A GFP-equipped bidirectional expression module well suited for monitoring tetracycline-regulated gene expression in mouse

H. E. Krestel, M. R. Mayford[1], P. H. Seeburg and R. Sprengel*

Department of Molecular Neurobiology, Max-Planck-Institute for Medical Research, Jahnstrasse. 29, D-69120 Heidelberg, Germany and [1]Department of Cell Biology, Scripps Research Institute, La Jolla, CA 92037, USA

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ABSTRACT

Doxycycline (Dox)-sensitive co-regulation of two transcriptionally coupled transgenes was investigated in the mouse. For this, we generated four independent mouse lines carrying coding regions for green fluorescent protein (GFP) and β-galactosidase in a bicistronic, bidirectional module. In all four lines the expression module was silent but was activated when transcription factor tTA was provided by the α-CaMKII-tTA transgene. *In vivo* analysis of GFP fluorescence, β-galactosidase and immunochemical stainings revealed differences in GFP and β-galactosidase levels between the lines, but comparable patterns of expression. Strong signals were found in neurons of the olfactory system, neocortical, limbic lobe and basal ganglia structures. Weaker expression was limited to thalamic, pontine and medullary structures, the spinal cord, the eye and to some Purkinje cells in the cerebellum. Strong GFP signals were always accompanied by intense β-galactosidase activity, both of which could be co-regulated by Dox. We conclude that the tTA-sensitive bidirectional expression module is well suited to express genes of interest in a regulated manner and that GFP can be used to track transcriptional activity of the module in the living mouse.

INTRODUCTION

Currently, the tetracyline (Tet)-regulated expression system is the only system for reliable reversibly controlled gene expression in the mouse (1–6). It is based on two components (7): First, a Tet-dependent transcription activator (tTA), which is a fusion between the Tet repressor of transposon TN10 and transcription factor binding domains of the herpes simplex protein VP16, and secondly, a tTA-responsive promoter, composed of seven Tet repressor binding sites ($TetO_7$) immediately upstream of an RNA polymerase II transcriptional start site of the cytomegalovirus IE promoter (CMVm).

When both elements are present in the cell, tTA binds to $TetO_7$ and activates transcription at its neighboring initiation site. By flanking $TetO_7$ with two CMVm fragments, Baron *et al.* (8) modified the tTA responsive promoter for co-expression of two facing transcription units. Both are co-regulated by tTA as demonstrated in cell culture for coupled luciferase and β-galactosidase (8). Next we analyzed the bidirectional promoter in genetically manipulated mice. We replaced luciferase with green fluorescent protein (GFP) and compared GFP and β-galactosidase expression level, pattern and its Tet regulation. Activation of the tTA responsive promoter was achieved by the α-CaMKII-tTA transgene of line $Tg^{CaMKIItTA}$ (5). In this mouse, a promoter fragment of the α-subunit gene of calcium–calmodulin dependent kinase II (α-CaMKII) determines the expression of tTA.

Our results indicate that the tTA-sensitive bidirectional transgenes yield tTA-responsive mice with high efficiency. In all lines analyzed, the expression levels of the transcriptionally coupled reporters were tTA dependent and could be regulated by Doxycycline (Dox). The expression levels of reporters were dependent on the chromosomal integration site, whereas the expression pattern seemed integration-independent. With α-CaMKII-tTA as activator, reporter expression was induced in many different cell types and mosaic expression was observed in homogeneous neuronal populations. Animals with high reporter gene expression died young.

MATERIALS AND METHODS

Animal experiments

Animal care was in compliance with the institutional guidelines at the animal facility of the Center for Molecular Biology (ZMBH, INF 282, D-69120 Heidelberg, Germany). Manipulations of the mouse embryos were performed according to a licence (37-9185.81/35/97) of the Regierungspräsidium Karlsruhe, Germany.

Preparation of the DNA transgene

The humanized GFP expression unit together with a simian virus 40 (SV40) splice donor–splice acceptor intron and polyadenylation signal was released by *Xho*I from plasmid pTR-UF2 (9). The 1123 bp fragment was first inserted into pBluescriptII, then released by partial *Sal*I/*Pst*I digestion and cloned into pUB-2A, a modified version of the *lacZ* gene containing plasmid pBI-1 (8), to create plasmid pGFPlacZ. The final

*To whom correspondence should be addressed. Tel: +49 6221 486101; Fax: +49 6221 486110; Email: sprengel@mpimf-heidelberg.mpg.de

plasmid pGFPlacZ contained $TetO_7$ flanked by two CMVm promoters and encoded β-galactosidase and the GFP.

Generation of transgenic mice

The bidirectional minigene was released as an *Ase*I fragment of 7691 bp from plasmid pGFPlacZ and purified by sucrose density gradient centrifugation in a Beckman LE-80K using an SW41 TI rotor (10–40 % sucrose, 35 000 r.p.m., 16 h at 15°C). In fractions containing the GFPlacZ minigene, sucrose was removed by Amicon Microcon 50 filters. Microinjection of the purified DNA into C57B16/DBA hybrid mice-derived pronuclei of fertilized oocytes and transplantation of the oocytes to the oviducts of pseudopregnant NMRI outbred foster mothers were performed as described (10).

Mice that carried the transgene were identified by PCR of genomic mouse tail DNA using primer hGFP13 (GCG GAG AGG GTG AAG GTG ATG C) and hGFP12 (CAG GGC CGT CGC CGA TTG G), which amplified a 489 bp DNA fragment specific for the GFP transgene. To activate the tTA-dependent promoter, transgenic mice were bred with $Tg^{CaMK2tTA}$ mice (5). In offspring, the presence of the α-CaMKII-tTA transgene was detected with primer pair tTA1 (GTG ATT AAC AGC GCA TTA GAG C) and tTA4 (GAA GGC TGG CTC TGC ACC TTG GTG) amplifying a 534 bp DNA fragment. Litters were screened at P1 for GFP fluorescence, visualized through the skull of living pups, using a fluorescence stereo-microscope (Leica MZ FLIII, Chroma excitation filter HQ470/40× and emission filter HQ 500LP; Leica Bensheim, Germany). Alternatively, newborn pups were placed in the visualization chamber of a Luminescent Image Analyser (LAS-1000, Fuji Photo Film Co., Ltd, Japan). Blue light excited GFP fluorescence through the skull, which could be recorded digitally (GFP emission filter FL-Filter Y515-Di in front of a CCD camera; Fujifilm, Japan).

Southern blot

Copy numer was determincd by Southern blot of genomic DNA, isolated out of the liver of double transgenic mice ($Tg^{CaMK2tTA/GFPtetO7lacZ}$). On each lane ~10 µg of *Sca*I-digested DNA was separated and transferred onto nitrocellulose (Schleicher and Schuel, Germany). After transfer the membrane was probed with a PCR-generated α-^{32}P-labeled fragment containing the GFP coding area. The position of the probe was visualized in a Bio-Imaging Analyzer BAS-1800 (Fuji Photo Film Co., Ltd, Japan) and quantified by appropriate software (Image Reader V1.4E and Image Gauge V3.0).

Immunohistochemistry

Mice were anesthetized with halothane (Hoechst, Frankfurt, Germany) and sacrificed. Brains were removed and postfixed for 2 h in phosphate-buffered saline (PBS) pH 7.4 containing 4% paraformaldehyde, at 4°C. Then, the brains were rinsed in PBS, embedded in 2% agarose in PBS and were cut coronally in 100 µm sections on a vibratome (Leica VT 1000S; Leica Instruments, Nussloch, Germany). Slices were immunostained for β-galactosidase using a modification of the procedure described previously (11). Briefly, slices were incubated for 1 h in day 1 buffer [PBS supplemented with 1% (w/v) bovine serum albumin (BSA) and 0.3% (v/v) Triton X-100] plus 2% normal goat serum. Subsequently, slices were incubated overnight in day 1 buffer containing the anti β-galactosidase rabbit IgG (1:8000; ICN, Eschwege, Germany). The next day, slices were washed twice in day 2 buffer (PBS with 0.3% BSA and 0.1% Triton X-100), incubated for 1 h in day 2 buffer containing Texas Red-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch, West Grove, PA), washed twice in day 2 buffer, twice in PBS, and then mounted on glass slides using an aqueous mounting medium (Mowiol 4.88; Polysciences, Warrington, PA). Coverslips suitable for confocal laser microscopy (0.17 ± 0.01 mm; Assistent Nr 1014, Sondheim/Rhön, Germany) were used.

An overview of immunostained slices was obtained with a fluorescence stereo-microscope (see above) equipped with a GFP filter set (MZ FL III). Details of brain regions were recorded with an upright fluorescence microscope (Axioplan2; Carl Zeiss, Göttingen, Germany) equipped with Zeiss filter set 10 (excitation filter BP 450–490, dichroic mirror FT 510 and emission filter BP 515–565) and filter set 15 (excitation filter BP 546/12, dichroic mirror FT 580 and emission filter LP 590).

Confocal laser scanning microscopy and image analysis

A Leica TCS-NT laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany) with a PLAPO 63X/1.4 oil immersion objective was used. An argon/krypton laser excited GFP at 488 nm and Texas Red at 568 nm. Emitted fluorescence was filtered using 515–550 nm bandpass and 590 nm longpass filters, respectively. The brain slices were scanned at x/y/z voxel size settings of 300/300/120 nm. Each plane of the image stack was recorded simultaneously for the red and green channel. Two corresponding planes of each channel were overlayed for co-localization analysis.

Reporter positive and negative cells were quantified for GFP and fluorescently labeled β-galactosidase in defined regions of the pyramidal cell layer in CA1. Both confocal and high magnification fluorescent images were used for counting.

β-galactosidase staining

Expression of β-galactosidase was visualized in brain slices after incubating in X-Gal staining solution [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ and 2 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) in dimethylformamide/PBS] in the dark at room temperature for 2 h. Subsequently, slices were washed twice in PBS, mounted, counterstained in eosine and embedded in the xylene-containing mounting medium Eukitt (O. Kindler GmbH, Freiburg, Germany).

Regulation of the Tet system with Dox

Doxycycline hydrochloride (Sigma-Aldrich, Deisenhofen, Germany) at a concentration of 20 mg/l, supplemented with 1% sucrose, was dissolved in water and provided to the parental mice in light-protected bottles. GFP and β-galactosidase levels were downregulated from conception until postnatal day 21 (P21). At P21 Dox was removed to induce GFP and β-galactosidase expression. The mice were analyzed at P42.

Immunoblotting

Isolated forebrains were homogenized in 25 mM HEPES pH 7.4 containing a cocktail of protease inhibitors (Complete™; Boehringer Mannheim, Germany) and kept on ice for 10 min. The homogenate was centrifuged for 5 min at 2000 r.p.m. (Biofuge fresco; Heraeus, Germany). The supernatant was

centrifuged again for 30 min at 13 000 r.p.m. The amount of total protein was determined in the supernatant (BCA Protein Assay Kit; Pierce, Rockford, IL). For each sample, 10 µg of total protein in 20 µl 1× SDS gel-loading buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was resolved on an 8% SDS–polyacrylamide gel for probing β-galactosidase. For probing GFP, a 12% SDS–polyacrylamide gel was used. Subsequently, proteins were transferred to nitrocellulose membranes and incubated with polyclonal anti-GFP (1:4000; Molecular Probes, Göttingen, Germany) and polyclonal anti β-galactosidase (1:10 000; ICN, Eschwege, Germany), followed by horseradish peroxidase-linked anti-rabbit secondary antibodies. The enhanced chemoluminence method (ECL, Amersham, Buckingamshire, UK) was applied to detect the secondary antibodies. To verify that equal amounts of protein were loaded in each lane, the nitrocellulose membranes were probed a second time with a monoclonal antibody against β-actin (1:20 000; Sigma, Deisenhofen, Germany).

RESULTS

tTA-regulated GFP and β-galactosidase expression in four transgenic lines

The bidirectional expression module GFPtetO$_7$lacZ, containing transcription units for GFP and β-galactosidase, was used to generate six independent transgenic mouse lines Tg^{GFPtetO7lacZ} (Fig. 1A). In the brains of two lines, neither GFP nor β-galactosidase was expressed whereas in four lines (G3, 7, 8 and 9), GFP and β-galactosidase were detected only in the presence of tTA, which was provided by the α-CaMKII-tTA transgene. In heads of double-transgenic Tg$^{CaMK2tTA/GFPtetO7lacZ}$ living pups, GFP fluorescence displayed the olfactory epithelium, the olfactory bulb and the cortex. This permitted rapid identification of double transgenic animals up to 5 days after birth (Fig. 1B). It demonstrated functional activity of the α-CaMKII-tTA transgene already in newborn mice (Fig. 1B) and indicated different reporter levels in lines G3, 7, 8 and 9 with lowest levels in mice of line G8. The reporter expression level was not correlated in a linear manner with the relative copy number of the transgene, which was found in Southern blots to be 2:2:1 between the lines G3, G9 and G8.

In low expressing mice G8, coronal brain sections showed β-galactosidase in striatal neurons and in some dispersed hippocampal and cortical cells (Fig. 2B). In lines G3, 7, and 9, GFP and β-galactosidase activity were intense in the cortex, olfactory system, limbic lobe and basal ganglia structures (Fig. 2B) and weak in thalamic, pontine and medullary structures, the spinal cord and the eye.

Thus, bidirectional transgenes suffer from strong expression variability, as do other transgenes, including the unidirectional Tet-responders (5), which is presumably due to integration site-specific effects.

Co-regulation of GFP and β-galactosidase expression in the mouse

The co-localization of GFP and β-galactosidase observed in the histological analysis of coronal brain sections (Fig. 2B) suggested that both reporters are co-regulated. This was verified by GFP and β-galactosidase immunoblots (Fig. 2A),

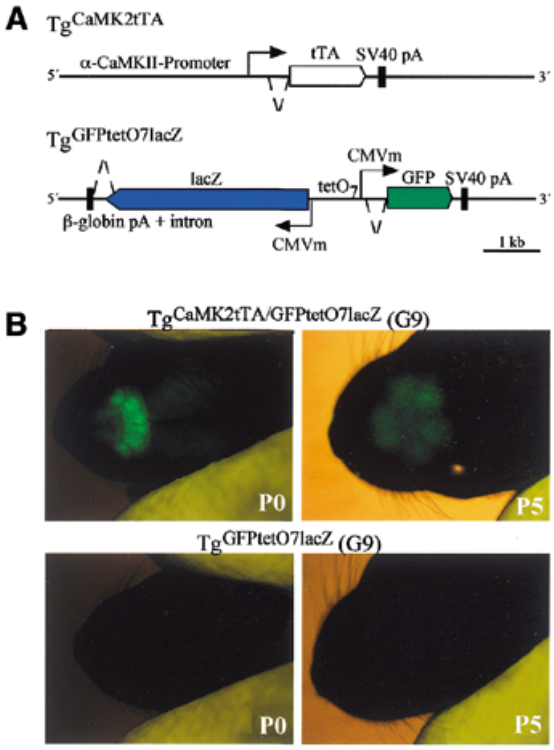

Figure 1. α-CaMKII-promoter controlled GFP expression in the mouse. (**A**) Schematic drawing of transgenes for regulated GFP and β-galactosidase expression. The upper line shows the tTA activator transgene from line Tg^{CaMK2tTA} and the lower line describes the tTA-responsive minigenes used to produce lines G3, 7, 8 and 9 (Tg^{GFPtetO7lacZ}). CMVm shows the position of transcriptional start sites after tTA has bound to the TetO$_7$. Dashed lines, intronic sequences; arrows, transcriptional starts, black boxes, transcriptional stops; open arrows, open reading frames. (**B**) Stereomicroscopic identification of GFP expressing Tg$^{CaMK2tTA/GFPtetO7lacZ}$ mice compared to non-activated Tg^{GFPtetO7lacZ} living pups of line G9 at P0 and P5. The overview shows GFP fluorescence in regions of the olfactory epithelium, the olfactory bulb and the cortex. At P5, exposure time was increased 4-fold to monitor GFP fluorescence.

which showed that high GFP levels were accompanied by strong β-galactosidase signals in the brain of α-CaMKII-tTA activated Tg^{GFPtetO7lacZ} mice. Thus, it looks like both transcriptional start sites of the bidirectional promoter are activated simultaneously by tTA. The immunoblot further confirmed that lines G3 and G9 achieve highest GFP and β-galactosidase levels, followed by lines G7 and G8 (Fig. 2A).

To take the co-localization analysis of GFP and β-galactosidase to a cellular level, we studied the hippocampus and especially the CA1 pyramidal cell layer by regular and confocal microscopy. Texas Red immuno-labeled β-galactosidase was visualized together with GFP fluorescence in coronal brain sections of α-CaMKII-tTA activated Tg^{GFPtetO7lacZ} mice (Fig. 3). A mosaic pattern was observed, with CA1 pyramidal neurons negative for both GFP and β galactosidase scattered among fluorescent CA1 cells labeled by both reporters. Depending on the responder line, 30 (G3) to 70% (G8) of CA1 neurons did not express GFP or β-galactosidase (Table 1). Reporter positive CA1 pyramidals show different intensities for both GFP and β-galactosidase (Fig. 3), which indicates that differences in tTA-induced reporter gene

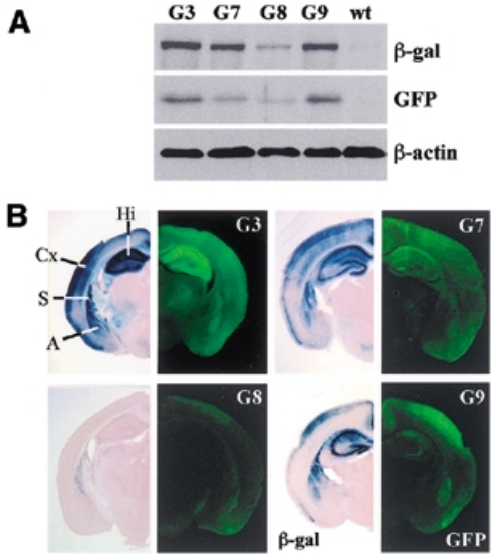

Figure 2. GFP and β-galactosidase expression in mice derived from lines G3, 7, 8 and 9. Immunoblot analysis of GFP, β-galactosidase and β-actin in whole brain extracts of mice derived from G3, 7, 8 and 9 compared to wild-type mice (wt). Proteins were resolved on SDS–polyacrylamide gels, transferred onto a nitrocellulose membrane and probed with antibodies that recognize GFP, β-galactosidase and β-actin. Antibodies were visualized by chemoluminence method. (**B**) Stereomicroscopic visualization of β-galactosidase (left) and GFP (right) expression in alternate coronal brain sections of the mice derived from G3, 7, 8 and 9 at P15. The β-galactosidase staining is co-localized with GFP fluorescence in all four lines. It is restricted to the cortex (Cx, layer 2,3 and 5), hippocampus (Hi), striatum (S) and amygdala (A). Line G8 shows moderate expression and GFP fluorescence in the overview is comparable to autofluorescence of brain sections of a wild-type mouse.

Table 1. Percentage of CA1 pyramidal cells positive for GFP, β-galactosidase (β-gal) and for both reporters

Line	β-gal	GFP	β-gal/GFP
G3	74	72	72
G7	62	60	58
G8	37	34	34
G9	72	72	72
G9+/–Dox	21	17	12

expression in mouse brains are mediated by the variable number of reporter gene expressing cells as well as the variable expression levels within positive cells. However, presence and absence of GFP and β-galactosidase were coherent in all lines; both reporters either co-localized or were both absent in individual cells.

Gene expression of the bidirectional module is regulated by Dox

In the absence of tTA, neither GFP nor β-galactosidase signals could be detected in $Tg^{GFPtetO7lacZ}$ mice. We investigated the Tet sensitivity of α-CaMKII-tTA activated $Tg^{GFPtetO7lacZ}$ mice by Dox treatment. When double-transgenic animals of all four

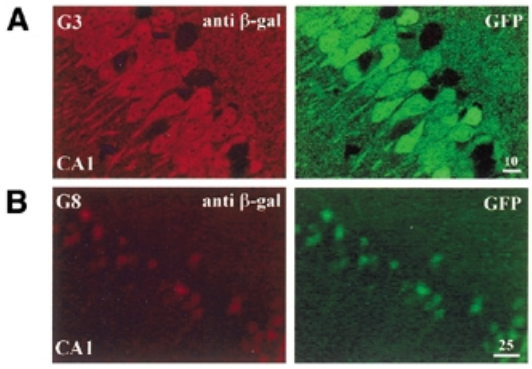

Figure 3. Mosaic expression of GFP and β-galactosidase monitored in CA1 pyramidal cells. CA1 pyramidal cells of mice derived from line G3 (**A**) and G8 (**B**) were analyzed for GFP and β-galactosidase expression in coronal brain sections by Texas Red-labeled β-galactosidase antibodies (left) and GFP fluorescence (right) in confocal images (A) and microscopical pictures (B). Brains were prepared from animals at P15 and fluorescent signals were monitored through Rhodamine and FITC filters, respectively. Scale bars are given in μm.

lines were raised with Dox from conception, tTA induction was prevented, keeping β-galactosidase and GFP at undetectable levels (Fig. 4A and B). Removal of Dox at P21 for 3 weeks induced weak expression of the reporters when compared with the untreated line G7 at P42 in immunoblots and histochemical stains (Fig. 4A and B). Since tTA activated G9 mice die between P15 and P25 (see below), this line can not serve as control. Subsequent confocal analysis of hippocampi of G9 derived mice, which had undergone Dox treatment, confirmed the weak induction. The number of GFP and β-galactosidase-negative cells was high (Fig. 3C, Table 1), perhaps due to slow clearance of Dox and thus incomplete tTA induction.

Bidirectionally coupled GFP and β-galactosidase are good reporter lines for tTA activity

To evaluate the sensitivity of the tTA reporter line $Tg^{GFPtetO7lacZ}$, we compared the β-galactosidase expression profile of high expressing line G9 and the expression of unidirectional tTA reporter lines (5), when mice of both lines are activated by the transgene α-CaMKII-tTA. In addition to the reported profile (5), we found GFP and β-galactosidase expression in the primary olfactory neurons, the vomeronasal organ, the eye, the medulla and especially in the area postrema of the reticular formation and in ventral horn neurons of the spinal cord (Fig. 5).

Differences to the reported tTA profile of line $Tg^{CaMK2tTA}$ might be explained by integration dependence of unidirectional tTA responders (5). Thus, in comparison to unidirectional reporter lines, the transgenic $Tg^{GFPtetO7lacZ}$ reporter lines appear to be more reliable for monitoring cell types with functional tTA activity at macroscopic as well as cellular levels.

DISCUSSION

In our study we describe that bidirectional tTA responder mice were obtained at high frequency and that most of these founders are transmitting tTA-inducible genes. Upon one

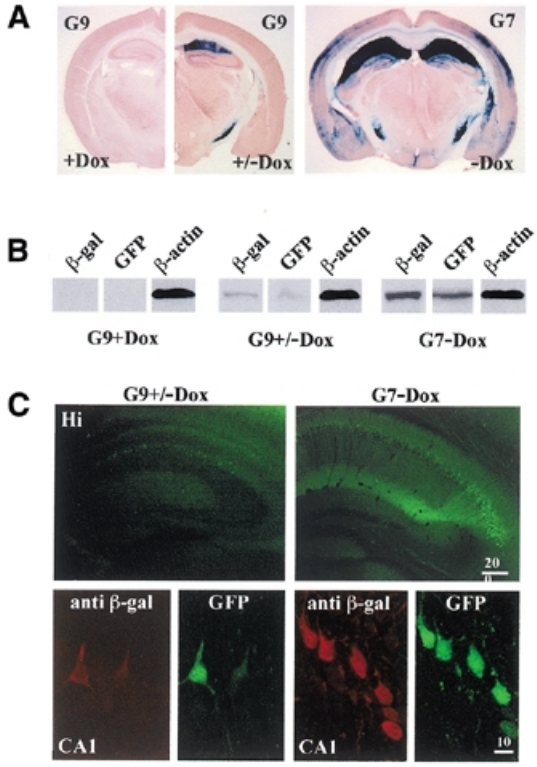

Figure 4. Dox-regulated tTA activity, monitored in responder line G9. (**A**) Coronal brain sections of $Tg^{CaMK2tTA/GFPtetO7lacZ}$ mice derived from line G9 and G7 at P42, stained for β-galactosidase activity. Activation by tTA is compared between animals raised under Dox (G9+Dox) to littermates for which Dox was removed at P21 (G9+/–Dox) and to mice of line G7 (G7–Dox). Without Dox, high expressing mice of line G9 die at ~3 weeks of age and cannot be used as controls. (**B**) Immunoblot analysis of whole mouse brain extracts, which were obtained from mice raised under identical experimental conditions as in (A). Each lane represents three separate experiments and antibodies used for the detection are indicated in the top of each panel. β-Actin served as internal standard. (**C**) GFP and β-galactosidase expression in hippocampi (Hi) of tTA-induced G9 (G9+/–Dox) and untreated G7 mice (G7–Dox) at P42. Confocal analysis (lower part) was performed in CA1 pyramidal cell layers. Dox treatment and removal (G9+/–Dox) leads to GFP and β-galactosidase induction in few CA1 pyramidal neurons. An increased number of CA1 neurons were observed in the untreated G7 (G7–Dox) mouse. Scale bars are given in μm.

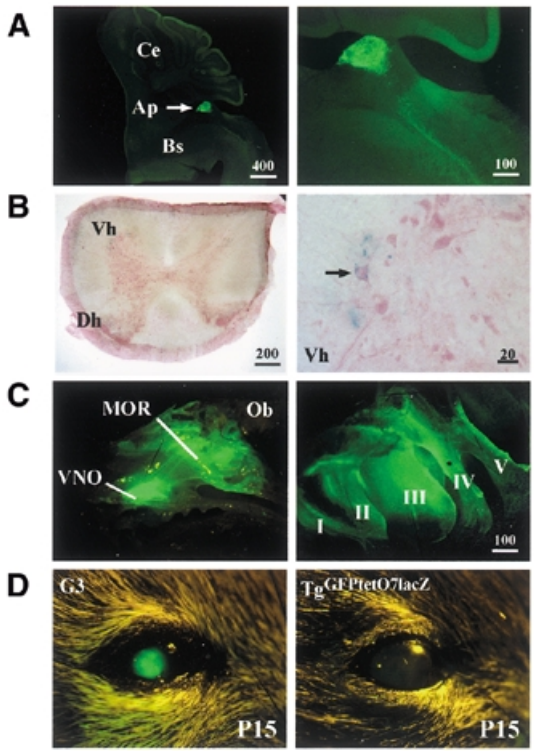

Figure 5. α-CaMKII promoter controlled tTA activity at P15–22, visualized by GFP and β-galactosidase expression in offspring of reporter line G3 in (**A**) the area postrema (Ap) of the reticular formation visualized in sagittal sections of brainstem (Bs) and cerebellum (Ce); (**B**) ventral horn neurons in horizontal sections of the spinal cord (Vh, ventral horn; Dh, dorsal horn); and (**C**) primary olfactory neurons lining the main olfactory region (MOR), the vomeronasal organ of the septum (VNO; Ob indicates the position of the olfactory bulb) and the turbinates in the lateral side of the nasal cavity (roman numbers I–V) visualized after a sagittal cut through the skull. Scale bars are in μm. (**D**) shows fluorescent primary lens fibers detectable through the pupil of the mouse eye.

pronucleus injection four out of six transgenic founders provided useful tTA responder lines. Similar efficiencies were obtained with other bidirectional constructs employed by us (12). However, the occurrence of non-responding founders suggested that expression and/or induction level of the bidirectional tTA responding minigenes are affected by the locus of transgenic insertion. The variability of tTA-induced GFP and β-galactosidase levels in four different $Tg^{GFPtetO7lacZ}$ lines supported the model of integration dependence.

In all our lines the bidirectional promoter was activated by tTA of line $Tg^{CaMK2tTA}$. In low responders G7 and G8, reporter expression was limited to regions that showed in good responders, G3 and G9, strong reporter levels. In mice of lines G3 and G9, GFP and β-galactosidase were expressed in many regions of the forebrain, with highest expression in CA1, dentate gyrus, cortex, striatum, amygdala, olfactory bulb and olfactory epithelium. Thus, the bidirectional promoter seems to be accessible to tTA activation in all neurons of the brain, but its activity is dependent first on the amount of available tTA, and second on the integration site of the transgene. This speculation, raised by the macroscopic comparison of the four different lines, could be substantiated in $Tg^{CaMK2tTA/GFPtetO7lacZ}$ mice by the heterogeneous reporter expression in CA1 pyramidal cells. Here, we found that weak tTA responsive mice of G8 had the lowest number of GFP and β-galactosidase positive cells. Similarly, in good tTA responder mice G9 impaired tTA induction, as observed in mice raised under Dox and then induced by Dox removal, was accompanied by a decreased number of GFP and β-galactosidase positive CA1 cells. Currently, the nature of this mosaic expression remains unsolved. Our efforts to image cellular tTA expression by different antibodies or by *in situ* hybridization remained unsuccessful. Therefore, we are unable to demonstrate a direct involvement of the amount of intracellular tTA in the mosaic expression, and we cannot exclude the fact that mosaic expression is an intrinsic property of the bidirectional promoter. However, another study on reporter gene activation in mouse

liver (1) does not report mosaic expression of bidirectional modules.

The microscopic analysis of the mosaic GFP and β-galactosidase expression patterns in CA1 pyramidal cells showed further that both reporters are co-regulated in single cells. An increase in GFP was accompanied by an increase in β-galactosidase expression. This co-regulation is of exceptional importance as it validates the use of a reporter in a bidirectional module as an indicator of Tet-regulated gene activity. Our data confirm GFP as a useful reporter in the mouse. It is less sensitive than luciferase (1), but can still be monitored in living rodents at the single-cell level, as shown for cortical layer 2/3 neurons (13).

As of today, GFP has been used in many studies to visualize organs or cell populations in live animals (14–17). However, Huang *et al.* reported cardiomyopathy associated with GFP overexpression in the heart of mice (18), and cytotoxic effects in cell culture were also described for strongly GFP-expressing cell lines (19,20). In our mice, we found that high responders of lines G3 and G9 developed a lethal phenotype. These lines showed highest GFP and β-galactosidase levels in the brain, and double transgenic $Tg^{CamK2tTA/GFPtetO7lacZ}$ mice died between P18 and P30. In mice of both lines, the phenotype was dependent on active tTA and was suppressed when tTA was inactivated by Dox. At this point it is unclear if the phenotype results from GFP toxicity. Over-expression of proteins in α-CaMKII positive neurons in general might be another cause of the lethal phenotype. In bidirectional responder lines with GFP replaced by a mutated Huntingtin or by the NMDAR1 subunit of the *N*-methyl-D-aspartate receptor, pathological phenotypes were observed as well (2,12). Further studies are required to explain the premature lethality in any of these mice.

In the four lines investigated, GFP and β-galactosidase were down-regulated to undetectable levels by Dox and were subsequently induced by Dox removal, as reported for cultured cells (21) and transgenic mice (1,22). In contrast to the findings of Kistner *et al.* (1) and Furth *et al.* (22), our mice received Dox from conception until a chosen age. Upon Dox removal GFP and β-galactosidase were not induced to their full extent. Failure to reach full expression probably results from Dox treatment during embryogenesis, as Dox penetrates the maternal placenta (23). Dox has unrestricted access to the embryonic brain because of the lack of a mature blood–brain barrier and because it can be stored in the brain tissue due to its high lipophilia (24). The permeation of Dox across a mature blood–brain barrier is rather low with 10% of Dox blood serum levels found in the cerebral spinal fluid (25). Therefore, the maturation of the blood–brain barrier can be regarded as a sealing effect against circulation-based Dox depletion. Even after Dox withdrawal, Dox levels in the CNS may be high enough to allow GFP and β-galactosidase to be induced only in cell populations with the strongest tTA expression. If Dox is administered after conception at the postnatal stage, when the blood–brain barrier is functionally active, the kinetics of Dox-dependent activation and inactivation of the bidirectional expression modules seem to be more effective. Within 5 days of Dox treatment (2 g/l) reporter gene expression is abolished. It is fully restored within 10 days upon Dox withdrawal (26).

In our analysis, GFP served as a sensitive marker in the central nervous system of $Tg^{CaMK2tTA/GFPtetO7lacZ}$ mice. Indeed, we identified GFP and β-galactosidase signals at developmental times and in regions where activity from the α-CaMKII transgene was not found before (5). Thus, we observed functional tTA already in newborns, visible through the heads of pups. In addition, in juvenile mice we found GFP and β-galactosidase in the reticular formation of the brainstem, in primary olfactory neurons, in ventral neurons of the spinal cord and in the eye. Some of these areas might reflect ectopic expression of the α-CaMKII-tTA transgene. However, in cell types such as primary olfactory (27) and spinal cord (28,29) neurons, presence of the α-CaMKII subunit or its mRNA has been reported.

In summary, we demonstrated that the GFP-containing bidirectional expression module provides a useful tool for Dox-regulated gene expression in the mouse. Transgenic responder lines are obtained at high efficiency; the expression pattern from the module shows little integration dependence and the genes on the module are co-regulated and controlled by Dox.

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