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

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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a virtually complete remission of transgene activity in both the kidney and liver was observed when doxycycline was withdrawn.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 (rTA), 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 rTA systems; induction kinetics, however, were slower with the rTA 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 (rTA^{S}-S2), which reaches the same activation levels as the original rTA, 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 (rTA^{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-
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 nLacZ 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 PsI and SalI sites of the pbI3 plasmid (10).

After the resulting construct was linearized with AseI, 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 nLacZ and PKD2 cDNA by PCR. The following oligonucleotide pairs were used: 5'-CAAACTCATCGAAGTGACCAG-3' and 5'-CAATTTAACCGCCACTC-3' to amplify a 388-bp fragment of the CATCGAAGTGACCAG-3' and 5'-CAATTTAACCGCCACTC-3' sites of the pBI3 plasmid (10).

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 MgCl2, 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 Vectorstain ABC kit instructions (Vector Laboratories, Burlington, 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

HT1A-1 cells, which are HeLa cells producing tTA, were stably transfected with the AseI-linearized pbI3 plasmid containing the full-length PKD2 cDNA and the nLacZ 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

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 nLacZ and luciferase transgenes, 10 μg of genomic DNA was digested with BamHI and hybridized with the respective fragments; to detect the reverse transactivator transgene, genomic DNA was digested with EcoRI.

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 of-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 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
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 MgCl₂, 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, rTA¹LAP-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 rTA¹LAP-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.
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).

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

<table>
<thead>
<tr>
<th>Site</th>
<th>No Doxycycline</th>
<th>Doxycycline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal gland</td>
<td>22.0</td>
<td>302.5</td>
</tr>
<tr>
<td>Brain</td>
<td>8.7</td>
<td>72.3</td>
</tr>
<tr>
<td>Heart</td>
<td>13.2</td>
<td>241.3</td>
</tr>
<tr>
<td>Intestine</td>
<td>16.7</td>
<td>812.9</td>
</tr>
<tr>
<td>Left kidney</td>
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<td>255,382.0</td>
</tr>
<tr>
<td>Right kidney</td>
<td>5.5</td>
<td>207,783.1</td>
</tr>
<tr>
<td>Liver</td>
<td>10.4</td>
<td>32,075.5</td>
</tr>
<tr>
<td>Lung</td>
<td>15.3</td>
<td>91.7</td>
</tr>
<tr>
<td>Pancreas</td>
<td>17.4</td>
<td>1397.2</td>
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<tr>
<td>Skeletal muscle</td>
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<tr>
<td>Spleen</td>
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</tr>
<tr>
<td>Testis</td>
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</tr>
<tr>
<td>Thymus</td>
<td>25.1</td>
<td>78.5</td>
</tr>
</tbody>
</table>

a Values are given in relative light units/μg total protein (n = 2 mice without doxycycline, n = 3 mice with doxycycline).
Induction Kinetics of Gene Expression

To test the induction kinetics of the rtTA\(^{1-AP-1}\) transgene in the kidney, female mice containing the LAP/rtTA\(^{2S-S2}\) 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/rtTA\(^{2S-S2}\) 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/rtTA2S-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 nLacZ 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/nLacZ 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 rTA4LAP-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 rTA2S-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
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 4. Activity of the reverse transactivator during development. (a through c) Female mice carrying a liver activator protein (LAP) promoter/rtTA2S-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.
expression in proximal tubules, in many cases, not every nucleus in a given tubular profile was blue. This mosaic pattern of \( \text{LacZ} \) 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 \textit{in vitro} the rtTA2S-S2 reverse transactivator shows full activation already at approximately 1 \( \mu \)g/ml of doxycycline (5). It is likely that the concentration of

\[ \text{Luciferase activity (nM/kg total protein)} \]

\[ \text{Length of induction and withdrawal} \]

**Figure 5.** Persistence of reverse transactivator activity. Adult double-transgenic mice containing liver activator protein (LAP) promoter/rtTA2S-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.

**Figure 6.** Simultaneous induction of polycystin-2 and \( \beta \)-galactosidase in stably transfected cells. (a through c) A bidirectional expression cassette consisting of the human PKD2 cDNA and a nLacZ 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 \( \beta \)-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).
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.

Figure 7. Inducible expression of the human PKD2 cDNA in the rTA \textsuperscript{LAP}-1 mouse line. The bidirectional PKD2/\textit{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).
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\textsuperscript{LAP},1 mouse line to inducibly produce human polycystin-2 in the kidney. Similar to what had been described for a human \textit{PKD1} transgene (22), we had hoped that the expression of the human \textit{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 \textit{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 \textit{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.

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\textbf{References}


