFP (0.125 ml/100 g body wt; titer, 1 × 10^10/ml infectious units, IU; n = 7) was injected through the left renal artery into the kidney. In initial pilot experiments, lower doses of 1 × 10^8 to 1 × 10^9 IU/ml showed no transduction. The vector was allowed to dwell in the kidney for 45 min before removal of the cannula and suturing of the incisions. The rats were sacrificed at 2 wk (n = 2 in saline and rAAV2-GFP group, respectively), 4 wk (n = 2 in each group), and 6 wk (n = 3 in each group) after transduction. Both the left and right kidneys of experimental animals were preserved by immersion in 10% neutral buffered formalin and processed for evaluation of transgene expression by immunohistochemistry for GFP and H^+^-ATPase, staining with hematoxylin and eosin for routine histologic evaluation, and in situ hybridization. We also performed experiments in which a higher titer of the vector was administered via intrarenal arterial administration. In these studies, male Lewis rats were injected with 0.125 ml/100 g body wt of 1 × 10^11 IU/ml of rAAV2-GFP or saline (n = 3 in each group). Animals were sacrificed at 6 wk after transduction, and the liver, spleen, heart, lung, and both kidneys were harvested for routine histologic examination and evaluation of transgene expression by PCR (qualitative and quantitative real-time PCR), immunofluorescence, and immunohistochemistry. For immunofluorescence, a slice of the kidney was fixed by immersion in 10% neutral buffered formalin overnight and then cryopreserved in 18% sucrose for 16 h. The tissue was embedded in OCT, and 5-μm-thick frozen sections were cut. Slides were mounted using Vector Shield (Vector Labs, Burlington, CA) and examined under an Olympus Provis fluorescence microscope. The animal experiments were conducted in accord with the NIH Guide for the Care of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee, University of Florida, Gainesville, Florida.

Immunohistochemical Analyses

Additional pieces of formalin-fixed kidney and other organs (liver, heart, spleen, lung) were embedded in paraffin. Four-micron-thick sections were deparaffinized, blocked for endogenous peroxidases, and incubated for 25 min at 95°C in Trilogy solution (Cell Marque, Hot Springs, AK) for antigen retrieval. After preliminary antibody titration studies, slides were stained for GFP using a rabbit anti-GFP antibody as a negative control. Detection was achieved using the Vector Elite Rabbit ABC kit, with 3,3′-diaminobenzidine (DAB) chromagen. Slides were counterstained with Light Green (Sigma Chemical Co., St Louis, MO).

Identification of transduced intercalated cells was determined by two strategies. First, serial sections were stained for H^+^-ATPase using a rabbit polyclonal antibody against the 70-kD subunit of bovine brain clathrin-coated vesicle H^+^-ATPase (1:400 dilution; kindly provided by Dr. Dennis Stone, University of Texas Health Sciences Center, Dallas, Texas) or GFP using a rabbit anti-GFP antibody (1:1000 dilution; Novus, Littleton, CO). The H^+^-ATPase antibody is known to label intercalated cells in the rat, mouse, and rabbit kidney (25,26).

Detection was achieved using a Vector Elite Rabbit ABC kit, with DAB as the chromagen and hematoxylin QS as the counterstain.

Co-localization studies were also performed on 4-μm paraffin sections. Slides were deparaffinized and then blocked for both endogenous peroxidases and biotin. Sections were then stained for GFP, followed by staining for H^+^-ATPase. Rabbit anti-GFP antibody (Novus, Littleton, CO) was diluted at 1:1000 and applied to the slides for 1 h at room temperature. Detection was achieved using a Vector Elite Rabbit ABC kit with 3-amo-no-9-ethylcarbazole (AEC) as the chromagen. Slides were re-equilibrated in buffer and incubated in 1:400 dilution of the H^+^-ATPase antibody for 1 h at room temperature. A Vector Elite Rabbit ABC Alkaline Phosphatase kit was used for detection. Vector Blue substrate was applied to the slides in the presence of levamisole to produce color contrast. No counterstaining agent was used. Slides were also stained with GFP alone using AEC as the chromagen as a color control. Images were taken with an Olympus Provis microscope using a digital color camera and Magnafire software.

In Situ Hybridization

A 657-bp fragment of the GFP transgene was amplified by PCR using DIG-labeled UTP (Roche Biochemicals, Germany) with the following primers: sense 5′-GGCGTCACAATTCCTCTGGGAAC-3′ and antisense 5′-GGCGTCAACAATTCCTCTGGGAAC-3′. Four-micron-thick paraffin sections were immersed in xylene, 100% ethanol, and 95% ethanol and washed briefly with diethylpyrocarbonate-treated water. Tissue sections were then digested with proteinase K (10 μg/ml) at 37°C for 10 min. The DIG-labeled GFP probe was denatured and hybridized performed in CEP hybe solution (Vysis, Downer’s Grove, IL) at 37°C overnight. Slides were washed three times for 2 min each in 50% formamidex2 × SSC, once for 2 min in 2 × SSC and again for 2 min in 2 × SSC + 0.1% Igepal (Sigma), and rinsed in TBS. Colorimetric detection was performed using an anti-DIG antibody conjugated to alkaline phosphatase (Roche) followed by incubation with Vector Blue Substrate and Nuclear Fast Red counterstaining. The slides were assessed by light microscopy, and images captured using a digital color camera and Magnafire software.

DNA Extraction and PCR for Detection of Vector Genomes

Genomic DNA was extracted from the different tissues using a commercially available Qiagen kit (Valencia, CA) and quantified by spectrophotometry. GFP-specific primers, sense 5′-GGCGTGGTC-CCAATTCTCGTGGAAC-3′ and antisense 5′-GGCGTCAACAAATCCCTCTGGGAAC-3′, were used to amplify a single copy 258-bp fragment of the GFP transgene using 100 ng of genomic DNA as template and the following PCR parameters: 5 min at 94°C; 30 s at 62°C; final extension period of 10 min at 72°C. Ten microliters of the PCR reaction was subjected to electrophoresis on a 2% agarose gel to

rAAV2-GFP. No staining was observed in blood vessels and glomeruli (C, D, and E). Saline-injected animals demonstrated background staining reaction (A). In addition, a section from the opposite right kidney (H) had background staining. Rabbit IgG was used instead of the primary antibody as an additional control (B). Representative low (A, B, C, and H [×20]) and higher magnification (D, E, and G [×80] and F [×250]) images are shown with dense cytoplasmic staining in the regions of the S3 segment of the proximal tubule (C through F) and cells with the morphologic appearance of type A intercalated cells in the medulla (C and G).

rAAV2-GFP. No staining was observed in blood vessels and glomeruli (C, D, and E). Saline-injected animals demonstrated background staining reaction (A). In addition, a section from the opposite right kidney (H) had background staining. Rabbit IgG was used instead of the primary antibody as an additional control (B). Representative low (A, B, C, and H [×20]) and higher magnification (D, E, and G [×80] and F [×250]) images are shown with dense cytoplasmic staining in the regions of the S3 segment of the proximal tubule (C through F) and cells with the morphologic appearance of type A intercalated cells in the medulla (C and G).
verify the presence and the size of the amplified product. rAAV2-GFP plasmid DNA was used as a positive control in the PCR reactions.

For real-time quantitative PCR, the following primer probe set was used to determine the number of copies of rAAV2-GFP vector DNA: sense 5'-TTTCAAGATGGCGGAACTACA-3' and antisense 5'-TCAATGCCTCTAGCTCGAT-3'. Genomic DNA (100 ng) was used in each assay. Real-time PCR was performed using the conditions recommended by Perkin-Elmer/Applied Biosystems (Foster City, CA) as described previously (27). The standard curve for real-time PCR was generated by using known quantities of rAAV2-GFP plasmid DNA. An internal control consisted of a standard GAPDH primer-probe set provided by Perkin-Elmer/Applied Biosystems. The below threshold for any detectable signal for this assay was between 40 to 400 copies per sample. Results are expressed as mean ± SEM of vector genomes per microgram DNA.

**Statistical Analyses**

Data are expressed as the means ± SEM. Statistical analyses were performed using the t test or ANOVA and the Student-Newman-Keuls test. All results are considered significant at P < 0.05.

**Results**

**Histologic Evaluation by Hematoxylin and Eosin Staining**

The protocol for intrarenal arterial administration of gene delivery involved an ischemia time of approximately 45 min, during which time vector was allowed to dwell in the kidney, followed by reperfusion. To evaluate the renal morphologic changes secondary to the procedure, we examined hematoxylin and eosin-stained kidney sections by light microscopy. Focal areas of chronic interstitial infiltration with mononuclear cells, tubular atrophy, and scar formation (Figure 2, arrowheads) were observed in both saline- and rAAV2-GFP (1 × 10^10 IU/ml)–injected kidneys at 6 wk after injection. Tubular cell regeneration and occasional casts were observed at earlier time points (2 to 4 wk; data not shown). No difference was observed between the saline- and rAAV2-GFP–injected kidneys, suggesting that these changes were most likely the result of ischemic injury inherent in the procedure and not a consequence of vector administration or an adverse immune response to rAAV or the transgene.

**Immunohistochemical Detection of Transgene Expression**

We examined the kidneys for expression of the GFP transgene by immunohistochemistry. Only minimal expression was identifiable in tubular cells at 2 wk after transduction. The number of positive cells increased to approximately 8 to 10 cells/40× field at 4 wk (data not shown). However, at 6 wk after transduction, we observed significant expression of GFP in renal tubules in all kidneys injected with rAAV2-GFP (1 × 10^10 IU/ml; n = 3), while no expression was observed in the non-injected right kidney or the saline-injected left kidneys (Figure 3). The expression of GFP was restricted to the outer medullary region of the kidney and was localized to proximal tubule epithelial cells identified by the presence of a brush border (Figure 3, C through F). No transduction was observed in glomeruli, blood vessels, or interstitial cells.

Interestingly, a subpopulation of collecting duct cells in the medullary region of the kidney with the histologic appearance of intercalated cells was also positive for GFP (Figure 3G). These cells were identified as type A intercalated cells by positive apical immunoreactivity for H^+-ATPase in serial sections that were also positive for GFP (Figure 4, A through D). Co-localization of GFP with H^+-ATPase staining was also confirmed by double labeling, as shown in Figure 4F. H^+-ATPase was also expressed in the proximal convoluted tubule cells, where it localized to the base of the brush border as reported previously (28). Examination of serial sections revealed that proximal tubules with apical H^+-ATPase staining were not positive for GFP, while tubules that contained GFP positive cells were negative for H^+-ATPase. These findings led to the conclusion that the cells of the S3 segment of the proximal tubule were the most frequently transduced cell population. The presence of GFP was also confirmed by in situ hybridization. As shown in Figure 5A, no signal was observed in the kidney of saline-injected animals. However, positive signal was detected in proximal tubular and intercalated cells in the rAAV2-GFP–injected kidneys (Figure 5, B and C).

**Evaluation of Transgene Expression after High-Dose Vector Administration**

To evaluate whether rAAV-mediated transduction was associated with any toxicity, we examined the kidney and other organs at 6 wk after a higher dose (0.125 ml/100 g body wt, 1 × 10^{11} IU/ml) of rAAV2-GFP or an equal volume of saline administered via intrarenal arterial injection as described above. The weight gain in the saline-injected animals was similar to the weight gain in the animals treated with the high dose vector during the 6 wk period (saline, 164.3 ± 9.9 g; vector, 177 ± 15.9 g; n = 3 in each group; P > 0.05). As shown in Figure 6, significantly higher transduction was observed in renal tubular cells by immunofluorescence for GFP, which was also confirmed by immunohistochemistry (Figure 7A, lower panel). Again, the predominant cells transduced were proximal tubular (Figures 6B, 6D, and 7A, lower panel) and intercalated cells (Figures 6C and 7A, inset). Glomeruli, vasculature, and interstitial cells were not transduced. Histologic examination of the transduced left kidney revealed focal mononuclear infiltration and tubular atrophy (Figure 7A, upper panel), which was also seen in the left kidneys of saline-injected animals. No histologic changes in the form of immune cell infiltration or cell injury were observed in other organs, including the right kidney, spleen, lung, liver and heart in animals injected with the vector (Figure 7, B through F, upper panel) compared with saline-injected animals. Evaluation for transgene expression by immunohistochemistry failed to detect any GFP in the other organs (Figure 7, B through F, lower panel). We also evaluated other organs for the presence or absence of vector DNA by both qualitative and quantitative real-time PCR. As expected, a positive PCR product (258 bp) was amplified from the transduced left kidney as well as from the liver, lung,
Figure 4. Co-localization of GFP with \( \text{H}^+ \)-ATPase. Serial sections were stained with either GFP (A and C) or the intercalated cell marker \( \text{H}^+ \)-ATPase (B and D) as described in the Materials and Methods. For easy comparison, both GFP and \( \text{H}^+ \)-ATPase were stained brown (arrows) in the serial sections (A through D). There were no GFP-positive cells in the saline-injected kidney (A). Some but not all intercalated cells in rAAV2-GFP-transduced kidneys were selectively stained by the GFP antibody (C). For co-localization studies, dual-labeling with GFP and \( \text{H}^+ \)-ATPase (E and F) was performed as described in Materials and Methods. The inset in F shows an rAAV2-GFP–injected kidney section stained with GFP alone (red). The apical dark blue staining of cells in (E) represents \( \text{H}^+ \)-ATPase–positive cells, and the purple-stained cells (arrows) in F represent cells positive for both GFP and \( \text{H}^+ \)-ATPase, indicating transduction of intercalated cells. The saline-injected kidney has no red or purple staining (E). Significant number of doubly positive purple cells was observed in rAAV2-GFP-transduced kidneys (F). All kidneys were positive for \( \text{H}^+ \)-ATPase staining. Magnification, ×400 for all images.
and spleen of rAAV2-GFP–injected animals (data not shown), while no product was obtained in the saline-injected animals. High copies of vector genomes were detected from the transduced left kidney (Figure 8). Vector DNA was also detectable in the liver (approximately threefold lower) and to a lesser extent in the lung and spleen; none was detectable in the heart and the right kidney of vector-treated animals (Figure 8B). The number of vector genomes was below the threshold for detection in the saline-injected animals.

Discussion

The results of this study demonstrate successful transduction of renal tubular epithelial cells after intrarenal arterial administration of a rAAV2-GFP vector. Proximal tubule cells, specifically in the S3 segment, as well as intercalated cells in the medullary collecting duct were transduced. No transduction was observed in blood vessels, glomeruli, or the interstitium. The ischemia associated with the procedure of gene delivery caused mild changes of tubular injury and interstitial mononuclear cell infiltration and was observed in both saline and vector-treated animals, suggesting that these morphologic changes were not due to an adverse immune response to the viral vector or the transgene.

Over the past decade, significant advances in molecular biology have led scientists to initiate gene therapy trials in humans; indeed, this modality has been attempted in several clinical situations, especially in the treatment of metabolic disorders, cancer, hypertension, and sepsis. The potential for the application of gene therapy in diseases related to the kidney as well as the techniques of gene delivery to renal cells has been the subject of several recent reviews (29–32). Our results showing rAAV-mediated transduction of renal tubular epithelial cells are clinically relevant for the development of targeted delivery of genes with renoprotective actions such as hepatocyte growth factor, CTLA4Ig, and antioxidants/antiapoptotic genes (e.g., heme oxygenase-1, manganese superoxide dismutase, Bcl2), molecules that have great promise in ameliorating renal injury after ischemia-reperfusion and exposure to nephrotoxins or in the setting of transplantation (33–35).

Our studies are also applicable to the treatment of renal tubular disorders, where progress with respect to gene therapy strategies has been hampered by the transient nature of gene expression. Successful correction of inherited renal diseases, including carbonic anhydrase II (36) and aquaporin-1 deficiency (37), have been reported. Lai et al. (36) utilized cationic liposomes to deliver carbonic anhydrase II to mice deficient in this enzyme by retrograde injection and demonstrated transgene expression in tubular cells of the outer medulla and at the corticomedullary junction. Transient improvement in urinary acidification after ammonium chloride treatment was observed in these studies. More recently, Yang et al. (37) reported partial correction of the urinary concentrating defect in response to water deprivation in aquaporin-1–deficient mice, by treatment with an adenoviral vector containing aquaporin-1. The vector was delivered by tail vein injection and resulted in transgene expression in proximal tubules and medullary vasa recta. However, aquaporin-1 expression and the functional effects were lost over 3 to 5 wk. Our findings of transgene expression in intercalated cells using rAAV and the ability of rAAV to provide long-term persistence of transgenes after a single application (9–11) make these vectors ideal candidates for investigation in renal tubular disorders. While our studies have evaluated transgene expression up to 6 wk, we anticipate that long-term persistent transgene expression will be possible with rAAV vectors, as has been reported previously in other tissues (9–11). It is possible that further modifications in vector design using specific promoters, alternate serotypes, or capsid modifications would enhance transduction efficiency.

Previous studies using adenoviral vectors delivered either by intrarenal arterial infusion or retrograde infusion into the renal pelvis have reported successful transduction of renal tubular cells (38). Predominantly proximal tubule cells were trans-
duced with intrarenal arterial administration, while tubular cells in the papilla and medulla were transduced by retrograde infusion of these vectors (38). Recent studies have demonstrated transduction of renal tubules using lentiviral vectors (39). Intrarenal arterial or venous administration of the lentiviral vector resulted in $\beta$-galactosidase staining of occasional inner medullary collecting duct cells, while retrograde infusion into the ureter resulted in weak transduction of renal tubules in the medulla and the corticomedullary junction. Similar to our studies using rAAV, no transduction was observed in glomeruli, intrarenal blood vessels, or the interstitium with these methods. Some of these limitations have been overcome by recently described modifications using adenoviral vectors (40, 41). Nahman et al. (40) developed microsphere-conjugated adenoviral complexes delivered by aortic injections and reported successful transduction of rat glomerular endothelial and mesangial cells in vivo. McDonald et al. (41) utilized a fiber-modified adenoviral vector containing the RGD integrin binding motif and observed selective transgene expression in the renal cortical vasculature. More recently, Mah et al. (42) have used heparan sulfate proteoglycan-coated microspheres conjugated with rAAV2 vectors and reported significantly higher transduction efficiencies in several tissues including the kidney, particularly with small microspheres (diameter, 4.4 μm).

We observed selective transduction of proximal tubule and intercalated cells in our studies. The reason(s) for this preferential transduction is not entirely clear. Several possible mechanisms may be implicated. The early steps of AAV infection involve attachment to a variety of cell surface receptors (heparan sulfate proteoglycan, fibroblast growth factor receptor, and $\alpha_v\beta_3$ integrin) followed by a clathrin-dependent or -independent internalization process (43–45). AAV2 generally requires a helper virus (usually an adenovirus or herpes virus) or a physical or chemical insult to undergo second strand synthesis and productive replication in vitro and in vivo (46). Relative enhancement of AAV-mediated transduction also occurs in renal cells in vitro after pretreatment with agents such as cisplatin and hydroxyurea (22). It is possible that ischemic cell injury results in increased surface expression of receptors that

Figure 6. Immunofluorescence for GFP in rat kidneys 6 wk after transduction with either saline (control; A) or rAAV2-GFP $1 \times 10^{11}$ IU/ml (B through D). Frozen sections of the kidney were examined by immunofluorescence microscopy. Positive green fluorescence is seen in renal tubular cells in animals transduced with rAAV2-GFP (B through D), while faint background autofluorescence is seen in the saline-injected kidney (A). Proximal tubular cells in the outer medullary region (B) and intercalated cells in the medullary collecting ducts (C) were positive (A through C, ×80). A higher magnification (×600) of a proximal tubule with positive green fluorescence is shown in D.
facilitate AAV uptake in the proximal tubule, particularly in the S3 segment, a region that is more susceptible to ischemic injury (47). It is also possible that regenerating cells have increased DNA synthesis and these are the cells that demonstrate more transduction. Selective transduction of intercalated cells in the collecting duct was also observed. Because these cells are capable of apical endocytosis (48) it is tempting to speculate that the vector might have gained access to the tubule lumen and subsequently been absorbed by endocytosis. However, it is also possible that transduction occurred via the basolateral surface through the peritubular capillaries.

While some investigators involved in gene therapy report efficient rAAV-mediated transduction, others have found a strong dependence of transduction upon the Ad helper virus, the presence of wild-type AAV contaminants, or the growth rate of cells being transduced. Some of the variability in rAAV transduction in vivo is undoubtedly related to the intrinsic properties of the target cells, specifically the presence or absence of receptors for rAAV uptake. However, much of the variation may also be due to the methods used to purify rAAV and hence contaminants that may be present in the final preparation. In general, there has been a correlation between the success of transduction with rAAV vectors and the ability to generate high-titer virus free of contaminants. Many improvements in the upstream packaging process have increased the overall yield (4). Among these innovations have been a switch from infectious Ad to plasmids containing the genes necessary for rAAV helper function, and a switch to plasmids that express less of the longer rep proteins (rep78 and rep68) relative to the shorter rep proteins (rep52 and rep40).

In summary, our studies demonstrate successful transgene expression in tubular epithelial cells after the intrarenal administration of rAAV2-GFP and provide the impetus for further studies to exploit its use as a tool for gene therapy in the kidney. These results also provide the first demonstration of rAAV-mediated transduction of intercalated cells in the kidney, findings that have not been previously reported with any other modality of gene delivery. Future studies to evaluate expression of transgenes using the different AAV serotypes and capsid mutants as well as specific promoter systems to optimize cell-specific delivery of transgenes will be important in maximizing the efficacy of rAAV-mediated gene delivery in the kidney.
mean GFP vector injected animals at 6 wk after transduction. 1, liver; 2, gram of genomic DNA isolated from tissues of saline and rAAV2-PCR was used to quantify the number of vector genomes per micro-

Figure 8. Quantitative PCR for detection of vector DNA in animals injected with high-dose rAAV2-GFP (1 × 10^{11} IU/ml). Real-time PCR was used to quantify the number of vector genomes per microgram of genomic DNA isolated from tissues of saline and rAAV2-GFP vector injected animals at 6 wk after transduction. 1, liver; 2, lung; 3, spleen; 4, heart; 5, right kidney; 6, left kidney. Values are mean ± SEM, n = 3 in each group. *P < 0.001 by ANOVA and Student-Newman-Keuls test.

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WWH is an inventor on patents related to recombinant AAV technology and owns equity in a company that is commercializing AAV for gene therapy applications. To the extent that the work in this article increases the value of these holdings, WWH has a potential conflict of interest.

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