A System for Inducible Gene Expression in Retinal Ganglion Cells

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PURPOSE. To develop a system for inducible gene expression in retinal ganglion cells, Thy1 and ckit promoters were used to direct expression of a second-generation reverse tetracycline transactivator (rtTA2(S)-M2).

METHODS. Transgenic mice were generated that harbor rtTA2(S)-M2 under the control of either the Thy1 or ckit promoter. These animals were crossed with mice transgenic for the LacZ gene downstream of a cassette of tet operator (TRE) binding sites. Induction of the LacZ reporter gene in vivo after either oral or subcutaneous doxycycline administration and in vitro in cultured retinal cells was assessed. To examine induction of a secreted protein, expression of pigment epithelium-derived factor (PEDF) in mice harboring Thy1-rtTA and TRE-PEDF constructs was quantified.

RESULTS. Five of seven Thy1-rtTA lines showed induction with subcutaneous doxycycline: maximum induction in one line (Thy1-C), moderate in one line (Thy1-F), and minimal in three lines. There was no detectable retinal LacZ expression in the absence of doxycycline.11

CONCLUSIONS. A transgenic system for inducible RGC expression has been developed that demonstrates minimal leakiness and significant induction with doxycycline. This system will be useful for several applications. (Invest Ophthalmol Vis Sci. 2005;46:2932–2939) DOI:10.1167/iovs.04-1237

Modulation of gene expression in experimental animals provides a means of studying basic biological processes and mechanisms, developing disease models, and testing new therapeutic strategies. A traditional approach for altering gene expression in the mouse has been the generation of transgenic animals, often driving transgene expression with cell-specific promoters. A wide variety of tissues and cell types within the eye have been studied with this method.1–6

A refinement of this constitutive approach is the use of inducible promoter systems that have the advantage of controlling the timing and level of expression of the transgene. Among several systems developed for this purpose, perhaps the most widely used is the tetracycline (Tet)-inducible system.7,8 The Tet system is based on a fusion of the Escherichia coli tetracycline repressor, which binds intracellular tetracycline or doxycycline, with the transcriptional activation domain of the viral transactivator VP16.6,10–12 The fusion results in a tetracycline-dependent transactivator (tTA), which, in the absence of tetracycline (or doxycycline) constitutively activates expression of transgenes that contain tetracycline response elements (TREs) in their promoter. Addition of tetracycline prevents the binding of the tTA to the TREs, turning off expression (the tet-off system). In contrast, the reverse tetracycline-dependent transactivator (rtTA), which is a mutated form of the tTA, activates gene expression by binding the tetracycline response element (TRE) in the presence of tetracycline (the tet-on system). Levels of induction have been reported with the tTA of as high as 105 over background and with the rtTA of 103 over background.7 A second-generation transactivator, rtTA2(S)-M2, has been described that functions at a 10-fold lower doxycycline concentration than rtTA, is more stable in eukaryotic cells, and has little background expression in the absence of doxycycline.11

Within the eye, the primary use of the tet system has been for the study of the retina. Inducible systems for modulation of retinal gene expression have been developed with both the rhodopsin and IRBP promoters.12 Such constructs have been used to study retinal neovascularization13 and retinal degeneration.14 A tyrosinase promoter-based system was used to analyze chiasmal development in albinism.15 More recently, the VM2Δ2 promoter was used to develop an inducible system for RPE cells, applicable to the study of choroidal neovascularization.16 In a related approach, the tetracycline system has also been used to control expression in viral-based gene transfer methods.17,18

Some success has been obtained in developing transgenic systems for studying and altering gene expression in retinal ganglion cells (RGCs), but success has been limited by the lack of well-described promoters that are both robust and RGC specific. RGC expression has been reported with Thy1,19 ckit,20,21 and Brn3b promoters.22 In a study of more than 25 independently generated Thy1 transgenic lines expressing red,
green, yellow, or cyan fluorescent proteins, it was observed that each line had a unique expression pattern, and specificity of RGC expression was, in general, inversely related to the level of expression. In a transgenic study with the ckit promoter, retinal expression was noted with a 4.2-kb 5′-flanking region promoter fragment, and it was further observed that a 300-bp portion of this fragment was sufficient to direct expression to RGCs. With regard to Brn3b, a 4.5-kb 5′-flanking region of Brn3b demonstrates low efficiency and stringency in directing expression to RGCs, whereas a 13-kb genomic clone successfully directed expression to RGCs among other cells of the retina. The investigation of RGC biology would be significantly advanced by the development of an in vivo, inducible system of gene transcription that is specific for RGCs.

This goal was achieved with the Thy1 and ckit promoters for their ability to direct inducible expression in RGCs.

METHODS

Constructs and Generation of Transgenic Mice

Thy1-rtTA was constructed from the Thy1.2-containing plasmid pUC18-Thy1.2 (a kind gift from Pico Caroni, Friedrich Miescher Institute, Basel, Switzerland). pUC18-Thy1.2 was linearized at its single XhoI site and then treated with alkaline phosphatase. In parallel, rtTA2(S)-M2, measuring 0.75 kb, from plasmid pUHrT62-1 (a kind gift from Hermann Bujard, Zentrum für Molekulare Biologie, Universitàt Heidelberg, Heidelberg, Germany) was PCR amplified with primers containing XhoI, EcRI and HindIII, gel purified as we have been observed to interfere with LacZ expression. The rtTA2(S)-M2 fragment with primers containing NotI and EcoRI was cloned into pCR-II-TOPO, sequenced, digested, and used to generate transgenic mice, as described.

For the ckit-rtTA construct, Prokit-Laz plasmid (a kind gift from Pico Caroni, Friedrich Miescher Institute, Basel, Switzerland) was PCR amplified with primers containing NotI and EcoRI, and cloned into XhoI and EcoRI digested pTRE, a plasmid containing the tetracycline response element (Clontech, Palo Alto, CA). Successful subcloning was confirmed by DNA sequencing at both the 5′ and 3′ ends of PEDF. This plasmid was used to generate transgenic mice, as described. Several founder lines were generated. Offspring from the 211 line were crossed with the Thy1-rtTA line to obtain mice that were doubly heterozygous for Thy1-rtTA and TRE-PEDF.

Mouse Care and Genotype Analysis

All animal studies and procedures were performed in accordance with the Johns Hopkins Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology. Positive founder animals were identified by genotype analysis of DNA prepared from two independent tail clippings per mouse, followed by PCR with two sets of primers: one set within the rtTA region common to both constructs and another specific to the individual promoter regions. Genomic DNA was obtained from a 3- to 4-mm portion of mouse tail that was digested overnight in 0.5 mg/mL proteinase K, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM EDTA, and 2% Triton-X at 55°C. Primers used for rtTA amplification for both lines were forward 5′-GAG AGA CAC CAT CTA CCG AT3′ and reverse 5′-AGC AGG CAA ACT GAA GAG CT-3′. For expression analysis, positive F2 offspring were crossed with a mouse that was heterozygous for PEDF

For induction of PEDF expression, positive F2 offspring were crossed with a reporter line that contains the LacZ gene downstream of TRE. Although this line contains an independent transgene, Ro1, it has not been observed to interfere with LacZ induction in the retina when
used as a reporter for other rtTA animals.12 Primers used to genotype were forward 5'-GGC GTG TAC GGT GGG AGG-3' and reverse 5'-GGG AAG GGC GAT CGG-3'.

**Doxycycline Administration**

The tetracycline derivative doxycycline was administered orally, by subcutaneous injection, or both. Oral doxycycline was administered in the drinking water at a concentration of 3 mg/mL in 5% sucrose for 5 days, followed by death on day 6. Subcutaneous doxycycline was administered in a dose of 0.5 mg/g per day subcutaneously for 3 days followed by death on day 4. For maximum induction, mice were administered oral doxycycline for 5 days and subcutaneous injections on days 2 and 4 with death on day 6.

**LacZ Histochemistry and Immunohistochemistry**

After enucleation and removal of the anterior segment, the retina was detached from the posterior pole and fixed for 5 minutes in 0.5% glutaraldehyde. After a rinse in phosphate-buffered saline (PBS), the retina was incubated in X-gal staining solution (1 mg/mL X-gal, 2 mM MgCl₂, 10 mM K₂Fe(CN)₆, 10 mM K₃Fe(CN)₆·3H₂O in PBS) for 72 hours. A dissecting microscope with a camera attachment was used to take images.

Immediately after enucleation and 5 minutes of fixation in 0.5% glutaraldehyde, eyes were embedded in optimal cutting temperature compound (OCT; Sakura Finetek, Torrance, CA) and snap frozen. Using a cryotome, 10-µm-thick sections were cut and placed on glass slides (Fisher Scientific, Pittsburgh, PA). Before staining, sections were incubated for 5 minutes in 0.5% glutaraldehyde, rinsed with PBS, and incubated in X-gal staining solution at room temperature. For immunohistochemistry, globes were immediately embedded in OCT and snap frozen, sectioned, and postfixed in 4% paraformaldehyde in PBS for 5 minutes followed by two washes in PBS. After incubation in 20% serum, 0.1% Triton, and PBS for 1 hour, overnight incubation was performed in 2% serum, 0.1% Triton, and PBS containing either 1:1500 rabbit anti-β-galactosidase antibody (U.S. Biological, Swampscott, MA) alone or in combination with either 1:1000 goat anti-mouse calretinin

**FIGURE 2.** Retinal flatmounts and optic nerve wholemounts. Weaned mice that harbored both the Thy1-rtTA and the TRE-lacZ transgenes were induced with subcutaneous (SQ) doxycycline injection. Retinas were evaluated in flatmounts with X-gal staining. Five of seven Thy1rtTA lines showed induction: maximum induction in one line (line C), moderate in one line (line F), and minimal in three lines (lines A, B, and E). (A–C) Flatmount and optic nerve from Thy1rtTA/TRE-lacZ (line C). (D–F) Flatmount and optic nerve from Thy1rtTA/TRE-lacZ (line F). (G, H) Flatmount from Thy1rtTA/TRE-lacZ (line A) (lines B and E not shown). (I) No induction was observed in Thy1rtTA/TRE-lacZ (line C) that did not receive doxycycline. Comparison of lacZ induction with oral (K; 3 mg/mL for 6 days) versus SQ injection (L; 0.5 mg/gram SQ for 3 days) was performed in Thy1rtTA/TRE-lacZ mice demonstrating more robust expression with SQ administration.

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antibody (Chemicon International, Temecula, CA) or 1:100 sheep anti-mouse chx10 antibody (Exalpha Biologicals, Inc., Maynard, MA). After three rinses in PBS, incubation for 1 hour was performed with, for double staining, either 1:500 donkey anti-goat Cy3 antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) or 1:500 goat anti-sheep Cy3 antibody (Jackson ImmunoResearch Laboratories) followed by three rinses in PBS and a 1-hour incubation in 1:500 goat anti-rabbit IgG Alexa 488 (Molecular Probes, Eugene, OR). For single staining, only the 1:500 goat anti-rabbit IgG Alexa 488 was used. Imaging was performed with a confocal microscope (510 Meta; Carl Zeiss Meditec, Inc., Thornwood, NY).

Immunopurification of Thy-1-Bearing Cells

For quantification of the number of Thy-1-immunopositive cells that express the transgene, retinas from an induced double transgenic and wild-type mouse were digested and triturated as described in the following paragraph. Immunomagnetic selection of Thy-1-expressing cells was performed by incubating the cell suspension with magnetic microbeads conjugated to Thy-1 antibodies (Miltenyi Biotec, Auburn, CA) followed by passage over a magnetized metal column. Cells bound by the Thy-1 conjugated microbeads were retained within the column, whereas the remaining retinal cells passed through the column. After removal of the column from the magnet, the purified Thy-1 immunoreactive cells were eluted, spun down on a cytospin centrifuge, briefly fixed in 4% paraformaldehyde, and immunostained with 1:1500 rabbit anti-β-galactosidase antibody followed by 1:500 goat anti-rabbit IgG Alexa 488 and Hoechst nuclear stain (Molecular Probes).

Cell Culture

For primary cell culture, retinas were dissected from a weaned mouse harboring both the Thy1-rtTA and TRE-LacZ transgenes and digested with activated papain (Worthington Biochemical Corp., Lakewood, NJ) and DNase (Sigma-Aldrich, St. Louis, MO) before dissociation by trituration. Cells were resuspended in growth medium (Neurobasal medium [Invitrogen], B27 supplement, glutamine [2 mM], and penicillin-streptomycin [1 U/mL], supplemented with brain-derived neurotrophic factor (BDNF, 50 ng/mL; Peprotech, Inc., Rocky Hill, NJ) 2 hours after seeding. Cells were seeded at a density of 5000 cells/well (150/mm²) in 96-well culture dishes (Falcon; BD Biosciences, Bedford, MA) that had been sequentially coated with poly-D-lysine (0.01 mg/mL) and laminin (0.01 µg/µL; Sigma-Aldrich). Seven wells were seeded for each concentration of doxycycline. Two hours after plating, doxycycline was added to final concentrations of 0, 5, 10, and 20 µg/mL. After 3 days in culture, cells were triple stained with the nuclear stain Hoechst 33342 (5 µM; Molecular Probes), the fluorescent β-galactosidase substrate fluorescein di-β-d-galactopyranoside (2 µM; Marker Gene Technologies, Inc., Eugene, OR), and the dead-cell stain Toto-3 (1 µM; Molecular Probes). After a 20-minute incubation and brief rinsing with PBS, the wells were imaged with an automated fluorescence microscope (KineticScan HCS, Cellomics, Inc., Pittsburgh, PA). In a standardized fashion that was identical for each well, 20 fields were imaged on the reader (KineticScan HCS Reader, Cellomics) using epifluorescence and filter sets corresponding to each of the three dyes. Using the target-activation program (Cellomics), those cells with a fluorescence value greater than 200 were considered to be expressing LacZ and were counted. The data were normalized to
counts from wells with cells without doxycycline and compared using a two-tailed t-test.

**Induction of PEDF Expression**

Five-week-old mice doubly heterozygous for Thy1-rtTA and TRE-PEDF were treated with control water or doxycycline. The doxycycline-treated mice received doxycycline (3 mg/ml) in their drinking water for 5 days. In addition, these mice received subcutaneous injection of doxycycline (0.3 μL of 0.5 g/μL doxycycline) at days 2 and 4 after initiation of oral doxycycline. The animals were then killed on day 6.

Retinal PEDF levels were measured in the Thy1-rtTA/TRE-PEDF double heterozygotes as well as nontransgenic littermates. Retinas were removed from both eyes and individually snap-frozen in liquid nitrogen. Total protein was extracted from retinas, and a two-antibody sandwich PEDF ELISA was performed as described.28,29

**RESULTS**

In an effort to develop a system for inducible expression of transgenes in murine RGCs, Thy1 and ckit promoters were used to direct expression of a second-generation rtTA to RGCs. Plasmids harboring Thy1-rtTA and ckit-rtTA (Fig. 1) were used to generate separate lines of transgenic mice. Seven Thy1-rtTA founders and seven ckit-rtTA founders were generated. F2 generation animals were crossed with a TRE-lacZ reporter line.30

Weaned F3-generation mice that harbored both the Thy1-rtTA and the TRE-lacZ transgenes were induced with subcutaneous doxycycline injection. Retinas were evaluated in a flat-mount preparation using X-gal staining. Five of the seven Thy1-rtTA lines showed detectable induction. Maximum expression was observed with the Thy1-C line, less but still moderate expression was observed with the Thy1-F line, and minimal but clearly present expression was seen with lines A, B, and E (Fig. 2). Flatmounts from double heterozygotes (Thy1-rtTA line C/TRE-PEDF or Thy1-rtTA line F/TRE-PEDF) animals that did not receive doxycycline did not demonstrate detectable LacZ expression.

Even with maximum doxycycline induction, none of the ckit-rtTA lines demonstrated detectable expression in flatmounts (data not shown). Of note, evaluation using reverse transcription-PCR and transgene-specific primers demonstrated RNA expression of the ckit-rtTA transgene in the retina of induced mice despite this lack of detectable lacZ expression (data not shown). The reason for the dichotomy between rtTA RNA expression and β-galactosidase activity remains unclear.

β-Galactosidase expression as assessed by LacZ histochecmistry in the Thy1-rtTA/TRE-lacZ animals tended to be evenly dispersed throughout all quadrants of the retina (Fig. 2). Furthermore, the pattern of expression was distributed evenly in the peripapillary and peripheral retina. Both large and small cell bodies stained intensely with the reaction product. Staining was apparent in the dendritic arbors of several cells in addition to their axons within the retina and optic nerve. Histologic sections demonstrated staining of dispersed RGCs and their axons within the nerve fiber layer (Fig. 3). In addition to staining of RGCs, labeling of some inner nuclear layer cells was also observed. Immunostaining for β-galactosidase showed more extensive staining of cells in the ganglion cell layer and inner aspect of the inner nuclear layer (Fig. 4). Double labeling with anti-calretinin antibody showed that some of the cells at the inner aspect of the inner nuclear layer were consistent with amacrine cells (Fig. 4).47 Estimation of the number of cells bearing the Thy1 epitope that expressed the induced transgene was performed by immunomagnetic purification of Thy1-positive cells and immunostaining for β-galactosidase (Fig. 5).

This demonstrated that more than 95% of Thy-1-positive cells expressed the induced transgene.

Comparison of β-galactosidase induction with oral (3 mg/ml for 6 days) versus subcutaneous injection (0.5 mg/g subcutaneous for 3 days) was performed (Figs. 2K, 2L). The mice in general rejected higher concentrations of doxycycline in their drinking water despite 5% sucrose. Subcutaneous doxycycline resulted in a more robust induction of β-galactosidase than that seen with oral administration.

β-Galactosidase serves as a model for the inducible expression of an intracellular protein. In some cases, it may be desirable to induce expression of a secreted molecule. To examine induction of such a secreted molecule and better quantify the level of induction, we examined PEDF as a model system. Mice doubly heterozygous for Thy1-rtTA (line C) and TRE-PEDF were treated with control water or doxycycline, administered orally, and supplemented with two subcutaneous injections. Retinal PEDF levels were measured in these mice as well as in nontransgenic littermates. As shown in Table 1, noninduced Thy1-rtTA/TRE-PEDF mice
had levels of PEDF per unit protein that were similar to those in nontransgenic littermates. In contrast, Thy1-rtTA/PEDF mice induced with doxycycline demonstrated a substantial increase in their retinal PEDF expression, approximately 1000-fold over nontransgenic animals and 600-fold over noninduced control subjects.

Because of the ability to culture and study RGCs in vitro,32-33 it is easy to envision experimental paradigms in which one might want to induce gene expression in culture. We therefore tested the ability to induce transgene expression in retinal cells cultured from Thy1-rtTA (line C)/TRE-lacZ mice (Fig. 5). Retinal cultures were performed without doxycycline and with three increasing concentrations of doxycycline. After 72 hours in culture, cells were imaged in the presence of a fluorescent substrate for β-galactosidase to quantify the number of cells expressing LacZ. The number of cells above our defined threshold in the noninduced wells was 0.36% (range 0%-0.78%). Treatment with doxycycline increased the number of cells expressing LacZ. The number of cells above our defined threshold in the noninduced wells was 0.36% (range 0%-0.78%). Treatment with doxycycline increased the number of positive cells that was 3.4 times that of background, corresponding to approximately 1% of total cells.

**DISCUSSION**

We have developed an inducible system for RGC gene expression in transgenic mice. The system offers the ability not only to control the timing of expression but also to modulate it in a dose-dependent manner. It shows low levels of background expression and significant induction in vivo, for both intracellular and secreted proteins, and in vitro as assessed in terms of the population of cells induced. Although expression was predominantly in RGCs as evidenced by nerve fiber layer and optic nerve staining, it was not totally RGC-specific as shown by the finding of some labeling of cells in the inner nuclear layer. This pattern of staining was similar to that of Feng et al.19 By LacZ histochemistry, only a subpopulation of RGCs was labeled, representing a fraction of the overall population of cells in the retina. Nevertheless, despite the limited subpopulation of RGCs labeled, induction of PEDF was almost 1000-fold over nontransgenic littermates. This finding is in keeping with the expected degree of induction with the tet-on system in which levels of induction of 103 over background have been observed.7 As the strong induction with PEDF did not appear consistent with the limited induction with LacZ histochemistry, we performed immunostaining with antibodies to β-galactosidase and observed more extensive expression, which we estimated to involve more than 95% of Thy-1 cells. As LacZ histochemistry requires the formation of the active β-galactosidase tetramer, higher concentrations of subunits may be needed to form the tetramer compared with the amount needed for detection by immunohistochemistry. We suspect that the degree of expression was only high enough to form the tetramer in a limited population of cells.


demonstrating in vitro dose-related induction allowed us to quantify the population of cells that were induced, but the particular benefit of this system is the ability to modulate gene expression in vivo with minimal manipulation and

**Table 1. In Vivo Induction of PEDF**

<table>
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<tr>
<th>Treatment</th>
<th>Eye</th>
<th>PEDF (ng/mL)</th>
<th>Total Protein (µg/mL)</th>
<th>PEDF/Total Protein (ng/µg)</th>
<th>Avg PEDF/Total Protein (ng/µg)</th>
<th>(x-fold) under Induction</th>
<th>t-Test Compared with NT</th>
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<tr>
<td>Nontransgenics (NT)</td>
<td>0 mg/mL</td>
<td>RE</td>
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<td>LE</td>
<td>0.83168</td>
<td>886.1</td>
<td>0.0009386</td>
<td>0.0007744</td>
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<td>0.0002223</td>
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Mice doubly heterozygous for Thy1-rtTA (line C) and TRE-PEDF were administered water or oral doxycycline, supplemented with subcutaneous injection. These animals, as well as noninduced, nontransgenic littermates, were evaluated for PEDF expression by ELISA. The Thy1-rtTA/PEDF mice treated with doxycycline demonstrated 1000-fold induction in their retinal PEDF expression, in comparison with nontransgenic mice, and 600-fold induction over noninduced Thy1-rtTA/PEDF mice.
toxicity. We anticipate this inducible system to be useful in studies intending to modify in vivo gene expression in RGCs or the inner retina. Such studies might include morphologic analysis in which labeling a subpopulation of cells is of particular benefit,34 new animal models of disease, modulating gene expression in response to injury, and testing new neuroprotective strategies. For example, the ability to express growth factors in the inner retina may be useful in efforts to promote RGC survival as well as in studies with oxygen-induced models of retinal neovascularization involving the superficial retina. The level of induction is sufficient for assessment of gene expression change as well as levels of protein expression.

The choice of promoter to place upstream of the rtTA is critical to the pattern of expression. The Thy1 promoter was reliable in directing expression to RGCs and the inner retina. The exploration of other potential promoters that might direct expression more exclusively to RGCs would be worthwhile.

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References


