Ksp-cadherin gene promoter.
II. Kidney-specific activity in transgenic mice

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Igarashi, Peter, Coovualli S. Shashikant, R. Brent Thomson, Dilys A. Whyte, Shuxian Liu-Chen, Frank H. Ruddle, and Peter S. Aronson. Ksp-cadherin gene promoter. II. Kidney-specific activity in transgenic mice. Am. J. Physiol. 277 (Renal Physiol. 46): F599–F610, 1999.—Kidney-specific cadherin (Ksp-cadherin, cadherin 16) is a tissue-specific member of the cadherin superfamily that is expressed exclusively in the basolateral membrane of tubular epithelial cells in the kidney. To determine the basis for tissue-specific expression of Ksp-cadherin in vivo, we evaluated the activity of the promoter in transgenic mice. Transgenic mice containing 3.3 kb of the mouse Ksp-cadherin promoter and an Escherichia coli lacZ reporter gene were generated by pronuclear microinjection. Assays of β-galactosidase enzyme activity showed that the transgene was expressed exclusively in the kidney in both adult and developing mice. Within the kidney, the transgene was expressed in a subset of renal tubular epithelial cells that endogenously expressed Ksp-cadherin and that were identified as collecting ducts by colabeling with Dolichos biflorus agglutinin. In the developing metanephros, expression of the transgene in the branching ureteric bud correlated with the developmental expression of Ksp-cadherin. Identical patterns of expression were observed in multiple founder mice, indicating that kidney specificity was independent of transgene integration site. However, heterocellular expression was observed consistent with repeat-induced gene silencing. We conclude that the Ksp-cadherin gene promoter directs kidney-specific expression in vivo. Regulatory elements that are sufficient to recapitulate the tissue- and differentiation-specific expression of Ksp-cadherin in the renal collecting duct are located within 3.3 kb upstream of the transcriptional start site.

gene regulation; β-galactosidase; pronuclear microinjection; collecting duct; kidney development; epithelial cell differentiation

KIDNEY-SPECIFIC CADHERIN (Ksp-cadherin, cadherin 16) is a novel, kidney-specific member of the cadherin family of Ca2+-dependent cell adhesion molecules (30, 31). Cadherins are integral plasma membrane proteins that mediate homotypic cell-cell interactions and are involved in morphogenetic processes such as cell compaction, epithelial differentiation, and cell migration (12, 28). Ksp-cadherin is a structurally distinct member of the cadherin family that lacks an amino-terminal prosequence and contains a highly truncated cytoplasmic domain (30, 31). Ksp-cadherin is further distinguished by its unique tissue distribution. Studies in the rabbit, mouse, and human have shown that Ksp-cadherin is exclusively expressed in the kidney (30, 31). Within the kidney, Ksp-cadherin has been immunolocalized to the basolateral membrane of renal tubular epithelial cells but is not expressed in glomeruli, blood vessels, or renal interstitial cells (30). In addition to tissue specificity, the expression of Ksp-cadherin is developmentally regulated. Northern blot analysis of embryonic mouse kidneys has shown that Ksp-cadherin is first detected at 14.5 days postcoitus (pc), which is after the formation of the first S-shaped bodies (Vanden Heuvel and Igarashi, unpublished observations). Expression increases during late gestation and remains high in the adult kidney. Immunolocalization studies in the developing human and rabbit kidney have shown that Ksp-cadherin is expressed in tubular epithelial cells of maturing nephrons but not in metanephric mesenchyme, renal vesicles, comma-shaped bodies, or S-shaped bodies (9, 29). Likewise, in the developing renal collecting system, Ksp-cadherin is expressed in the maturing collecting ducts but not in the ampullae of the ureteric buds. Thus the expression of Ksp-cadherin in renal tubular epithelial cells is differentiation specific as well as tissue specific. The abundant expression of Ksp-cadherin in mature renal tubular epithelial cells suggests that it may have a role in maintaining the differentiated state.

Although the function of Ksp-cadherin remains unclear, it has proven to be a useful model of kidney-specific gene expression. In the companion study (33), we report the cloning and characterization of the mouse Ksp-cadherin gene promoter. The promoter is TATA-less but contains other consensus eukaryotic promoter elements including an initiator, GC boxes, and CAAT box. Several consensus binding sites for transcription factors that mediate tissue-specific gene expression were identified, including activator protein-2 (AP-2), hepatocyte nuclear factor-3 (HNF-3), CCAAT/enhancer-binding protein (C/EBP), basic helix-loop-helix (bHLH) proteins, and GATA factors. Using reporter gene assays in transfected cells, we showed that 2.6 kb of the proximal 5’ flanking region of the Ksp-cadherin gene contained a functional promoter that was orientation specific. Moreover, the promoter was highly active in renal epithelial cells (MDCK and mIMCD-3) but not in mesenchymal cells (NIH 3T3 or MMR1), suggesting that the Ksp-cadherin promoter was kidney epithelial

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cell specific in vitro. Studies using electrophoretic mobility-shift assay (EMSA) showed that renal epithelial cells contain nuclear proteins that bind specifically to the proximal Ksp-cadherin promoter. As a rigorous proof of tissue specificity, the present study utilized reporter gene assays in transgenic mice to verify whether the activity of the Ksp-cadherin promoter was kidney specific in vivo. We also examined the temporal pattern of expression of the transgene to evaluate whether the promoter could direct differentiation-specific expression in renal epithelial cells. A preliminary account of this work has been published in abstract form (16).

MATERIALS AND METHODS

Materials. FVB/N mice were obtained from Taconic (Germantown, NY). CD-1 mice and B6CBA hybrid mice were from Charles River Laboratories (Wilmington, MA) or The Jackson Laboratory (Bar Harbor, ME). Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs (Beverly, MA) or Boehringer-Mannheim (Indianapolis, IN). Galacto-Star chemiluminescent substrate was obtained from Tropix (Bedford, MA). Plasmid and genomic DNA preparation kits were from Qiagen (Valencia, CA). Fluorescein-conjugated Dolichos biflorus agglutinin (DBA) was from Vector Laboratories (Burlingame, CA). Other reagents were of molecular biological grade from Sigma (St. Louis, MO), Promega (Madison, WI), Boehringer-Mannheim, or US Biochemicals (Cleveland, OH). Oligonucleotides were synthesized by the Yale Pathology Department Program in Critical Technologies and were purified by PAGE or OPC columns (Perkin-Elmer, Norwalk, CT).

Construction of the reporter plasmid. The β-galactosidase reporter plasmid used in this study was derived from pNLacF (19), which was a generous gift from Dr. Josephine Briggs (National Institute of Diabetes and Digestive and Kidney Diseases). pNLacF encodes Escherichia coli β-galactosidase containing a nuclear localization signal from simian virus 40 (Pro-Lys-Lys-Lys-Arg-Lys-Val) fused to the amino terminus. A PCR product containing the sequence of the mouse Ksp-cadherin promoter, from nucleotides 1–3458 to 5′ endonuclease I, and a unique SalI site of pNLacF to generate the plasmid pKsp-nlacZ was digested with SalI and cloned in the sense orientation into the unique SalI site of pNLacF to generate the plasmid pKsp-nlacZ. Plasmids were transformed into XL-2 Blue competent cells and purified using alkaline lysis maxiprep and anion-exchange chromatography (Qiagen Endofree plasmid kits). The sequence of the insert was verified by automated DNA sequencing, which was performed by the W. H. Keck Foundation Biotechnology Resource Laboratory at Yale University. pKsp-nlacZ was digested with PstI and ScaI, and a 6.9-kb restriction fragment (containing 3,350 bp of the Ksp-cadherin 5′ flanking region, the E. coli lacZ reporter gene, and a mouse protamine-1 intron and polyadenylation signal) was purified from vector-derived fragments by centrifugation through 10–40% sucrose density gradients. Fractions containing the 6.9-kb fragment were collected, dialyzed exhaustively against microinjection buffer (10 mM Tris-Cl (pH 7.4), 0.25 mM EDTA), and concentrated to 4 µg/ml using Microcon-30 filters (Millipore, Bedford, MA). DNA solutions were filtered through 0.2 µm filters (Vanguard, Neptune, NJ) prior to microinjection.

Generation of transgenic mice. Transgenic mice were generated by pronuclear microinjection as described previously (13). All experiments involving mice were performed in accordance with the National Institutes of Health (NIH) “Guide for the Care and Use of Laboratory Animals” (DHHS Publication No. (NIH) 85–23, Revised 1985, Office of Science and Health Reports, Bethesda, MD 20892) and under the auspices of the Yale Animal Care and Use Committee. Briefly, 15–20 female donor mice (strain FVB/N) were superovulated with pregnant mare’s serum (10 U/30 g) 3 days prior to the experiment and human chorionic gonadotropin (hCG, 10 U ip) 1 day prior to the experiment. Donor females were mated with stud males (strain FVB/N) 18 h prior to the experiment. At the same time, 30–40 female mice (B6CBA hybrids or CD-1) were randomly mated with vasectomized CD-1 males to obtain at least 3–6 pseudopregnant foster mothers. Donor females with copulatory plugs were euthanized by cervical dislocation and ovarioctomized, and the fertilized single-cell embryos were harvested from the ampullae. Embryos were incubated with hyaluronidase to remove the cumulus cells, washed, then transferred to M16 culture medium. Three to seven hours after harvesting, the embryos were transferred to a drop of M2 culture medium overlaid with oil. A holding pipette was used to position the embryo while a glass injection needle was inserted into the male pronucleus. Injection of purified DNA (200–500 copies) was verified by visible swelling of the pronucleus. Embryos that survived microinjection were transferred into the oviduct of a pseudopregnant foster mother. Transgenic progeny were identified by Southern blot hybridization of genomic DNA extracted from tail biopsies or yolk sacs using a probe derived from the Ksp-cadherin promoter. From nucleotides 1–3458 to 5′ endonuclease I, and a unique SalI site of pNLacF to generate the plasmid pKsp-nlacZ. Plasmids were transformed into XL-2 Blue competent cells and purified using alkaline lysis maxiprep and anion-exchange chromatography (Qiagen Endofree plasmid kits). The sequence of the insert was verified by automated DNA sequencing, which was performed by the W. H. Keck Foundation Biotechnology Resource Laboratory at Yale University. pKsp-nlacZ was digested with PstI and ScaI, and a 6.9-kb restriction fragment (containing 3,350 bp of the Ksp-cadherin 5′ flanking region, the E. coli lacZ reporter gene, and a mouse protamine-1 intron and polyadenylation signal) was purified from vector-derived fragments by centrifugation through 10–40% sucrose density gradients. Fractions containing the 6.9-kb fragment were collected, dialyzed exhaustively against microinjection buffer (10 mM Tris-Cl (pH 7.4), 0.25 mM EDTA), and concentrated to 4 µg/ml using Microcon-30 filters (Millipore, Bedford, MA). DNA solutions were filtered through 0.2 µm filters (Vanguard, Neptune, NJ) prior to microinjection.

β-Galactosidase assays. Assays of β-galactosidase activity in tissue homogenates were performed using a chemiluminescent assay as described by Shaper et al. (27). Fresh tissues (100 mg) were dissected from transgenic and nontransgenic mice and were homogenized in 1 ml of lysis buffer containing 100 mM potassium phosphate (pH 7.8), 0.2% Nonidet P-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 5 µg/ml leupeptin. Homogenization was performed for 20 s on ice using a VirTis homogenizer (Gardiner, NY). After centrifugation at 12,500 g for 10 min, the supernatants were heated at 48°C for 50 min to inactivate endogenous mammalian β-galactosidase-like activity (34). Twenty microliters of heat-inactivated lysate was incubated for 60 min with 300 µl of reaction buffer containing Galacto-Star (Tropix), 100 mM sodium phosphate (pH 7.5), 1 mM MgCl₂, and 5% Sarcophy-I (Tropix). Light output was integrated over 5 s at room temperature using an Optocomp I photon counting luminometer (MGM Instruments, Hamden, CT). β-Galactosidase activity was normalized to protein concentration, which was determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL) with BSA as the standard.
Assays of β-galactosidase activity in situ were performed by staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). Whole mount staining with X-Gal was performed as described previously (2). Embryos (up to 16.5 days pc) or isolated metanephroi were dissected and washed in ice-cold PBS (150 mM NaCl, 15 mM sodium phosphate, pH 7.3). To ensure adequate exposure to the substrate, the metanephroi were bisected, and the peritoneal cavities of late-gestation embryos were opened and the overlying livers were removed. Samples were fixed by immersion in PBS containing 0.25% glutaraldehyde for 30 min on ice, then washed three times with PBS. Samples were then incubated overnight in staining solution (PBS containing 20 mM Tris-Cl (pH 7.3), 1.8 mM spermidine, 2 mM MgCl₂, 0.02% Nonidet P-40, 0.01% sodium deoxycholate, 5 mM potassium ferriyanide, 5 mM potassium ferricyanide, and 1 mg/ml X-Gal). Staining with X-Gal was performed at 37°C in the dark with continuous agitation. Stained specimens were rinsed with PBS, then photographed under incident light with a Wild M420 microscope (Leica, Deerfield, IL) and Kodak 160T film.

Tissue sections were stained with X-Gal using an adaptation of the method of Hogan et al. (15). Embryos and neonatal metanephroi were dissected and fixed by immersion for 1 h on ice in PBS containing 2% paraformaldehyde. Adult kidneys were first perfused with the same fixative, bisected, then immersed in 2% paraformaldehyde for 2 h. After fixation, samples were rinsed with PBS, then incubated overnight at 4°C in PBS containing 30% sucrose. Cryoprotected samples were embedded in OCT medium, frozen in isopentane, sectioned at 10 μm, and mounted on Vectabond-coated slides. Specimens were overlaid with staining solution (above), then covered with a Fisher Probe-On slide (Pittsburgh, PA) to create an incubation chamber. Slides were incubated overnight at 37°C in the dark with continuous agitation. Stained sections were rinsed with PBS and mounted with aqueous medium (Vectorshield) to prevent diffusion of the reaction product. Photomicrographs were obtained under bright-field or phase-contrast illumination using a Zeiss Axioshot microscope (Thornwood, NJ) and Kodak 160T film. Sections of whole embryos were photographed under dark-field and bright-field illumination using a Wild microscope.

Indirect immunofluorescence. Indirect immunofluorescence was performed on 10-μm-thick cryosections as described previously (3). An affinity-purified rabbit antibody that recognizes mouse Ksp-cadherin was used at a dilution of 1:50. The generation and characterization of this antibody is described elsewhere (K. E. Earle, R. C. Kim, C. L. Yang, R. B. Thomson, and P. S. Aronson, unpublished observations). FITC-coupled goat anti-rabbit IgG was used as the secondary antibody at a dilution of 1:100 (Zymed Laboratories, South San Francisco, CA). In some experiments (see Figs. 5 and 6), staining with DBA was performed on the same sections following staining with X-Gal. Preliminary experiments verified that the DBA-staining protocol did not affect the X-Gal reaction product. Photomicrographs were obtained as described above.

Statistical analysis. Measurements of β-galactosidase activity were performed in triplicate, and activity is expressed as light output (arbitrary units) per milligram protein. Mean data of independent experiments using different tissue preparations are reported. Error bars represent SE of the mean. Statistical analysis was performed using paired t-tests. Statistical significance was defined as P < 0.05.

RESULTS

Generation of transgenic mice. To measure the activity of the Ksp-cadherin promoter in vivo, a transgene containing the promoter and a β-galactosidase reporter gene was created and expressed in mice. The Ksp-cadherin/n lacZ transgene (Fig. 1) contained 3,350 bp of the proximal 5′ flanking region of the mouse Ksp-cadherin gene and a portion of the first (noncoding) exon cloned upstream to an E. coli lacZ reporter gene in the plasmid pNLacF (19). Since pNLacF does not contain its own promoter, the expression of the reporter gene is dependent on the promoter and regulatory elements contained in the Ksp-cadherin sequence. The β-galactosidase gene in pNLacF contains a nuclear localization signal from simian virus 40 that permits the enzyme produced by the reporter gene (nuclear) to be unequivocally distinguished from endogenous β-galactosidase-like activity (cytoplasmic) (5). pNLacF also contains an intron and polyadenylation signal from the mouse protamine-1 gene to enhance transgene expression (6).

A 6.9-kb Pst I restriction fragment containing the Ksp-cadherin/n lacZ construct was isolated from vector sequences and microinjected into the pronuclei of fertilized one-cell embryos to generate transgenic mice. Transgenic progeny were identified by Southern blot analysis of genomic DNA isolated from tail biopsies or yolk sacs. Transgenic mice were then examined for expression of β-galactosidase using a chemiluminescent assay or by whole mount staining with X-Gal. The initial analysis was performed on F₀ founders in which each animal represents a unique transgene integration site and copy number. Of 28 independent embryos examined, 6 exhibited β-galactosidase activity exclusively in the kidney with a pattern that will be de-
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scribed below. To permit examination of genetically identical mice at different developmental stages, an additional set of founders was produced and bred to generate permanent lines. Two of the permanent lines (lines 8 and 26) were selected for detailed study. Genomic DNA from the two lines was digested with Xba I, which cuts once within the transgene, and hybridized with a probe derived from the lacZ gene. A 7-kb band was observed in both lines consistent with tandem integration in a head-to-tail orientation, which confirmed the absence of gross rearrangements of the transgene (data not shown). The number of integrated copies of the transgene was estimated as 3 copies in line 8 and 10 copies in line 26. With a few exceptions (noted below), the patterns of expression of the transgene were identical in the different founder animals and two permanent lines.

Expression of the transgene in the adult and developing mouse. To determine whether the expression of the Ksp-cadherin/nlacZ transgene was kidney specific, β-galactosidase activity was measured in tissue homogenates using a sensitive chemiluminescent assay. Since mammalian tissues contain endogenous β-galactosidase-like activity, lysates were pretreated by heating at 48°C, which inactivates the mammalian enzyme but does not affect bacterial β-galactosidase (34). Figure 2 shows the expression of β-galactosidase in various tissues obtained from adult transgenic mice (hatched bars) and their nontransgenic littermates (gray-shaded bars). Low activity was observed in most tissues, and there were no differences between transgenic and nontransgenic mice (P > 0.05). However, in the kidney there was a significant increase in β-galactosidase activity in transgenic mice compared with their nontransgenic littermates (P < 0.025). The results shown are for line 8; results in line 26 were similar (not shown). These results suggested that the expression of the transgene was kidney specific. However, since not all tissues could be tested in this manner, we examined the expression of the transgene in developing mouse embryos. Preliminary studies using whole mount staining of embryos suggested that the transgene was only expressed in the developing kidney (not shown). To examine this issue more completely, we prepared sections of entire mouse embryos and stained them with X-Gal. With this reagent, the appearance of insoluble blue reaction product indicates sites of β-galactosidase activity. Figure 3 shows sagittal sections of a mouse embryo at 15.5 days pc, a stage at which Ksp-cadherin is endogenously expressed in the metanephros. Figure 3, A and C, shows dark-field illumination of the head (A) and trunk (C) regions to provide orientation. Figure 3, B and D, shows bright-field illumination following staining with X-Gal. Note that the blue reaction product was only present in the developing kidney (metanephros), indicated by the arrows in Fig. 3, C–F. Higher magnification images (Fig. 3, E and F), showed expression in the developing kidney but no expression in surrounding tissues including the liver, stomach, adrenal gland, pancreas, spinal cord, and mesonephros. The β-galactosidase reaction product could be distinguished from osseous calcification (arrowheads, Fig. 3, C and F). Antibody labeling was restricted to the developing metanephros, indicated by the arrows in Fig. 3, D and F, which appeared gray on bright-field illumination and white on dark-field illumination. No expression of the transgene was observed at earlier stages of development (10.5 days pc or 11.5 days pc, data not shown). Taken together, these results demonstrated that the expression of the transgene was kidney specific in both the developing and adult mouse. These results also verified that kidney-specific expression of Ksp-cadherin was due, at least in part, to tissue-specific gene transcription.

Expression of the transgene in the developing metanephros. Figure 4 shows the expression of the Ksp-cadherin/nlacZ transgene in the developing metanephros at 15.5 days pc. To verify that the transgene was appropriately expressed in cells that endogenously produce Ksp-cadherin, colocalization studies were performed. Serial sections of the metanephros were prepared, and Fig. 4, A and B, shows adjacent sections stained with X-Gal (Fig. 4B) and an antibody to Ksp-cadherin (Fig. 4A). Figure 4A shows that the expression of Ksp-cadherin in the mouse metanephros was identical to the pattern observed in the human and rabbit (9, 29). Antibody labeling was restricted to tubular epithelial cells, and no expression of Ksp-cadherin was observed in glomeruli or in nonepithelial cells. The developing metanephros exhibits a centripetal gradient of nephron maturation in which the subcapsular nephrogenic zone contains uninduced mesenchyme, pretubular condensates, ampullae of the ureteric buds, and immature nephrons (renal vesicles, comma-shaped bodies, S-shaped bodies). Progressively more mature nephrons and segments of the collecting duct are located toward the medullary region in the center of the metanephros. Figure 4A shows that

Fig. 2. Expression of the Ksp-cadherin/nlacZ transgene in adult mouse tissues. Tissues from transgenic mice (hatched bars) and their nontransgenic littermates (gray-shaded bars) were homogenized, and β-galactosidase activity was measured in the heat-inactivated supernatants using a chemiluminescent assay. Data are means ± SE of 3 independent experiments on one of the permanent lines (line 8). Significant differences in β-galactosidase activity were only observed in kidney. *P < 0.025, paired t-test.
Ksp-cadherin was only expressed in maturing tubular epithelial cells located in the central region of the developing metanephros but was not significantly expressed in the subcapsular nephrogenic zone. Thus, in the developing mouse metanephros, as in the rabbit and human (9, 29), the expression of Ksp-cadherin was differentiation specific.

The arrows in Fig. 4, A and B, indicate that the Ksp-cadherin/nlacZ transgene was highly expressed in a subset of tubular epithelial cells that endogenously
expressed Ksp-cadherin protein. No β-galactosidase-positive tubules were Ksp-cadherin negative, and there was no expression of the transgene in mesenchymal cells, which do not express Ksp-cadherin. The nuclear localization of the blue reaction product demonstrated that the β-galactosidase activity originated from the transgene and was not due to endogenous lysosomal β-galactosidase-like activity. However, the arrowhead in Fig. 4A indicates that not all Ksp-cadherin-positive tubules expressed the transgene. On the basis of their location and characteristic branching, the tubules expressing the transgene were identified as branches of the ureteric bud. Figure 4, C and D, shows that the expression of the transgene in the branching ureteric bud was developmentally regulated. Abundant β-galactosidase activity was present in nuclei of the more mature branches of the ureteric bud located in the center of the metanephros (arrows). There was considerably less expression in the ampullae of the ureteric buds (arrowheads), which comprise relatively undifferentiated epithelial cells. The arrowhead in the top left corners of Fig. 4, C and D, indicates a ureteric bud that was sectioned longitudinally in which the proximal region (toward the right) exhibited positive staining with X-Gal. However, staining was reduced toward the renal capsule, and the ampulla adjacent to the renal vesicle was stained minimally. Taken together, these results demonstrated that the expression of the transgene was differentiation specific and recapitulated the expression of endogenous Ksp-cadherin in the developing collecting system.

Expression of the transgene in the neonatal kidney. Next, we examined the expression of the Ksp-cadherin/nlacZ transgene at 3 days postpartum (pp), a stage at which nephrogenesis is still ongoing in the mouse. Figure 5, A and B, shows results of whole mount staining of the metanephroi from two founder animals at 3 days pp. Figure 5A shows a kidney from a transgenic animal in which the blue reaction product was present primarily in the medullary region (arrow).
Figure 5B shows a kidney from a nontransgenic littermate, which was negative. Figure 5, C and D, shows staining of adjacent sections of the medullary region with X-Gal (Fig. 5D) and a Ksp-cadherin antibody (Fig. 5C). As indicated by the arrowheads in Fig. 5, C and D, β-galactosidase was present in the nuclei of collecting ducts that communicated with the renal pelvis. Note that Ksp-cadherin was uniformly expressed in the collecting duct but that the expression of the transgene varied widely between adjacent cells within the collecting duct (heterocellular expression). At this developmental stage, Ksp-cadherin and the transgene were also expressed in papillary surface epithelium (arrows in Figs. 5, C and D) and the ureter (not shown). To verify that the transgene was expressed in collecting ducts, we performed colocalization with DBA, a lectin that specifically labels collecting ducts and ureteric buds. A method was developed for staining the same section with both X-Gal and either DBA or an antibody to Ksp-cadherin. This method involved tissue fixation with 2% paraformaldehyde, staining first with X-Gal and then with antibody or lectin, postfixation after staining with DBA, and use of aqueous mounting medium to minimize diffusion of the X-Gal reaction product. Preliminary studies verified that these procedures did not affect the pattern or intensity of X-Gal staining (not shown). Figure 5, E and F, shows a transverse section of the renal medulla stained with both fluorescein-conjugated DBA (Fig. 5E) and X-Gal (Fig. 5F). As indicated by the arrows, all of the blue-stained nuclei were located within DBA-positive collecting ducts. Again, expression was heterocellular, since some collecting duct cells did not highly express the transgene. Figure 5, G and H, shows the cortical region of the neonatal mouse kidney; the arrows indicate that the expression of β-galactosidase in a branching collecting duct coincided with expression of Ksp-cadherin. Expression of both β-galactosidase and Ksp-cadherin ceased as the tubule entered the subcapsular nephrogenic zone. Figure 5, G and H, also illustrates that the renal cortex contained many other tubular epithelial cells that endogenously expressed Ksp-cadherin but did not express the transgene.

Expression of the transgene in the adult kidney. Next, we examined the expression of the Ksp-cadherin/nlacZ transgene in the adult kidney. Figure 6, A and B, shows results of whole mount staining of adult kidneys with X-Gal. The transgenic kidney (Fig. 6A) exhibited blue staining in a collecting duct pattern, whereas staining was absent in the kidney of a nontransgenic littermate (Fig. 6B). Figure 6, C and D, shows a section of the inner medulla stained with both X-Gal (Fig. 6D) and a Ksp-cadherin antibody (Fig. 6C); the arrows indicate that the transgene was expressed in the nuclei of inner medullary collecting duct cells that endogenously expressed Ksp-cadherin. Figure 6, E and F, shows the corticomedullary region stained with X-Gal (Fig. 6F) and DBA (Fig. 6E); as indicated by the arrows, the transgene was expressed exclusively in DBA-positive collecting ducts. Figure 6, G and H, shows the outer cortex stained with a Ksp-cadherin antibody (Fig. 6G) and X-Gal (Fig. 6H); the transgene was expressed in collecting ducts (arrows) that endogenously expressed Ksp-cadherin. However, there was no expression of the transgene in other tubular epithelial cells that also expressed Ksp-cadherin. The nonexpressing tubules were identified as proximal tubules, on the basis of their characteristic brush border, or as thick ascending limbs of loops of Henle, on the basis of their straight conformation, single cell type, absence of a brush border, and abundant expression of Ksp-cadherin. Neither Ksp-cadherin nor β-galactosidase was expressed in glomeruli.

The patterns of transgene expression described above were observed in six different founder animals and two independent permanent lines. The patterns of expression were identical in each of these animals with the exception of one of the founders, which exhibited a partial phenotype consisting of expression restricted to the renal papilla, and one of the permanent lines (line 8), which exhibited occasional expression in proximal tubules and thick ascending limbs of loops of Henle (not shown). No expression of the transgene was ever observed in cells that did not endogenously express Ksp-cadherin protein.

DISCUSSION

Studies of kidney-specific and developmentally regulated gene expression in the kidney may provide insights into transcriptional regulation of renal cell differentiation. In the liver, tissue-specific expression of genes such as albumin and α1-antitrypsin is mediated by liver-restricted transcription factors that bind to cognate sites in target genes and stimulate transcription. As an initial step toward understanding the mechanisms of tissue-specific gene expression in the kidney, we have attempted to identify the regions in kidney-specific genes that confer kidney specificity and that may contain binding sites for regulatory proteins. In the companion article (33), we report studies of the promoter of a kidney-specific cadherin (Ksp-cadherin) that is expressed exclusively in renal tubular epithelial cells. The Ksp-cadherin promoter was capable of directing high-level expression of a heterologous reporter gene in renal epithelial cells but not in mesenchymal cells, suggesting that the activity of the promoter was renal epithelial cell specific. As rigorous proof of tissue specificity, we have now performed reporter gene assays in transgenic mice. These studies demonstrate that the activity of the proximal Ksp-cadherin promoter is also kidney specific in vivo, verifying the in vitro results.

Within the kidney, the cloned 3.3-kb Ksp-cadherin promoter is active in renal tubular epithelial cells but not in glomeruli, blood vessels, or interstitial cells, which is identical to the expression of the endogenous Ksp-cadherin protein. Colocalization of β-galactosidase activity and Ksp-cadherin protein showed that the cloned promoter is only active in cells that endogenously express Ksp-cadherin. These cells were identified as collecting ducts by colocalization of DBA. Taken together, these results demonstrate that regulatory
elements that are sufficient for expression of Ksp-
cadherin in the renal collecting duct are located within
3.3 kb upstream to the transcription initiation site.
Deletion analysis of the Ksp-cadherin promoter in
cultured cells has identified an 82-bp region that is
critical for promoter activity in mIMCD-3 cells, which
are derived from the renal collecting duct. Further
studies will be required to determine whether this
region is also sufficient for kidney-specific expression in
vivo and to define the specific sequences that are
responsible for tissue specificity. These sequences would
represent candidate enhancers for binding to transcrip-
tional factors that mediate kidney-specific gene expres-
sion. The transgene containing 3.3 kb of 5’
upstream to the transcription initiation site. 

In addition to studies of kidney-specific gene regula-
tion, the cloned Ksp-cadherin promoter may be a useful
reagent for directing the expression of heterologous
genes in transgenic mice. An example of this type of
experiment has recently been reported by Nelson et al.
(22) in which the 5’ flanking region of the aquaporin-2
(AQP2) gene was used to drive Cre recombinase in
the kidney and male reproductive system. By mating Cre
transgenic mice with mice containing loxP sites incorpo-
rated by homologous recombination, tissue-specific gene
knockouts may be achieved. In addition to this type of
experiment, the Ksp-cadherin promoter may be useful
for creating kidney-specific and differentiation-specific
gain-of-function mutations of particular genes of inter-
est.

In addition to Ksp-cadherin, only a few kidney-
specific gene promoters have been shown to direct
appropriate expression in transgenic mice. Among the
kidney-specific promoters that have been validated in
vivo are erythropoietin, γ-glutamyl transpeptidase
(GGT), kidney androgen-regulated protein (KAP), vacu-
lar H+ ATPase (V-ATPase) B1 subunit, and AQP2. The
erthropoietin gene is expressed in the liver during
fetal life, but the kidney is the primary site of expres-
sion in response to hypoxia/anemia in the adult. Stud-
ies using transgenic mice have revealed that distinct
5’ and 3’ flanking sequences mediate hypoxia-inducible
expression in the kidney and liver (17, 25). GGT, an
important enzyme in glutathione metabolism, is ex-
pressed in many epithelial cells including the renal
proximal tubule. Six distinct GGT transcripts are pro-
duced from a single-copy gene through use of alterna-
tive promoters, of which the type II promoter is kidney
specific. Recently, a reporter gene containing 346 bp of
the type II promoter has been shown to be expressed
exclusively in renal proximal tubules in transgenic
mice (26). Another promoter that is expressed in the
renal proximal tubule is the KAP promoter. KAP is an
abundant protein of unknown function that is normally
expressed in proximal convoluted tubules in males but
not in females. Ding et al. (7) recently produced trans-
genomic mice in which the expression of the human
angiotensinogen gene was controlled by 1,542 bp of the
KAP promoter. Angiotensinogen expression was re-
stricted to renal proximal tubules and could be induced
in females by treatment with testosterone, indicating
that the KAP promoter contained elements that were
sufficient for both tissue specificity and androgen re-
sponsiveness.

Two other kidney-specific promoters have been shown
to direct expression in collecting ducts. The B1 isoform
of the 56-kDa subunit of the V-ATPase is expressed

Fig. 5. Expression of the Ksp-cadherin/nlacZ transgene in the neonatal kidney. A and B: images of the cut surface of
bisectioned kidneys from a transgenic founder animal (A) and a nontransgenic littermate (B) at 3 days postpartum (pp)
following whole-mount staining with X-Gal. In the transgenic kidney (A), blue reaction product is located primarily
in the medulla (arrow). Punctate appearance is due to nuclear localization. No blue reaction product is evident in
the nontransgenic kidney (B). C and D: indirect immunofluorescence (C) and bright-field (D) images of adjacent
sections of the renal papilla (line 26) following staining with a Ksp-cadherin-specific antibody (C) or X-Gal (D).
Ksp-cadherin and β-galactosidase are expressed in collecting duct cells (arrowheads) and papillary surface
epithelium (arrows). Note that X-Gal staining within the collecting ducts is heterocellular. E and F: epifluorescence
(Ε) and phase-contrast (F) images of the medullary region (line 8) stained sequentially with X-Gal (F) and Dolichos
biflorus agglutinin (DBA) (Ε). Arrows indicate blue-stained nuclei that are located exclusively within DBA-positive
collecting ducts. G and H: indirect immunofluorescence (G) and phase-contrast (H) images of the renal cortex (line
8) stained sequentially with X-Gal (H) and a Ksp-cadherin-specific antibody (G). Blue reaction product is located in
a branching collecting duct (arrow). Neither Ksp-cadherin nor β-galactosidase is produced in the subcapsular
nephrogenic zone (nz). Original magnifications, ×5 (A and B) and ×100 (C–H).
expressed exclusively in intercalated cells in the distal nephron and collecting duct (20). In a preliminary study, a reporter gene containing 3.5 kb of the 5′ flanking region of the human V-ATPase B1 subunit gene was expressed in renal intercalated cells in transgenic mice (21). AQP2 is expressed in principal cells in the renal collecting duct, and there are some sequence similarities between the Ksp-cadherin promoter and the AQP2 promoter (see companion study, Ref. 33). Nelson et al. (22) have shown that 14 kb of the human AQP2 promoter confers expression in the renal collecting ducts of transgenic mice. Unexpectedly, the AQP2 promoter was also active in seminiferous tubules in the testis and epithelial cells of the vas deferens, which were not previously known to be sites of endogenous AQP2 expression. However, follow-up studies confirmed that AQP2 protein was present in the apical membrane of the vas deferens, indicating that the transgene expression pattern was authentic (22). In contrast to AQP2, no activity of the Ksp-cadherin promoter was detected in the male reproductive system. In addition to the above kidney-specific promoters, several other promoters of genes that are not necessarily kidney specific have been shown to direct expression in specific nephron segments in transgenic mice. These promoters include dopamine- and cAMP-regulated phosphoprotein (DARPP-32) and prepro-epidermal growth factor (preproEGF), which direct expression in the loop of Henle (4, 24), and phosphoenolpyruvate carboxykinase (PEPCK) and plasmaglin activator inhibitor type 1 (PAI-1), which direct expression in the proximal tubule (1, 10, 23).

Kidney-specific activity of the Ksp-cadherin promoter was observed in multiple founder animals, indicating that tissue specificity was not dependent on the integration site of the transgene (position effects). However, a heterocellular pattern was observed in which the expression of the transgene varied widely between adjacent cells within the collecting duct. Heterocellular expression, also known as cellular mosaicism or variegation, is unlikely to be due to differences in cell type since the phenomenon was observed in the terminal inner medullary collecting duct, which comprises a single cell type (32). Although the magnitude varied between different lines, heterocellular expression was observed in all of the founder animals, which excluded inadvertent integration into the X chromosome (and lyonization) or integration of the transgene after the first cell division as causes of cellular mosaicism. Heterocellular expression has frequently been observed in the tissues of transgenic mice, including the kidney (4, 22), and may be particularly evident when the assay for expression of the transgene can discriminate between individual cells (14, 18). In the present study, the use of a reporter gene product targeted to the nucleus permitted expression in neighboring cells to be readily detected.

Heterocellular expression resembles the phenomenon of position-effect variegation (PEV), in which an active gene is integrated near inactive heterochromatin, and propagation of the heterochromatin state along the chromosome results in gene inactivation. Cell-to-cell variation in the extent of heterochromatin spread produces a heterocellular pattern of gene inactivation. Recent studies in Drosophila have shown that tandem arrays of transgenes can themselves induce the local formation of heterochromatin (8). Heterochromatization causes inactivation of genes within the array, and the magnitude of inactivation increases with higher copy number. A similar phenomenon called repeat-induced gene silencing (RIGS) occurs in mammals, and Garrick et al. (11) have found that reducing the copy number of a transgene without altering the integration site results in decreased chromatin compaction, decreased methylation, and increased expression. In our study, mice from line 8 had a lower copy number than line 26 but exhibited higher levels of β-galactosidase expression, consistent with RIGS as a cause of heterocellular expression.

In conclusion, the proximal 5′ flanking region of the mouse Ksp-cadherin gene contains a functional promoter and regulatory elements that are sufficient to direct kidney-specific expression in vivo. The 3.3-kb fragment contains regulatory elements that recapitulate the expression of Ksp-cadherin in the ureteric bud of the developing metanephros and the collecting ducts of the adult kidney. Elements that are required for high levels of expression in the proximal tubule, loop of Henle, and distal tubule appear to be located elsewhere in the gene locus. Kidney-specific expression of the transgene is not dependent on the site of integration. However, the transgene exhibits cellular mosaicism consistent with RIGS.

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Fig. 6. Expression of the Ksp-cadherin/nlacZ transgene in the adult kidney. A and B: images of the cut surface of bisected kidneys from an adult transgenic mouse (line 26) (A) and nontransgenic littermate (B) following whole mount staining with X-Gal. Punctate blue staining in collecting duct pattern is observed in the transgenic kidney (A) but not in the nontransgenic kidney (B). C and D: indirect immunofluorescence (C) and bright-field (D) images of inner medulla (line 26) stained sequentially with X-Gal (D) and a Ksp-cadherin-specific antibody (C). Blue reaction product is present in nuclei of Ksp-cadherin-expressing epithelial cells (arrows). E and F: epifluorescence (E) and bright-field (F) images of cortico-medullary region (line 26) stained sequentially with X-Gal (F) and DBA (E). Arrows indicate blue-stained nuclei that are located exclusively within DBA-positive collecting ducts. G and H: indirect immunofluorescence (G) and bright-field (H) images of renal cortex (line 26) stained sequentially with X-Gal (H) and a Ksp-cadherin-specific antibody (G). Blue reaction product is located in collecting duct (arrow). H is not in proximal tubules (pt) or thick ascending limbs of loops of Henle (tl), which also express Ksp-cadherin. Neither Ksp-cadherin nor β-galactosidase is produced in glomeruli (gl). Original magnifications, ×3.2 (A and B), ×100 (C, D, G, and H), and ×50 (E and F).
EXPRESSION OF KSP-CADHERIN PROMOTER IN TRANSGENIC MICE


