J Am Soc Nephrol 14: 2042–2051, 2003

Use of the Tetracycline System for Inducible Protein Synthesis in the Kidney

ANNA RACHEL GALLAGHER,* KAI SCHÖNIG,† NELSON BROWN,* HERMANN BUJARD,† and RALPH WITZGALL‡

*Institute for Anatomy and Cell Biology I and †Center for Molecular Biology, University of Heidelberg, and ‡Institute for Molecular and Cellular Anatomy, University of Regensburg, Germany.

Abstract. The great advantage of the tetracycline-inducible system lies in its ability to address a large variety of biological questions in a time-dependent and tissue-specific manner. This study describes a transgenic mouse line, rTA^{LAP}-1, which produces the reverse tetracycline transactivator under control of the liver activator protein (LAP) promoter. Two reporter lines with luciferase and *LacZ* reporter genes were used to demonstrate predominant expression in the kidney and liver when doxycycline was added to the drinking water. In the kidney, transgene expression was found primarily in cortical proximal tubules. No luciferase and β-galactosidase activity was detected in mice without doxycycline in the drinking water, which attests to the tight control of this system. One of the advantages of the tet system lies in its reversibility, and indeed, a virtually complete remission of transgene activity in both the kidney and liver was observed when doxycycline was withdrawn. Also examined was transactivator activity during development by exposing the mothers producing the reverse transactivator to doxycycline before mating. Transgene activity was detected in newborn kidneys and liver, indicating that sufficient amounts of doxycycline had crossed the placental barrier. During nephron development, the LAP promoter appeared to be only active in the more mature proximal tubules. Finally, the rTA^{LAP}-1 line was used to inducibly express the human PKD2 cDNA in proximal tubules of transgenic mice, but no cystic changes were detected, even after 6 mo of induction.

The understanding of gene function has been greatly aided by a multitude of transgenic mice. Conventional knockout mice, however, have the disadvantage that inactivation of the gene of interest may cause embryonic lethality or so severe a phenotype that it does not allow its analysis in all organs of adult mice. Therefore, a number of systems have been developed, which can either inactivate or overexpress genes conditionally in a tissue-specific and/or time-dependent manner. Of the few inducible expression systems that have been devised so far, the tetracycline-dependent system (tet system) probably has shown the greatest potential.

The tet system consists of two components. The first represents a tetracycline-dependent transactivator (tTA) driven by a specific promoter. The second contains tet operator sequences next to a minimal promoter, that control the transcription of the cDNA or gene of interest. Two versions of the tet-inducible system exist. In the original design, tTA binds to the tet operator only in the absence of tetracycline (1). In a subsequent version, the reverse tetracycline-inducible system (rtTA), a mutated tTA will bind to the tet operator only in the presence of tetracycline (2).

Although first established to study the function of genes in mammalian cell lines, the tet system soon also made a successful transition into animals (3,4). A similar extent of transgene expression has been achieved with both the tTA and rtTA systems; induction kinetics, however, were slower with the tTA system because withdrawal of doxycycline is necessary before transcription of the transgene can be induced. Recent modifications to the transactivator sequence have led to an improved tetracycline-dependent reverse transactivator (rtTA2^{S}-S2), which reaches the same activation levels as the original rtTA, while its regulatory range is tenfold enhanced as a result of its lower background activity (5).

A number of tet-inducible mouse lines have been generated (6), but none has been directed specifically to the kidney. For one line, in which the reverse transactivator is driven by the human cytomegalovirus promoter (rtTA^{CMV}-3), tet-inducible protein synthesis was described in many organs, among them the kidney (3). There, the transgene is specifically expressed in cortical and inner medullary collecting ducts (7). In this report, we describe another transgenic mouse line, in which an optimized reverse transactivator is only active in the liver and the kidney.

Materials and Methods

Transgenic Animals

Three strains of transgenic mice were used in our study. The reverse transactivator line rTA^{LAP}-1, in which the reverse transacti-

Received December 5, 2002. Accepted May 12, 2003

Correspondence to Ralph Witzgall, Institute for Molecular and Cellular Anatomy, University of Regensburg, Universitätsstr. 31, 93053 Regensburg, Germany. Phone: +49-941-9432821; Fax: +49-941-9432868; E-mail: ralph.witzgall@vkl.uni-regensburg.de

1046-6673/1408-2042
Journal of the American Society of Nephrology

DOI: 10.1097/01.ASN.0000079615.38843.4A

vator is driven by the liver activator protein (LAP) promoter, has been previously described (8,9). It contains an expression cassette that consists of 2.8 kbp of the LAP promoter and a cDNA encoding the S2 version of the reverse transactivator (5). The first reporter line, LC1, contains a bidirectional expression cassette of the luciferase gene and Cre cDNAs under control of tet operator sequences (9). A second reporter line again contains a bidirectional expression cassette, but this time with the *nLac*Z gene (encoding β-galactosidase with a nuclear localization signal) and the human PKD2 cDNA under control of tet operator sequences. The latter was generated by cloning the human PKD2 cDNA into the *Pst*I and *Sal*I sites of the pBI3 plasmid (10). After the resulting construct was linearized with *Ase*I, it was isolated by gel electrophoresis, passed twice through an Elutip D column (Schleicher & Schuell, Dassel, Germany), and injected into fertilized oocytes of C57Bl/6 x DBA mice. All transgenic lines were maintained on a C57Bl/6 background. Founders were identified by screening tail biopsy specimens for the presence of *nLac*Z and PKD2 cDNA by PCR. The following oligonucleotide pairs were used: 5'-CAAAC-CATCGAAGTGACCAG-3' and 5'-CAATTTAACCGCCACT-CAGG-3' to amplify a 388-bp fragment of the *nLacZ* gene; and 5'-ATTTGCAGATCTGTTCTCACATATCGG-3' and 5'-CTCT-CAATCCTGGGGGAA-3' to amplify a 407-bp fragment of the PKD2 cDNA. The reaction conditions were 94°C for 4 min, followed by 25 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. Thirteen out of 49 founders were positive for both *nLac*Z and PKD2. Of the 13 founders, only four were inducible for β-galactosidase activity.

Mice of either gender were used for our studies. For the induction kinetics (both for luciferase and β-galactosidase), mice were between 35 and 42 d old. Mice used in the withdrawal study were 6 mo old. For the dose-response experiments and the tissue survey, mice were 12 mo (no doxycycline) and 4 mo old (0.2 and 2 mg/ml of doxycycline).

Genotype Analysis

Genomic DNA was isolated from tail biopsy specimens of mice and subjected to Southern blot analysis according to standard protocols (11). To detect the presence of the *nLac*Z and luciferase transgenes, 10 μg of genomic DNA was digested with *Bam*HI and hybridized with the respective fragments; to detect the reverse transactivator transgene, genomic DNA was digested with *Eco*RI.

Doxycycline Administration

Doxycycline hydrochloride (Sigma, Deisenhofen, Germany) and sucrose (5% final concentration) were dissolved in water. The doxycycline-sucrose solution was prepared fresh every 3 to 4 d in a brown drinking bottle.

In Vivo *Bioluminescence Imaging*

Mice were anesthetized, injected intraperitoneally with 100 μg D-luciferin (Promega, Madison, WI) per gram of body weight, and immediately placed in a dark chamber. Luminescence was captured by a photon-counting camera (two-stage ICCD C2400-47; Hamamatsu Photonics Deutschland, Herrsching, Germany) fitted with a Nikon lens (35 mm/fl.2) for a period of 1 to 2 min. The images were then digitized with the Argus 20 image processor (Hamamatsu Photonics Deutschland) and later processed by Adobe Photoshop software (Adobe Systems, San Jose, CA).

Luciferase Assay

Tissues from adult mice were homogenized for 10 s in 500 μl of lysis buffer (20 mM DTT, 25 mM Tris pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100) immediately after removal; in the case of newborn mice tissues were sonicated for 10 s in 100 μl of lysis buffer. After homogenization, the tissues were snap-frozen in liquid nitrogen and stored at −80°C. When luciferase activity was measured, the samples were thawed and centrifuged for 15 min at 14,000 rpm and 4°C. Ten microliters of the supernatant were combined with 250 μl of 25 mM glycylglycine, 15 mM $MgSO_4$, 5 mM ATP, 0.5 mM D-luciferin and assayed for 10 s in a Lumat LB9501 (Berthold, Wildbad, Germany). All measurements were performed in duplicate. An aliquot of the lysate was used to determine the protein concentration by means of an improved Bradford assay (12).

β-Galactosidase Staining

Adult mice were perfused through the distal abdominal aorta with 4% paraformaldehyde,1x PBS for 3 min at a pressure of 180 mmHg. The relevant organs were removed, sliced, and immersed in 18% sucrose,1x PBS until they sank to the bottom. Organs from newborn mice were removed without prior perfusion, cut into slices, fixed in 1.25% paraformaldehyde, 0.2% glutaraldehyde, and 1x PBS for 15 min and then immersed twice in 30% sucrose,1x PBS for 15 min each. All tissues were subsequently frozen in liquid nitrogen-cooled isopentane. β-Galactosidase staining was performed on 7-μm cryosections. The sections were equilibrated in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 20 mM NaCl, 10 mM EGTA pH 8.0, 10 mM sodium phosphate pH 8.0) and then incubated overnight at 30°C in the presence of 1 mg/ml of X-gal. The sections were washed twice in PBS and then either counterstained with eosin and mounted, or used further for immunohistochemistry.

Immunohistochemistry and Alkaline Phosphatase Staining

Immunoperoxidase staining was carried out according to the Vectastain ABC kit instructions (Vector Laboratories, Burlingham, CA). Primary antibodies were a rabbit polyclonal anti–Tamm-Horsfall glycoprotein antibody (Biotrend, Cologne, Germany; diluted 1:100) and a rabbit polyclonal anti-HA antibody (Sigma; diluted 1:2,000). β-Galactosidase staining was carried out overnight before immunohistochemistry. Alkaline phosphatase histochemistry was performed by exposing cryosections to the appropriate reaction solution (0.3 mM nitro blue tetrazolium chloride, 0.3 mM 5-bromo-4-chloro-3-indolylphosphate, 4-toluidine salt in 0.2 M Tris·HCl, pH 9.5) for 25 min. The sections were subsequently washed twice with bidistilled water for 10 min and then mounted in bicarbonate-buffered glycerol pH 8.6. All sections were examined with a Leica Polyvar 2 microscope; pictures were taken with Nikon digital camera DXM1200. Finally, all images were processed with Adobe Photoshop software (Adobe Systems).

Cell Culture and Transfection Protocols

HtTA-1 cells, which are HeLa cells producing tTA, were stably transfected with the *Ase*I-linearized pBI3 plasmid containing the full-length PKD2 cDNA and the *nLac*Z gene by using a polyornithine protocol (13). Forty-eight hours after transfection, cells were plated onto 10-cm petri dishes and selected with puromycin (0.5 μg/ml; Calbiochem, Darmstadt, Germany). Approximately 2 wk later, resistant colonies were isolated and tested for β-galactosidase and polycystin-2 synthesis.

Protein Preparation and Western Blot Analysis

Proteins were prepared from cell lines by lysing the cells in a buffer containing 1% Triton X-100, 0.05% SDS, 150 mM NaCl, 10 mM Tris HCl pH 7.5, 2 mM EDTA pH 8.0, 1 μg/ml of leupeptin, 1 mM PMSF. After the protein concentration was determined, aliquots containing

30 μg of protein were analyzed by Western blot. To prepare protein from organs, 6-wk-old mice were placed on doxycycline for 2 wk; mice not receiving doxycycline were used as negative controls. Upon removal, kidneys and livers were added to 5 ml of a homogenization buffer containing 250 mM sucrose, 25 mM Tris pH 7.4, 5 mM EDTA, 2 μg/ml of leupeptin, 2 μg/ml of aprotinin, 1 mM benzamidine, 1 mM PMSF, 20 μg/ml TAME (*p*-tosyl-L-arginine methyl ester). After the tissues were homogenized with a motor-driven Teflon pestle, they were centrifuged for 15 min at 500 × *g* and 4°C to remove debris. An aliquot of the supernatant containing 50 μg of protein was analyzed by Western blot.

Proteins were run on polyacrylamide gels under denaturing and reducing conditions and then transferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated with the 12CA5 mouse monoclonal anti-HA epitope antibody (cell culture supernatant diluted 1:30). Final detection of the HA-epitope–tagged polycystin-2 protein was done with horseradish peroxidase–conjugated goat anti-mouse IgG Fab (diluted 1:10,000; Sigma) and the chemiluminescence reagents from NEN (Bad Homburg, Germany).

Immunocytochemistry and β-Galactosidase Staining of Cells

Cells were plated on glass coverslips and allowed to grow for an additional 3 d in the absence of doxycycline. After fixation with 2% PFA, 1x PBS, the cells were washed with 1x PBS and then stained for β-galactosidase activity in a solution containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mM $MgCl_2$, 1x PBS, and 1 mg/ml of X-gal. The next morning, the cells were washed with 1x PBS, permeabilized with 0.2% Triton X-100, 1x PBS, 2% BSA for 45 min and then incubated with the anti-HA–epitope antibody 12CA5 (diluted 1:30) for 2 h at room temperature. Subsequently, the cells were washed three times with 1x PBS and incubated for 60 min with Cy3-conjugated rat anti-mouse IgG antibody (Dianova, Germany; diluted 1:300). The coverslips were mounted and examined with a Leica Polyvar 2 microscope. Pictures were taken with Nikon digital camera DXM1200.

Results

In Vivo Imaging and Dose Dependency

The transactivator mouse line, rTA^{LAP}-1 (8,9), was created by using the optimized S2 version of the reverse transactivator (5) under control of the LAP promoter (14). Mice double-transgenic for $rtTA^{LAP}$-1 and the luciferase reporter gene were administered doxycycline at a concentration of 2 mg/ml in their drinking water. Using a noninvasive imaging system that can detect luminescence (8,15), it appeared that the LAP promoter was liver specific (Figure 1, a). However, after removing a number of organs and examining them for luciferase activity, pronounced luminescence also originated from the kidney (Figure 1, b), which could probably not be seen by noninvasive imaging because of the retroperitoneal location of the kidneys. The comparison of lysates from the kidneys with those from a number of other organs (*i.e.*, adrenal gland, brain, heart, intestine, lung, pancreas, skeletal muscle, spleen, testis, and thymus) showed at least 100-fold higher luciferase values in the kidneys (Table 1). A lower dose of 0.2 mg/ml of doxycycline in the drinking water resulted in a more than 90% reduction of luciferase activity in both the kidneys and the liver (Figure 1, c; note the logarithmic scale).

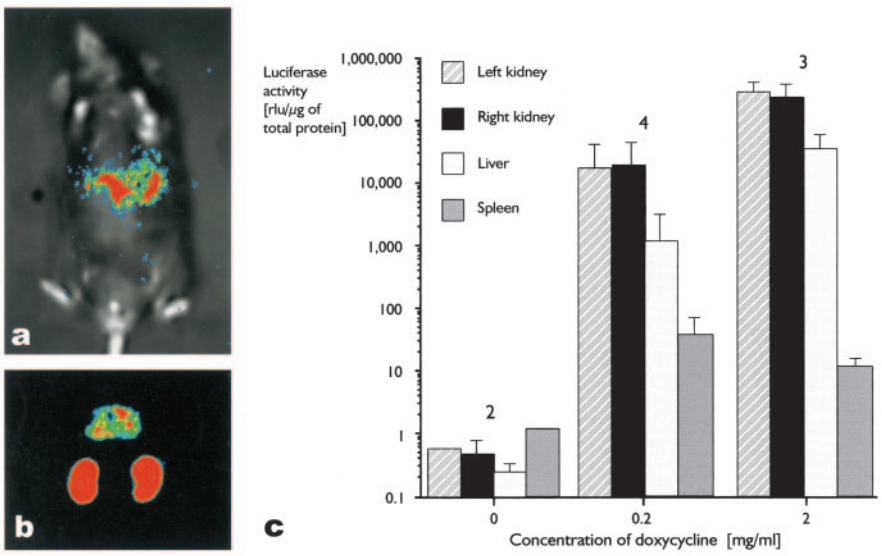

Figure 1. Imaging and dose dependency of luciferase activity. (a) Double-transgenic mice, which contained one expression cassette consisting of the reverse transactivator under control of the liver activator protein (LAP) promoter and a second expression cassette consisting of a luciferase cDNA under control of tetracycline-dependent system (tet) operator sequences, were administered doxycycline at a concentration of 2 mg/ml in the drinking water for 7 d. Then they were injected with D-luciferin and immediately placed into a dark chamber to collect photons. It can be seen by projecting the signal onto the mouse that most photons originated from the upper abdomen. (b) Upon removal of the liver and both kidneys, this luminescence could be attributed to the liver, but very strong luciferase activity was also present in the kidneys. (c) A dose-response experiment showed that administering doxycycline for 14 d at a concentration of only 0.2 mg/ml in the drinking water resulted in a pronounced decrease of luciferase activity. Data are presented as mean + SD; the number of mice is given above the bars.

Table 1. Luciferase activities in various tissues after 14 days of administration of doxycycline (2 mg/ml) in drinking water[a]

Site	No Doxycycline	Doxycycline
Adrenal gland	22.0	302.5
Brain	8.7	72.3
Heart	13.2	241.3
Intestine	16.7	812.9
Left kidney	5.4	255,382.0
Right kidney	5.5	207,783.1
Liver	10.4	32,075.5
Lung	15.3	91.7
Pancreas	17.4	1397.2
Skeletal muscle	36.4	38.2
Spleen	17.2	11.2
Testis	15.5	62.3
Thymus	25.1	78.5

[a] Values are given in relative light units/μg total protein ($n = 2$ mice without doxycycline, $n = 3$ mice with doxycycline).

Localization of the Tet Activator in the Kidney

To determine in which cells of the kidney the LAP promoter is active, mice double-transgenic for the reverse transactivator and the *nLacZ* gene were administered doxycycline at a concentration of 2 mg/ml in the drinking water for 14 d. Cryosections of the kidneys were first stained for β-galactosidase activity overnight, and then either subjected to alkaline phosphatase histochemistry or stained with antibodies against markers for the various nephron segments. β-Galactosidase activity was most prominent in the cortical portion of the proximal tubule (Figure 2, a), which became evident when costained for the brush border enzyme alkaline phosphatase, thus strongly suggesting that the LAP promoter was only active in the S1 and S2 but not in the S3 portion of the proximal tubule (Figure 2, b). It is noteworthy that quite often not all cells in the same tubular profile showed β-galactosidase activity; this mosaic pattern was seen in all animals examined. Only in approximately 20% of alkaline phosphatase–positive profiles all nuclei of a given profile produced β-galactosidase, whereas in approximately the same percentage of profiles no β-galactosidase-positive nuclei were detected (Figure 2, e). In addition to the cortex, there was some β-galactosidase activity in the inner stripe, suggesting transgene induction also in thick ascending limbs. Indeed, by costaining with an antibody against the Tamm-Horsfall glycoprotein, thick ascending limb profiles in the inner stripe and the cortex were identified that contained blue nuclei (Figure 2, c and d). We found no profile in the inner stripe in which all nuclei produced β-galactosidase, whereas in approximately 90% of the profiles, none of the nuclei was positive for β-galactosidase, which is in stark contrast to the situation in proximal tubules (Figure 2, e). Using antibodies against the thiazide-sensitive NaCl cotransporter (a marker of distal convoluted tubules) and against aquaporin-2 (a marker of collecting ducts), blue nuclei could very rarely be detected in cortical distal convoluted tubules and collecting ducts (data not shown).

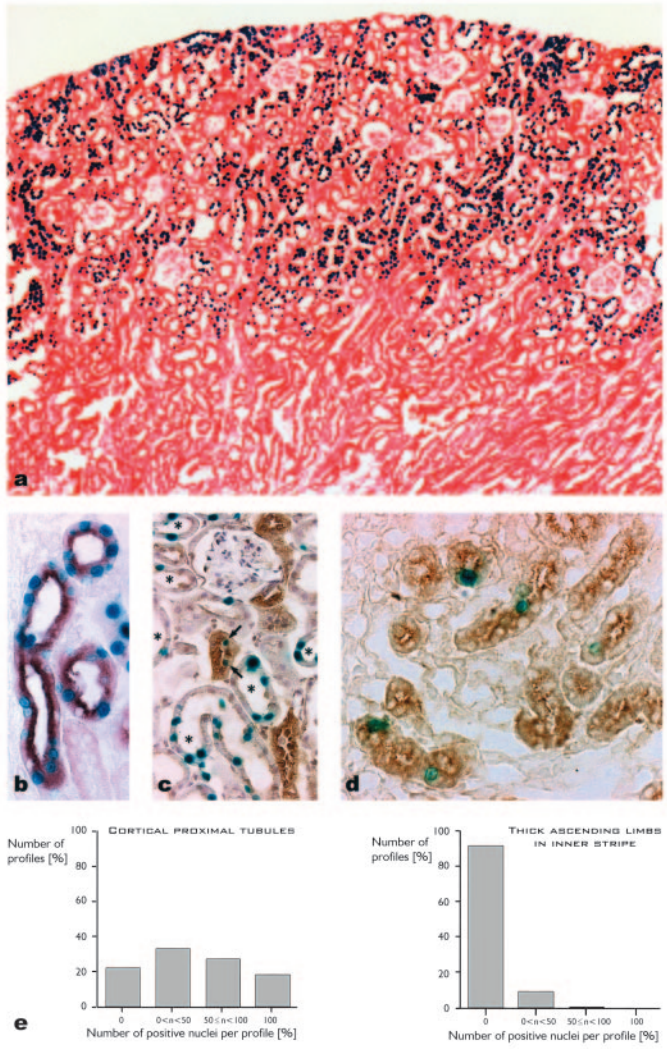

Figure 2. Localization of β-galactosidase activity in the kidney. Double-transgenic mice, which contained liver activator protein (LAP) promoter/rtTA2^S-S2 and tetracycline-dependent (tet) operator/*nLacZ* expression cassettes, were fed doxycycline at a concentration of 2 mg/ml in the drinking water for 14 d. (a) Histochemical staining for β-galactosidase activity and subsequent counterstaining with eosin demonstrates many blue nuclei in the cortex of the kidney. (b) Staining for β-galactosidase and alkaline phosphatase, a brush border enzyme, reveals that most β-galactosidase–positive nuclei are present in proximal tubules. Note that there are also some nuclei in proximal tubules, which did not turn blue. (c, d) When histochemical staining for β-galactosidase was combined with immunostaining against Tamm-Horsfall protein, a marker of thick ascending limbs, some blue nuclei were also detected in thick ascending limb cells in the cortex (arrows in c) and the inner stripe (d). Asterisks in c mark proximal tubules with many blue nuclei. (e) A careful quantitation substantiates that β-galactosidase–positive nuclei were much more frequent in proximal tubules than in thick ascending limbs, but it also demonstrates the considerable degree of mosaicism. Data are presented as mean values.

Induction Kinetics of Gene Expression

To test the induction kinetics of the rtTA^{LAP}-1 transgene in the kidney, female mice containing the LAP/rtTA2^{S}-S2 cassette were mated with males containing the luciferase and *nLacZ* reporter genes under the control of the tet operator. When the offspring were approximately 5 wk old, they were exposed to doxycycline for 1 to 14 d. Luciferase activity was then measured in the lysates from the right and left kidney, liver, and spleen of double- and single-transgenic mice (Figure 3).

As expected, instrument background was detected for the transgenic animals containing only the reverse transactivator. The tightness of the system became evident by the fact that the organs containing only the luciferase gene, and the organs from double-transgenic animals with no exposure to doxycycline also only showed background activity. After 1 d of doxycycline administration, however, a high luciferase activity of remarkably similar levels was seen in the right and left kidney of double-transgenic mice, thus demonstrating a uniform induction and validating our approach. As expected, luciferase values rose with increasing exposure to doxycycline (Figure 3, a). The same was seen for the liver, where induction was at

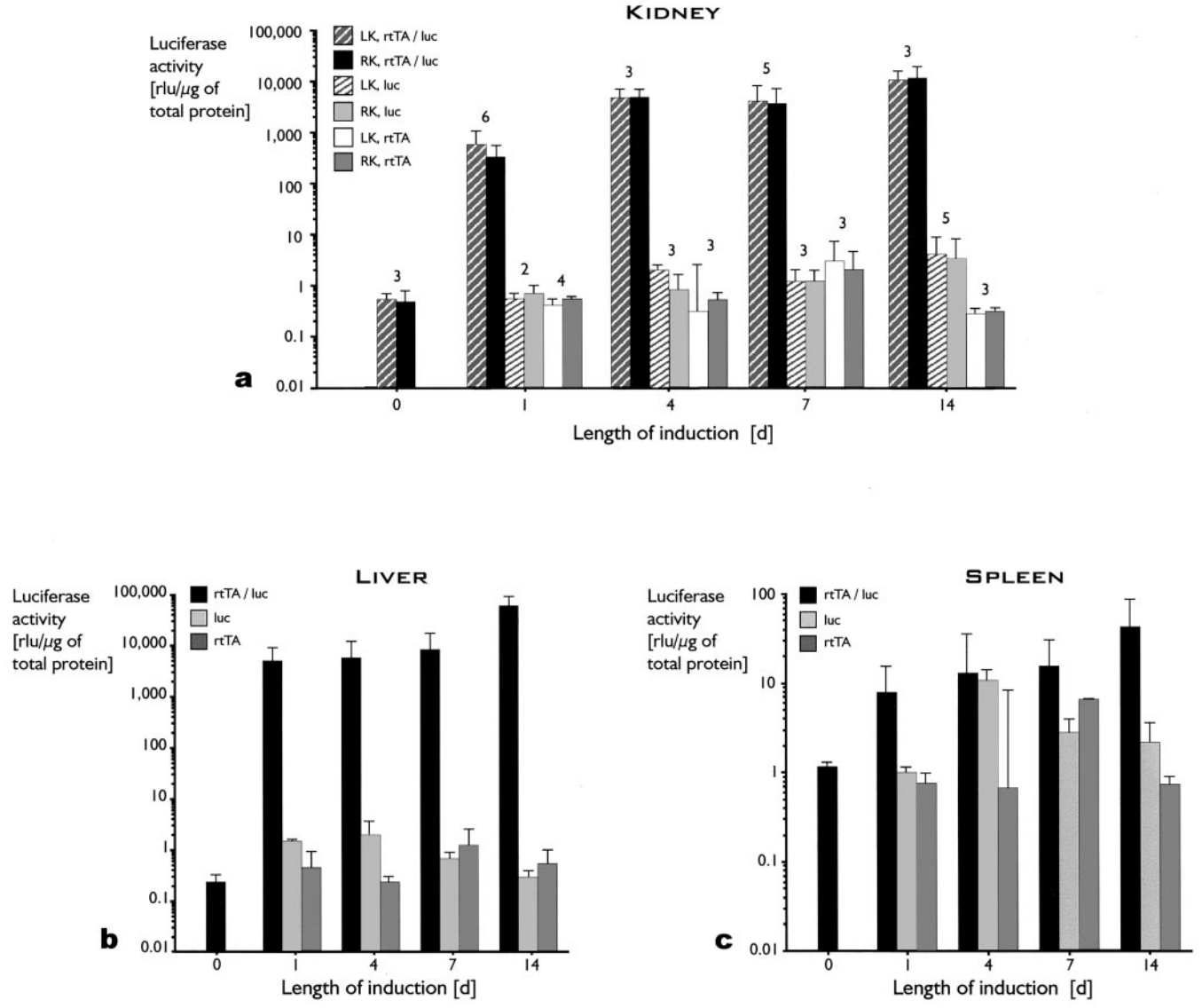

Figure 3. Kinetics of luciferase induction in adult mice. Mice containing only the liver activator protein (LAP) promoter/rtTA2^{S}-S2 expression cassette (rtTA), only the tetracycline-dependent (tet) operator/luciferase expression cassette (luc), or both expression cassettes (rtTA/luc) were fed doxycycline in the drinking water for 1, 4, 7, and 14 d before both kidneys, the liver, and spleen were removed. Already after 24 h on doxycycline, pronounced luciferase activities were observed in the kidneys (a) and the liver (b), whereas only a very modest luciferase activity was measured in the spleen, even after 14 d of doxycycline administration (c). The tightness of the system is evident from the very low luciferase activity of rtTA/luc mice without exposure to doxycycline and from equally low luciferase activity of luc mice exposed to doxycycline for 14 d. RK and LK, right and left kidney, respectively. Data are presented as mean + SD; the number of mice is given above the bars.

least 10,000-fold higher than background after 14 d of doxycycline administration (Figure 3, b). The spleen, which was used as a control tissue, showed less than 1% of the hepatic and renal luciferase values (Figure 3, c).

The same time course was used to investigate the induction and location of β-galactosidase activity. β-Galactosidase positive nuclei were already seen after 1 d of doxycycline administration, although we could not detect more than five blue nuclei per section in the cortex of the kidney. The longer the exposure to doxycycline, the more cells became induced, which correlated well with the luciferase data. Apparently tubular cells in the cortex reacted first, whereas β-galactosidase positive cells were fewer in the inner stripe and took longer to be induced (data not shown).

Developmental Kinetics of Gene Expression

Because we were able to control gene expression with doxycycline in adult kidneys, we also wanted to see if this was possible during development. For this part of the study, the mothers carrying the LAP/rtTA2^{S}-S2 transgene were placed on doxycycline 1 wk before mating. The offspring were analyzed on the day of birth as well as 7, 14, and 35 d after birth. A high level of luciferase activity was detected in newborn mice, indicating that sufficient amounts of doxycycline had crossed the placental barrier. Luciferase activity in the kidneys of newborn mice was similar to that in the kidneys of adult mice after 1 d of exposure to doxycycline. However, 1 and 2 wk postpartum, luciferase activity was markedly reduced, suggesting that doxycycline was not transduced at sufficiently high concentrations through the mothers' milk (Figure 4, a). In livers of newborn mice, luciferase activity was equivalent to that of livers from adult mice after being induced for 14 d. A pronounced decline in activity was also seen in the liver over the first couple of weeks, similar to that in the kidney (Figure 4, b). However, in both hepatic and renal tissues, a subsequent increase can be seen in 35-d-old mice similar to the levels seen in tissues from adult mice after 14 d of doxycycline administration (Figure 4, a and b). A similar pattern, although at much lower levels, was also observed in the spleen (Figure 4, c). β-Galactosidase histochemistry revealed that the induced cells in newborn kidney were limited to the more mature tubular profiles because no blue nuclei could be detected in the nephrogenic zone below the capsule (Figure 4, d). No blue nuclei could be detected in the kidneys of 14-d-old mice (data not shown).

Persistence of Transgene Expression

One of the attractive features of the tet system lies in its reversibility, which depends on the decrease of doxycycline levels after removal as well as on the half-life of the mRNA and protein encoded by the transgene. We therefore analyzed the decline in luciferase levels of double-transgenic mice, which were treated with doxycycline for 14 d. When doxycycline was removed from the drinking water, luciferase activity in the liver decreased to background levels within 14 d after discontinuation of doxycycline (Figure 5). Luciferase activity in the kidneys had fallen 100-fold after 14 d and to just above background levels after 35 d. Again, β-galactosidase activity mimicked the results of the luciferase assays because blue nuclei were still present in the kidneys 14 d after removal of doxycycline; no blue nuclei were detected in the kidney after 35 d of withdrawal (data not shown).

Inducible Expression of PKD2

To test the inducibility of the bidirectional expression cassette containing the human PKD2 cDNA and the *nLac*Z gene in the tet system, stably transfected HeLa cell lines were generated that inducibly produce full-length HA-tagged polycystin-2 and β-galactosidase in the absence of doxycycline. The clones were tested first for β-galactosidase activity and then for polycystin-2 immunoreactivity. All β-galactosidase-positive cells also stained positive for HA-tagged polycystin-2 protein (Figure 6, a and b). These immunocytochemical findings were subsequently confirmed by Western blot analysis. It can be clearly seen that the PKD2 cDNA is switched off in the presence of doxycycline and induced upon withdrawal of doxycycline (Figure 6, c).

Mice double-transgenic for the reverse transactivator and the tet operator/PKD2/*nLac*Z expression cassette were administered doxycycline in the drinking water for 14 d, animals without doxycycline in the drinking water served as a negative control. When protein lysates from the kidney and liver were subjected to Western blot analysis, the epitope-tagged polycystin-2 protein was only detected in animals receiving doxycycline (Figure 7, a). Analysis of cryosections revealed that the expression of PKD2 was most pronounced in the proximal tubule, therefore mirroring the β-galactosidase staining (Figure 7, b and c). To investigate whether the overexpression of the human PKD2 cDNA would lead to alterations in the kidneys, we administered doxycycline long-term starting at 8 wk after birth. Even after 6 mo, however, no morphologic changes were visible (Figure 7, d and e).

Discussion

The aim of our study was to characterize the transgenic mouse line rTA^{LAP}-1, which allows the inducible and reversible expression of transgenes predominantly in proximal tubules. In this line the tetracycline-inducible transactivator is driven by the LAP promoter. This promoter based on the analysis of other transgenic lines, was previously thought to be liver-specific (3). It was therefore somewhat unexpected to detect transactivator activity in the kidney, which, however, was a stable effect because we observed renal expression in many crosses and over six generations. Whether this particular expression pattern of the rtTA2^{S}-S2 transgene is due to a positional effect of its integration site remains a matter of speculation.

Strongest transgene expression was detected in the cortical proximal tubules, although transactivator activity in other parts of the nephron was also observed. Expression in thick ascending limb cells was variable and seemed to depend on the length of induction and on the tet operator line used, while there was consistently strong expression in proximal tubules independent of the tet operator mouse lines. Such a pattern of tet operator

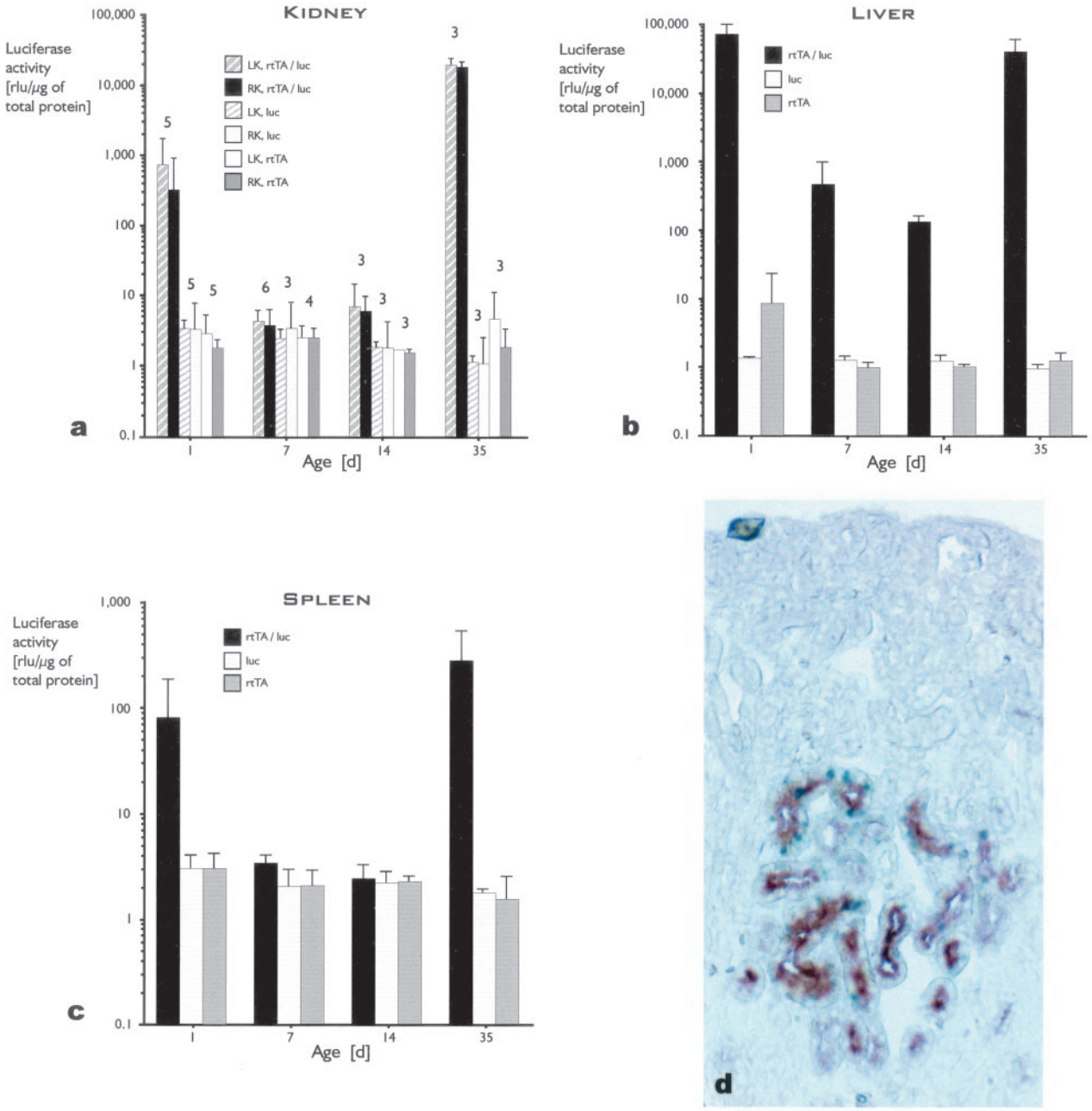

Figure 4. Activity of the reverse transactivator during development. (a through c) Female mice carrying a liver activator protein (LAP) promoter/rtTA2^{S}-S2 expression cassette (rtTA) were fed doxycycline in the drinking water from 7 d before being mated with male mice carrying a tetracycline-dependent (tet) operator/luciferase expression cassette (luc). Luciferase activity was determined in both kidneys, liver, and spleen of newborn mice and 7, 14, and 35 d after birth. High luciferase activity was observed in the kidneys and liver of newborn double-transgenic (rtTA/luc) mice and 35 d after birth, whereas it was considerably lower in the intermediate time points, suggesting that sufficient levels of doxycycline crossed the placenta, but that not enough doxycycline was provided with the mothers' milk. After weaning, when the offspring began to drink water with doxycycline, the reverse transactivator obviously became activated again. (d) Histological section of a kidney from a newborn mouse with β-galactosidase instead of luciferase as a reporter protein. Double staining for β-galactosidase and alkaline phosphatase, which marks differentiated proximal tubules, demonstrates that the LAP promoter is not active in the nephrogenic zone immediately below the capsule but only in mature proximal tubules. RK and LK, right and left kidney, respectively. Data are presented as mean + SD; the number of mice is given above the bars.

activity has already been seen in the brain, where a reverse transactivator line under the control of the CaMKII-α promoter showed expression in the striatum and septum when placed only shortly on doxycycline; with a longer induction period, expression could also be detected in the cortex and hippocampus (16). We also noticed that even though there was strong

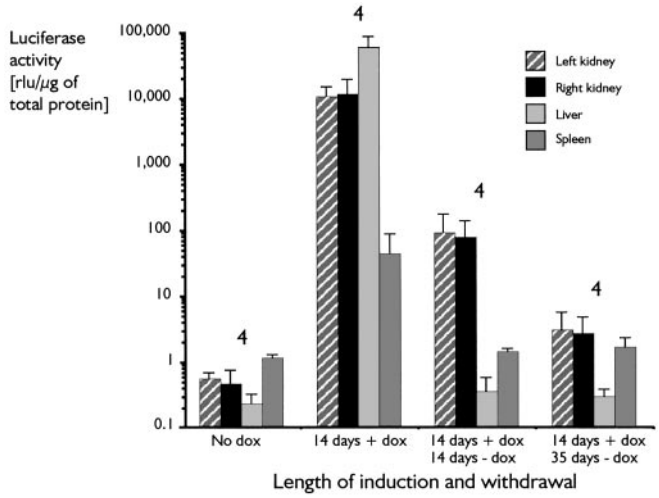

Figure 5. Persistence of reverse transactivator activity. Adult double-transgenic mice containing liver activator protein (LAP) promoter/rtTA2^S-S2 and tetracycline-dependent (tet) operator/luciferase expression cassettes were fed doxycycline in the drinking water for 14 d and then transferred to drinking water without doxycycline. It becomes evident that the activation of the reverse transactivator is reversible, but that it takes longer in the kidney than in the liver before the transactivator returns to its uninduced state. Data are given as mean + SD; the number of mice is given above the bars.

expression in proximal tubules, in many cases, not every nucleus in a given tubular profile was blue. This mosaic pattern of *Lac*Z expression has been noted previously (17), but the underlying mechanisms are poorly understood, although it is thought to depend on chromosomal context and transgene copy number (18).

Depending on the kind of study to be conducted, mosaicism represents a more or less serious problem. For example, if one wants to investigate tumor induction, the mosaic inactivation of a tumor suppressor gene upon the induction of Cre recombinase would still permit the development of tumors and might actually be advantageous because it would be possible to perform a genotype-phenotype correlation of normal and malignant tissue in the same organ (the prediction being that in tumors both copies of a tumor suppressor gene are inactivated, whereas in nontransformed tissue one or both alleles are still wild-type). On the other hand, physiologic studies may be severely hindered by mosaicism because wild-type cells would be able to compensate for the cells in which the inactivation event has taken place. Those investigations would therefore only be feasible at a low level of mosaicism.

The rate of induction in the kidney and liver was very fast: we could detect strong reporter gene expression already 1 d after administering doxycycline in the drinking water, which obviously is a convenient method of supplying the chemical. Upon discontinuation of doxycycline, the activity in the liver was reduced to background levels after 14 d of withdrawal. However, transgene expression in the kidney decreased more slowly, reaching background levels after 5-wk withdrawal. The difference between the kidney and liver is most likely the result of the pharmacokinetics of doxycycline in these tissues. Studies with other tetracyclines have demonstrated that higher levels of tetracyclines accumulate in the kidney than in the liver (19,20), which would account for the slower reduction of transgene expression in the kidney compared with the liver. A lower concentration of doxycycline in the drinking water may help to accelerate the return to background levels, although it would entail lower induction levels. The pronounced difference of induction levels between 0.2 and 2 mg/ml of doxycycline in the drinking water (approximately tenfold) was somewhat surprising because *in vitro* the rtTA2^S-S2 reverse transactivator shows full activation already at approximately 1 μg/ml of doxycycline (5). It is likely that the concentration of

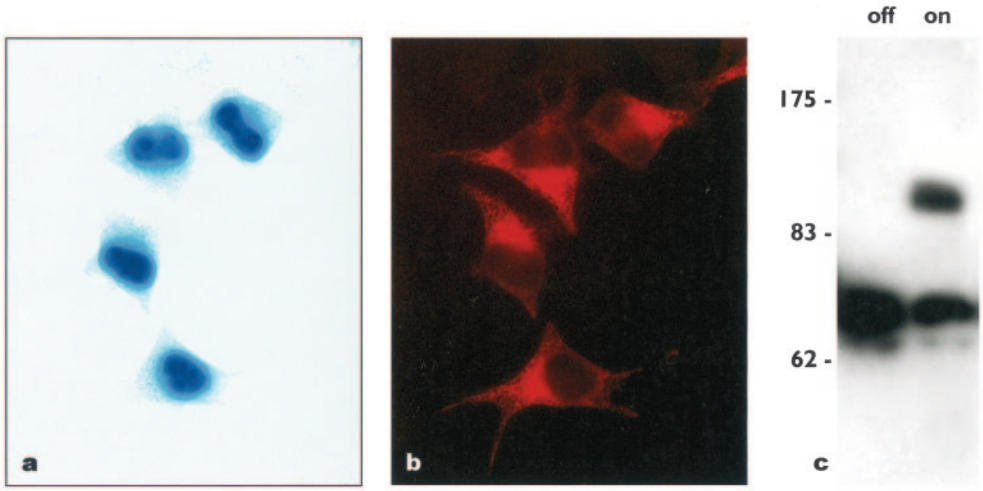

Figure 6. Simultaneous induction of polycystin-2 and β-galactosidase in stably transfected cells. (a through c) A bidirectional expression cassette consisting of the human PKD2 cDNA and a *nLac*Z gene under control of tetracycline-dependent (tet) operator sequences was stably transfected into HeLa cells producing a tTA. Upon induction, the nuclei of transfected cells stained positive for β-galactosidase (a), whereas the HA-tagged polycystin-2 protein was detected in the cytoplasm (b). In the case of polycystin-2, this immunocytochemical finding was subsequently confirmed by Western blot analysis (c; molecular weights are given in kDa).

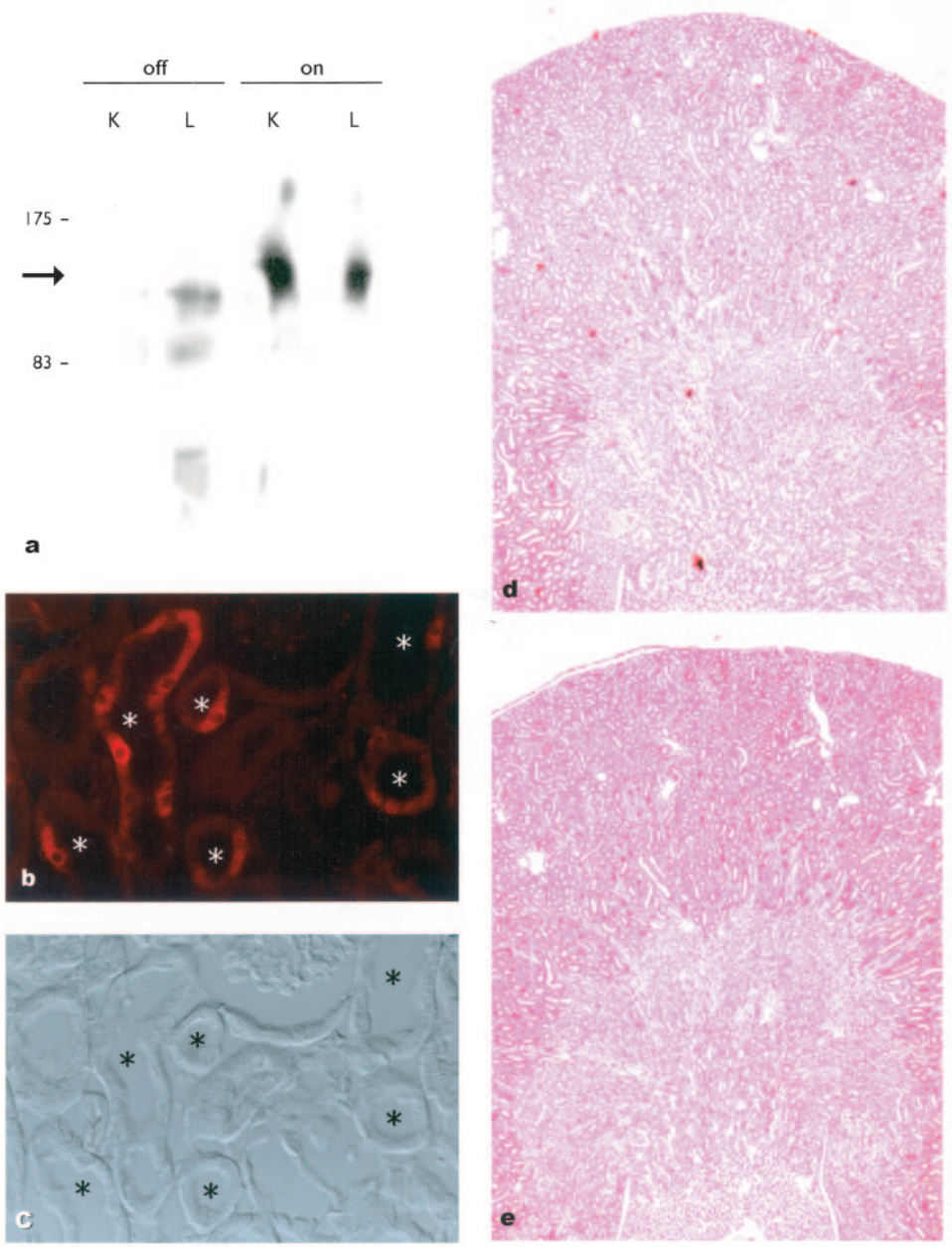

Figure 7. Inducible expression of the human PKD2 cDNA in the rTA^{LAP}-1 mouse line. The bidirectional PKD2/*nLacZ* expression cassette was used to establish transgenic mice, which were then mated with mice containing the reverse transactivator under control of the liver activator protein (LAP) promoter. (a) Western blot analysis was performed with 50 μg of protein prepared from kidney and liver. Only in those mice receiving doxycycline in the drinking water ("on"), polycystin-2 (arrow) can be detected in both the kidney (K) and the liver (L); without doxycycline ("off"), neither organ produces polycystin-2 (the numbers on the left indicate the molecular weight in kDa). (b, c) When kidney sections from double-transgenic mice that had been fed doxycycline in the drinking water were subjected to immunohistochemistry with the anti-HA epitope antibody, polycystin-2 was detected in cortical tubular profiles. Nomarski optics were used to identify these profiles as proximal tubules through the presence of their brush border (asterisks in b and c). (d, e) PAS staining of kidney sections from double-transgenic mice reveals no morphologic changes to nontreated mice (d) even after exposure to doxycycline for 6 mo (e).

doxycycline in the serum and extracellular fluid is much lower than that in the drinking water, possibly in the nanogram per milliliter range, but direct measurements would be necessary to make precise statements. If this explanation is correct, then the intraperitoneal administration of doxycycline may prove advantageous because the issue of uptake via the intestine is eliminated. Clearly, however, data obtained *in vitro* have to be transferred very cautiously to experiments with animals. On the other hand, our withdrawal study illustrates the advantage of the tet-on system in animals. If we had used the original tet-off system, a rapid induction after withdrawal of tetracycline would have been virtually impossible.

Consistent with previous observations that doxycycline can pass readily through the placental barrier (21), we noticed transgene activation in kidneys and livers of newborn mice. It is obvious, however, that sufficient levels of doxycycline do not pass through the mothers' milk, an interpretation based on the only very moderate reporter gene activity at 7 and 14 d postpartum. Therefore, a sustained induction in the postnatal period has to be obtained by intraperitoneal administration of doxycycline.

Finally, we made use of the rTA^{LAP}-1 mouse line to inducibly produce human polycystin-2 in the kidney. Similar to what had been described for a human *PKD1* transgene (22), we had hoped that the expression of the human PKD2 cDNA would lead to the development of cysts. Even after 6 mo, however, no cysts were observed. There may be several explanations for such a result. First, we did not reach high enough levels of polycystin-2 to induce cystogenesis. Second, there is no critical level above which wild-type polycystin-2 would lead to negative effects. Third, expression of the PKD2 cDNA in other nephron segments and not only in the S1 and S2 portions of the proximal tubule would have resulted in cysts. And fourth, as a result of the species difference (we used the human PKD2 cDNA), the cascade toward cyst formation was not turned on. At this point, it is not possible to determine what explanation is the correct one, but it could very well be a combination of these factors.

Acknowledgments

We thank Frank Zimmermann and Sascha Dlugosz for performing the microinjections to establish the transgenic mice. We thank Rainer Beck for his assistance in genotyping the mice and Dr. Mazahir Hasan for his scientific input. We acknowledge the technical expertise of Hiltraud Hosser, Ingrid Hartmann, and Brunhilde Hähnel, and the graphic work of Ton Maurer. The PKD2 cDNA was a kind gift of Stefan Somlo. Financial support was provided by the European Union (QLRT-2000-01104). Part of this work was presented at the 2002 meeting of the American Society of Nephrology in Philadelphia, Pennsylvania.

References

1. Gossen M, Bujard H: Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 89: 5547–5551, 1992
2. Gossen M, Freundlieb S, Bender G, Müller G, Hillen W, Bujard H: Transcriptional activation by tetracyclines in mammalian cells. *Science* 268: 1766–1769, 1995
3. Kistner A, Gossen M, Zimmermann F JJ, Ullmer C, Lübbert H, Bujard H: Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc Natl Acad Sci U S A* 93: 10933–10938, 1996
4. Furth PA, St. Onge L, Böger H, Gruss P, Gossen M, Kistner A, Bujard H, Hennighausen L: Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc Natl Acad Sci U S A* 91: 9302–9306, 1994
5. Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W: Exploring the sequence space for tetracycline-dependent transcriptional activators: Novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* 97: 7963–7968, 2000
6. Schönig K, Bujard H: Generating conditional mouse mutants via tetracycline-controlled gene expression. *Methods Mol Biol* 209: 69–104, 2003
7. Puttini S, Beggah AT, Ouvrard-Pascaud A, Legris C, Blot-Chabaud M, Farman N, Jaisser F: Tetracycline-inducible gene expression in cultured rat renal CD cells and in intact CD from transgenic mice. *Am J Physiol Renal Physiol* 281: F1164–F1172, 2001
8. Hasan MT, Schönig K, Berger S, Graewe W, Bujard H: Long-term, non-invasive imaging of regulated gene expression in living mice. *Genesis* 29: 116–122, 2001
9. Schönig K, Schwenk F, Rajewsky K, Bujard H: Stringent doxycycline dependent control of CRE recombinase *in vivo. Nucleic Acids Res* 30: e134, 2002
10. Baron U, Freundlieb S, Gossen M, Bujard H: Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res* 23: 3605–3606, 1995
11. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K: *Current Protocols in Molecular Biology,* New York, Wiley, 1996
12. Zor T, Selinger Z: Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. *Anal Biochem* 236: 302–308, 1996
13. Dong Y, Skoultchi AI, Pollard JW: Efficient DNA transfection of quiescent mammalian cells using poly-L-ornithine. *Nucleic Acids Res* 21: 771–772, 1993
14. Talbot D, Descombes P, Schibler U: The 5' flanking region of the rat LAP (C/EBPβ) gene can direct high-level, position-independent, copy number–dependent expression in multiple tissues in transgenic mice. *Nucleic Acids Res* 22: 756–766, 1994
15. Contag CH, Spilman SD, Contag PR, Oshiro M, Eames B, Dennery P, Stevenson DK, Benaron DA: Visualizing gene expression in living animals using a bioluminescent reporter. *Photochem Photobiol* 66: 523–531, 1997
16. Mansuy IM, Winder DG, Moallem TM, Osman M, Mayford M, Hawkins RD, Kandel ER: Inducible and reversible gene expression with the rtTA system for the study of memory. *Neuron* 21: 257–265, 1998
17. Montoliu L, Chávez S, Vidal M: Variegation associated with *Lac*Z in transgenic animals: A warning note. *Transgenic Res* 9: 237–239, 2000
18. Ramírez A, Milot E, Ponsa I, Marcos-Gutiérrez C, Page A, Santos M, Jorcano J, Vidal M: Sequence and chromosomal context effects on variegated expression of keratin 5/lacZ constructs in stratified epithelia of transgenic mice. *Genetics* 158: 341–350, 2001
19. Serrano JM, Moreno L, Rosado I, Guimera E, Escudero E: Biliary elimination kinetics and tissues concentrations of oxytetracycline after intravenous administration in hens. *J Vet Pharmacol Ther* 22: 148–152, 1999
20. Craigmill AL, Holland RE, Robinson D, Wetzlich S, Arndt T: Serum pharmacokinetics of oxytetracycline in sheep and calves and tissue residues in sheep following a single intramuscular injection of long-acting preparation. *J Vet Pharmacol Ther* 23: 345–352, 2000
21. Shin MK, Levorse JM, Ingram RS, Tilghman SM: The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature* 402: 496–501, 1999
22. Pritchard L, Sloane-Stanley JA, Sharpe JA, Aspinwall R, Lu W, Buckle V, Strmecki L, Walker D, Ward CJ, Alpers CE, Zhou J, Wood WG, Harris PC: A human *PKD1* transgene generates functional polycystin-1 in mice and is associated with a cystic phenotype. *Hum Mol Genet* 9: 2617–2627, 2000