

## RESEARCH ARTICLE

# Tetracycline-inducible transgene expression mediated by a single AAV vector

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*Regulated gene delivery systems are usually made of two elements: an inducible promoter and a transactivator. In order to optimize gene delivery and regulation, a single viral vector ensuring adequate stoichiometry of the two elements is required. However, efficient regulation is hampered by interferences between the inducible promoter and (i) the promoter used to express the transactivator and/or (ii) promoter/enhancer elements present in the viral vector backbone. We describe a single AAV vector in which transcription of both the reverse tetracycline transactivator (rtTA) and the transgene is initiated from a bidirectional tetracycline-responsive promoter and terminated at bidirectional SV40 polyadenylation sites flanking both ITRs. Up to 50-fold induction of gene expression in human tumor cell lines and 100-fold in primary cultures of rat Schwann cells*

*was demonstrated. In addition an 80-fold induction in vivo in the rat brain has been obtained. In vitro, the autoregulatory vector exhibits an induced expression level superior to that obtained using the constitutive CMV promoter. Although extinction of the transgene after removal of tetracycline was rapid (less than 3 days), inducibility after addition of tetracycline was slow (about 14 days). This kinetics is suitable for therapeutic gene expression in slowly progressive diseases while allowing rapid switch-off in case of undesirable effects. As compared to previously described autoregulatory tet-repressible (tetOFF) AAV vectors, the tet-inducible (tetON) vector prevents chronic antibiotic administration in the uninduced state.*

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## Introduction

Several promoter systems are available which are capable of regulating gene expression in eukaryotic cells. These include promoters whose activity is modified in response to heavy-metal ions,<sup>1,2</sup> isopropyl- $\beta$ -D-thiogalactoside,<sup>3</sup> hormones such as corticosteroids,<sup>4</sup> progesterone antagonists<sup>5</sup> or tetracycline.<sup>6</sup> In most of these systems, the pharmacological properties of the inducer drug are a major concern for potential clinical applications. Doxycycline, an analog of tetracycline, is widely accepted because of its safe use in humans,<sup>7</sup> its specificity for the bacterial tetracycline repressor (TetR), and the low dose of drug necessary to produce protein levels suitable for clinical use.<sup>8</sup>

The tetracycline-dependent regulatory system (tet system) is based upon the interaction between the tetracycline transactivator (tTA), consisting of the prokaryotic TetR fused to the activator domain of the herpes simplex virus VP16 protein, and the tetracycline-responsive element (TRE), consisting of seven copies of the prokaryotic tetracycline operator site (tetO) fused to a minimal CMV promoter.<sup>6</sup> In the presence of tetracycline (tet), tTA loses its ability to bind TRE and the expression

is shut off. A reverse transactivator (rtTA) has been derived from tTA by mutagenesis. In contrast to tTA, rtTA only binds TRE in the presence of tet.<sup>9</sup> The latter system is likely to be useful for clinical applications, especially for progressive diseases such as neurodegenerative diseases, in which repeated and intermittent treatments will probably be required.

Tet systems are usually based on two constructs, with the first one containing the gene of interest under the control of the TRE, and the second one containing the transactivator (tTA or rtTA) under the control of a constitutive<sup>6</sup> or a tissue-specific promoter.<sup>10</sup> Single constructs bearing both components suffer from interference between TRE and the promoter used to express the transactivator, resulting in a high basal expression of the gene of interest in the non-induced state. Autoregulatory systems that are devoid of a constitutive promoter and that express the transactivator under the control of the TRE provided a satisfactory tet-dependent regulation.<sup>11,12</sup> As compared to vectors in which the transactivator is expressed constitutively, the use of autoregulatory vectors furthermore allows one to restrict the toxicity<sup>13</sup> and potential immunogenicity of the transactivator to the time of induction.

Tet systems have been developed in viral vectors, including those based on adenovirus,<sup>14</sup> adeno-associated virus (AAV),<sup>15</sup> retrovirus<sup>16</sup> and herpes simplex virus.<sup>17</sup> In these vectors, viral non-coding sequences harboring

transcriptional promoter and/or enhancer activity drive tetracycline-independent transcription of the transgene.

The use of self-inactivating (SIN) vectors in which the viral 5' LTR enhancer and promoter control elements are deleted postinfection has allowed one to overcome the leakiness of tet-autoregulatory retroviral vectors.<sup>16,18</sup> In contrast, deletions in the AAV ITRs resulted in constructs that were deficient for replication and packaging of recombinant DNA.<sup>19</sup> Another strategy, consisting of a set of two AAV vectors (the first bearing the transgene under the control of TRE, and the second the rtTA transactivator and the tet silencer<sup>20</sup> under the control of constitutive promoters), has been proposed.<sup>21</sup> In this system, efficient tet-dependent regulation can be obtained by coinfection with these two vectors provided that their relative ratio is tightly adjusted. However, transduction with a single vector carrying both units and leading to homogenous expression would greatly facilitate gene therapy applications.

Recently, tet-repressible transgene expression from a single AAV vector has been improved by using a chicken  $\beta$ -globin insulator element<sup>22</sup> to shield the inducible promoter from viral enhancers.<sup>23</sup> However, the leakiness of the construct was only partially reduced *in vitro*.

Other systems for achieving regulatable transgene expression through the expression of heterologous hybrid transcription factors interacting with drug-responsive elements are under development. The rapamycin-inducible system has been incorporated in AAV vectors resulting in successful regulation of two different therapeutic genes: growth hormone<sup>24</sup> and Epo.<sup>25</sup> Similar to the tet system, the use of two separate constructs resulted in efficient regulation, whereas a single construct harboring the two transcription units in series was leaky<sup>24</sup>.

In this paper, we describe a single AAV vector containing an autoregulatory tightly controlled system for tet-inducible transgene expression. Transcription of both rtTA and the transgene is initiated at a bidirectional TRE and terminated at bidirectional polyadenylation sequences immediately adjacent to the ITRs. As compared to previously described autoregulatory tet-repressible (tetOFF) AAV vectors,<sup>15,23</sup> the tet-inducible vector avoids chronic antibiotic administration in the uninduced state.

## Material and methods

### Plasmids

The pTR-UF plasmid (a kind gift from N Muzyczka), an AAV vector containing the wild-type *Aequoria Victoria* GFP gene and neomycin-resistance expression cassettes,<sup>26</sup> was digested with XhoI and partially digested with BglII. The BglII-XhoI fragment containing the CMV promoter (pCMV), the GFP gene and an SV40 polyA signal was replaced by the 2248 bp HindIII-XhoI fragment from pTetON (Clontech, Palo Alto, CA, USA), containing the pCMV, the rtTA and an SV40 polyA, after conversion of the HindIII site into BglII, thus generating an intermediate plasmid, pATetON. Then, this plasmid was digested with XhoI and partially digested with BglII and the fragment containing the neomycin-resistance cassette, originally present in pTR-UF, was replaced by

the 2050 bp XhoI-BglII fragment from pArEGFP (Wosch, 2001: <http://www/ulb.uni-duesseldorf.de/diss/mathnat/2001/wosch.pdf>) consisting of (i) the TRE promoter (a 456 bp XhoI-EcoRI fragment from pTRE (Clontech) in which EcoRI was converted into HindIII), (ii) the enhanced green fluorescent protein (EGFP) gene (a 779 bp HindIII-NotI fragment from pEGFP1 (Clontech), and (iii) SV40 intron and polyA (an 863 bp NotI-BamHI fragment from pHGFP-S65T (Clontech), in which BamHI was replaced by BglII), generating pHUB1 (Figure 1a).

In order to generate a cell-type-specific and inducible construct, we used the promoter of the myelin protein P0.<sup>27</sup> The CMV promoter of pHUB1 was excised by partial EcoRI-XhoI digestion and replaced by the EcoRI-XhoI fragment of pPG-6 (kindly provided by G. Lemke, Molecular Neurobiology Laboratory, The Salk Institute, La Jolla, USA)<sup>28</sup> containing the 1100 bp P0 promoter sequence cloned between T3 and T7 in a pBS-backbone, generating pHUB2 (Figure 1b).

To generate the autoregulatory tet-inducible pAC1 plasmid, the 1222 bp EcoRI fragment from pHUB1 was replaced by the EcoRI fragment (map position 12-568) from pBI-GL (Clontech; gene bank accession #U89935) comprising two minimal CMV promoters in opposite orientations separated by seven copies of the 42 bp tetracycline operator (Figure 1c).

The pTR-EGFP plasmid is an AAV vector harboring EGFP (Clontech) and neomycin-resistance expression cassettes that has been derived from pTR-UF<sup>26</sup> as previously described.<sup>29</sup>

The pcDNA3 plasmid containing the neomycin-resistance gene under the control of the SV40 early promoter was purchased from Invitrogen (Gröningen, The Netherlands).

### Cell lines

HeLa and U87-MG cell lines were obtained from American Type Culture Collection (Bethesda, MD, USA). The 293T cell line was purchased from Q-One Biotech (Glasgow, UK). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS (Gibco BRL, Life Technologies, Merelbeke, Belgium).

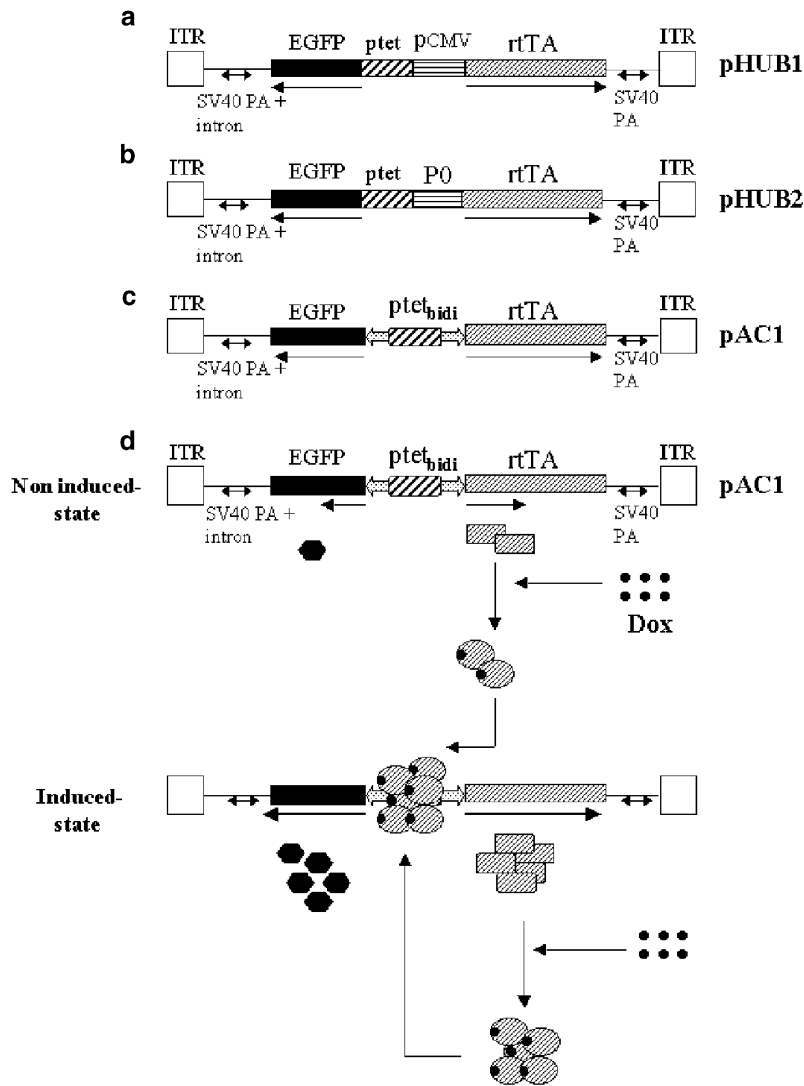
### Isolation and culture of rat Schwann cells

Rat Schwann cells (RSC) were isolated from the sciatic nerve of 1–3-day-old rats according to Brockes and colleagues<sup>30,31</sup> with slight modifications. Contaminating fibroblasts were eliminated by incubation with 10  $\mu$ M cytosine arabinoside (AraC; Sigma, Deisenhofen) for 5 days followed by two steps of complement lysis. The quality of the cultures was checked by performing immunostaining with anti-Thy1.1 (Sigma, Deisenhofen) and anti-S100 (Sigma, Deisenhofen) in order to visualize fibroblasts and Schwann cells.

RSC were cultured on dishes precoated with 0.1 mg/ml poly-L-lysine (Sigma, Deisenhofen) in DMEM containing 10% Tet-System approved FCS (Clontech) and 2  $\mu$ M forskolin.

### Transfections

293T cells were transfected using the calcium phosphate coprecipitation method. HeLa cells and RSC were transfected using 1  $\mu$ g DNA and 3  $\mu$ l FuGENE transfect-



**Figure 1** Schematic diagram of tetracycline-inducible AAV vectors used in this study. (a–c) ITR, inverted terminal repeat; polyA, SV40 fragment containing the early and late polyadenylation signals; intron, SV40 small intron; EGFP, enhanced green fluorescent protein gene; rtTA, reverse tetracycline transactivator; pCMV, early cytomegalovirus promoter; P0, myelin Schwann-cell-specific promoter; TRE, seven tetracycline operator sites fused to minimal CMV promoter;  $ptet_{bidi}$ , seven tetracycline operator sites flanked by two minimal CMV promoters. (d) Schematic representation of doxycycline-mediated induction of rtTA and EGFP expression. In the absence of dox, low basal levels of rtTA (hatched rectangles) and EGFP (filled hexagons) are expressed from the  $ptet_{bidi}$  promoter. Dox (filled circles) binds to rtTA, thus inducing a conformational change which results in binding of the activated rtTA (hatched ovals) to the tetracycline operator sites contained in  $ptet_{bidi}$  and activation of transcription of both rtTA and EGFP. The newly synthesized rtTA then becomes activated by dox and further upregulates  $ptet_{bidi}$ .

tion reagent (Boehringer Mannheim, Germany) according to the manufacturer's protocol.

For lipofection of U87-MG cells, cationic liposomes were prepared from  $3\beta$  N-(N',N'-dimethylaminoethane)-carbamoyl-cholesterol (DcChol: 3 mg, 6  $\mu$ mol) and dioleoyl-phosphatidylethanolamine (DOPE: 3 mg, 4  $\mu$ mol) by reverse phase evaporation as described.<sup>43</sup> Briefly, the dried lipids were resuspended in 5 ml sodium acetate buffer (20 mM, pH 6) containing 5% sorbitol and extruded 11 times through a polycarbonate filter of 100 nm pore diameter (Cyclopore, Whatman) using a LiposoFast device (Avestin). The resulting liposomes were stored at 4°C until use. In a typical experiment, 10  $\mu$ g DNA was combined with 50  $\mu$ l liposomes for a 10 cm culture plate for 4–5 h transfection at 37°C in

serum-free OptiMem culture medium (Life Technologies).

#### Establishment of stable cellular clones

To obtain U87-MG/ $ptet_{bidi}$ -EGFP clones, the pAC1 and the pcDNA3 plasmids were cotransfected at a ratio of 10:1 into U87-MG. Twenty-four hours post-transfection, the cells were trypsinized and diluted 1:5 into complete medium containing geneticin (650  $\mu$ g/ml). Neomycin-resistant clones were isolated after 2 weeks.

#### Fluorimetry

Cells were grown either in the presence or absence of 1  $\mu$ g/ml of doxycycline (dox; Sigma). Whole cell extracts were made by incubating cells for 1 h at 4°C in lysis

buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP40, 50 mM NaF). Lysates were cleared by centrifugation at 12 000g for 1 min. The protein content of extracts was quantified using the BioRad protein assay (Hercules, Belgium). Equivalent amounts of proteins from each plate were diluted to a final volume of 2 ml and fluorescence emission spectra were recorded using a Shimadzu RF-5001 PC spectrofluorimeter. Samples were excited at 488 nm and emitted fluorescence intensity was measured at 509 nm.

### Recombinant AAV (rAAV) viral preparations

Recombinant AAV-tet<sub>bid</sub>EGFP virus was obtained as follows. 293T cells ( $5.0 \times 10^6$  cells seeded on 10 cm plates) were cotransfected with the vector plasmid pAC1 together with the helper/packaging plasmid pDG<sup>32</sup> (a kind gift from Dr Kleinschmidt, Heidelberg), expressing the AAV viral genes and the adenoviral genes required for AAV replication and encapsidation, at a molar ratio of 1:1. Fifty hours post-transfection, the medium was discarded and the cells were harvested by low-speed centrifugation and resuspended in Tris, pH 8.0, NaCl 0.1 M. The rAAV vector stock was purified as previously described by Zolotukhin *et al*<sup>33</sup> with slight modifications. Briefly, after three cycles of freezing/thawing, the lysate was clarified by 30 min centrifugation at 10 000 g, treated with DNase at 37°C for 30 min, and centrifuged at 10 000 g for 30 min to eliminate the residual debris. The virus was further purified by iodixanol gradient and heparin agarose column chromatography, dialyzed against PBS 5% sorbitol and stored at -80°C. The titer, as measured by *in situ* focus hybridization assay,<sup>34</sup> was  $1.0 \times 10^8$  infections units per ml (IU/ml).

For *in vivo* injections, AAV-tet<sub>bid</sub>EGFP recombinant virus was produced at the Gene Vector Production Network (Laboratoire de Thérapie Génique, Nantes, France). The infectious titer, as measured by *in situ* focus hybridization assay,<sup>34</sup> was  $5.0 \times 10^8$  IU/ml.

Recombinant AAV-CMV-EGFP virus was produced at the Gene Vector Production Network (Généthon III, Evry, France) using the pTR-EGFP vector plasmid<sup>29</sup> and titrated by *in situ* focus hybridization assay as previously described.<sup>34</sup> The titer was  $8.9 \times 10^8$  IU/ml.

### Stereotaxic injections

Adult female Wistar rats (Iffa Credo, L'arbresle, France) weighing  $\pm 200$  g were housed and treated according to the Belgian law and all the experiments were accepted by the local ethical committee. The animals were anesthetized with a mixture of ketamine (Imalgène 1000, Merial; 100 mg/kg) and xylazine (Rompun, Bayer; 10 mg/kg) and placed in a Kopf stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). The injection coordinates were -1.0 mm posterior, 2.5 mm lateral to bregma, and 5.5 mm below the dural surface, and the injection rate was 0.2  $\mu$ l/min. The needle was left in place for 1 min before a slow withdrawal over an additional minute. Animals were fed either with Dox Diet containing doxycycline at a concentration of 6.0 g/kg (Bio-Serv, Frenchtown, New Jersey) or with unsupplemented rat food. Animals were killed 1 month after surgery and perfused intracardially first with saline, then with 4% paraformaldehyde (PF4). Brains were postfixed for 24 h in PF4. Fifty-micron vibratome sections were mounted

on glass slides using FluorSave Reagent (Calbiochem, La Jolla, CA, USA).

### Fluorescence-activated cell analysis and sorting (FACS)

Transfected HeLa cells and RSC were cultured without or with 1  $\mu$ g dox/ml and analyzed in a FACStar analyser/sorter (Becton Dickinson). Cells were sorted on the basis of gfp fluorescence 4 days after transfection. Kanamycin (100  $\mu$ g/ml) was added 1 day before and after sorting in order to avoid infection during FACS sorting. Half of the cells were further cultured with or without dox. For long-term experiments, the sorted cells were cultured as usual and split when confluent. Cells were reanalyzed in FACStar after 2, 4 and 6 weeks in culture.

### Fluorescence microscopy

Live EGFP-positive cells were visualized using an inverted Axiovert fluorescence microscope and an FITC filter (Carl Zeiss, Göttingen, Germany).

PF4-fixed transfected or infected cells were cover-slipped using FluorSave reagent (Calbiochem, La Jolla, CA, USA) and photographed using a Zeiss Axiophot-2 fluorescence microscope equipped with an FITC filter coupled with an AxioCam video camera (Carl Zeiss, Göttingen, Germany). Images were acquired as tiff files using the KS-300 software (Carl Zeiss, Göttingen, Germany), converted to gray scale and the fluorescence intensity was quantified using the Scion Image Beta 4.0.2 software (NIH, Bethesda).

## Results

### Construction of tetracycline-inducible AAV vectors

An AAV vector containing the rTA transactivator under the control of the CMV promoter and the EGFP reporter gene under the control of the TRE (hereafter called pHUB1) was first constructed (Figure 1a). AAV ITRs contain promoter/enhancer elements<sup>35,19</sup> that could (i) initiate transcription of an mRNA including the EGFP coding sequence and (ii) activate at distance the minimal CMV promoter contained in TRE. In order to reduce interference of the AAV ITRs on tet-dependent transcription, the two transcription cassettes were placed in opposite orientations starting from the middle of the vector and terminated by bidirectional SV40 polyadenylation sequences<sup>36,37</sup> flanking the ITRs.

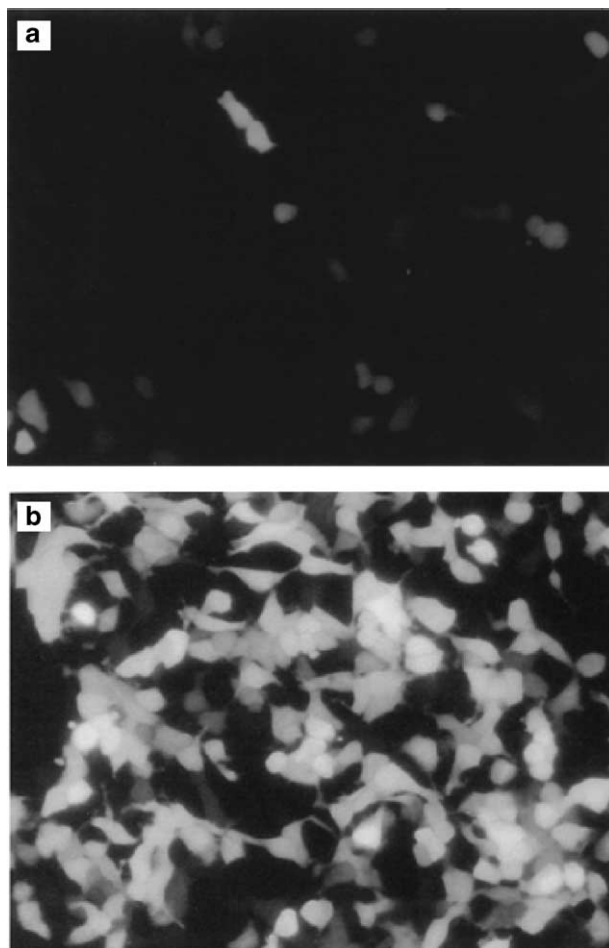
In order to induce tissue-specific and tet-regulatable gene expression, the CMV promoter was replaced by the myelin protein P0 promoter<sup>27</sup> which has been shown to drive Schwann-cell-specific expression,<sup>38</sup> thus creating pHUB2 (Figure 1b).

In order to avoid interference with enhancer sites contained in CMV or P0<sup>38</sup> promoters, an autoregulatory vector, devoid of enhancer sites, was designed by placing a bidirectional tet-inducible promoter (ptet<sub>bid</sub>)<sup>39</sup> in the middle of the vector to drive transcription of both rTA and EGFP (Figure 1c). This autoregulatory construct acts as a self-accelerating transcription cassette, reaching a plateau when the seven operator sites present in ptet<sub>bid</sub> are saturated (see Figure 1d).

### Short-term tetracycline-inducible gene expression

The tet-inducible vectors were transfected into various cell types including 293T immortalized human embryonic kidney cells, HeLa human cervical carcinoma cells, U87-MG human astrocytoma cells and primary cultures of RSC.

Fluorescent microscopic observation of transfected cells cultured in the presence or absence of dox (1  $\mu\text{g}/\text{ml}$ ) revealed an important regulation of transgene expression achieved by pAC1 (Figure 2). However, FACS analysis revealed that, with all plasmids, the percentage of gfp-positive cells was less than two-fold higher in the induced (+dox) than in the non-induced (-dox) states (see Table 1). The discrepancy between microscopic observation and FACS analysis possibly reflects the higher sensitivity of the FACS which detects all gfp-expressing cells including those with a level of fluorescence too low to be observed by microscopy. In favor of this hypothesis, the mean fluorescence of gfp-positive cells was significantly higher in the induced culture (data not shown). When the percentage of gfp-positive cells was multiplied by the mean fluorescence, a statistically significant factor of induction was obtained in all cell types for pHUB1 and pAC1 (Table 1). The Schwann-cell-specific vector, pHUB2, showed a four-fold regulation in



**Figure 2** Induction of EGFP expression after transient transfection into 293T cells. 293T cells were transfected with pAC1. Cells were cultured in the absence (a) or presence (b) of dox (1  $\mu\text{g}/\text{ml}$ ) for 48 h. Dishes were photographed using a fluorescence microscope. Original magnification was 20-fold.

RSC. In contrast, in HeLa cells gene expression from pHUB2 was nearly undetectable and not inducible (data not shown).

In order to evaluate more precisely the amount of gfp protein produced, cellular extracts were analyzed by fluorimetry. Measurement of fluorescence in cellular extracts is likely to better reflect the actual regulation of gene expression. Indeed, a linear relationship between the amount of protein and fluorescence units was demonstrated in the range of 10–350 arbitrary units (data not shown). Using pAC1, a 25-fold increase in fluorescence units was demonstrated upon dox induction in 293T cells (Figure 3).

### Long-term tetracycline-inducible EGFP expression

An excess of copies of the plasmids immediately after transient transfection (see above) could result in a high residual activity of the minimal CMV promoter contained in the TRE. Therefore we analyzed gene inducibility at long term.

Gfp-positive cells in induced cultures were FACS-sorted and further cultured in the presence or absence of dox. Although the number of gfp-positive cells in the induced state decreased with time, increasing induction could be demonstrated in both HeLa and RSC using pAC1, but not using pHUB1 and pHUB2 (Figure 4). Indeed, at 6 weeks post-transfection with pAC1, a 56- and a 102-fold induction was obtained, respectively, in HeLa and RSC. In contrast, when using pHUB1, only very limited although significant induction was obtained. The use of the P0 promoter to express rtTA in pHUB2 resulted in significant expression of EGFP in RSC (Figure 4) but not in HeLa cells (data not shown). A low but significant factor of induction similar to that obtained with pHUB1 was demonstrated using pHUB2 in RSC (Figure 4). These data suggest that enhancer sequences present in the CMV and P0 promoters prevent the establishment of efficient tetracycline-dependent regulation. Therefore only pAC1 was further studied.

Stable clones of U87-MG cells containing the pAC1 plasmid were obtained by cotransfection with the pcDNA3 plasmid harboring the neomycin-resistance gene and by geneticin selection. A representative clone (U87-MG/ptet<sub>bid</sub>-EGFP#1) was cultured in the presence and absence of dox for seven passages. Fluorimetric analysis revealed that emitted fluorescence was 13-fold higher in whole cell extracts ( $134.7 \pm 39.5$  arbitrary units,  $n=7$ ) from dox-treated cultures than in untreated cultures ( $10.5 \pm 3.2$  arbitrary units,  $n=7$ ). (Student's *t*-test showed that the difference between the fluorescence of induced versus non-induced cultures was highly significant ( $P < 0.005$ ).

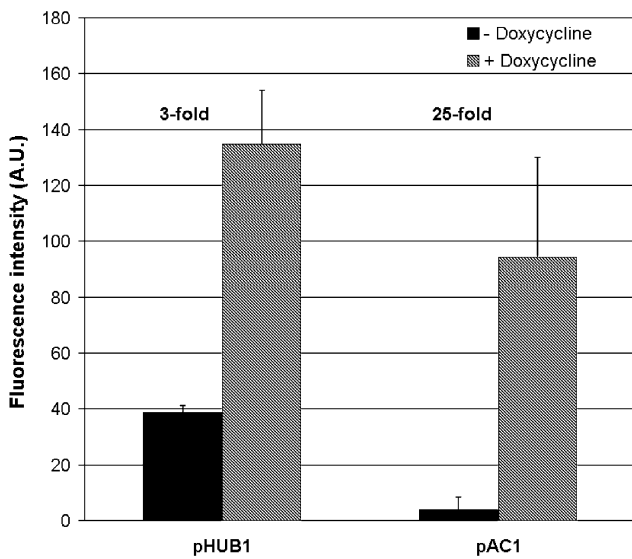
### Slow induction and rapid extinction of the autoregulatory AAV vector

We then studied the kinetics of gene expression upon addition/removal of antibiotics. To determine the time required for full induction of the autoregulatory system, dox was added to uninduced U87-MG/ptet<sub>bid</sub>-EGFP#1 at the time of cell plating (day 0). GFP expression reached the same level as a culture continuously left in the presence of dox 14 days after induction (Figure 5). In contrast, when dox was removed from an induced culture, a level of GFP expression similar to that of a

**Table 1** FACS analysis of HeLa, 293T and rat Schwann cells transiently transfected with tetracycline-inducible vectors

Cell type	Plasmid/treatment	% <i>gfp</i> <sup>+</sup> cells	% <i>gfp</i> <sup>+</sup> cells × mean fluorescence × 10 <sup>-5</sup>
HeLa cells	pHUB1 -dox	22.45 ± 2.13	6.29 ± 2.11
	pHUB1 +dox	29.26 ± 1.84*	29.45 ± 5.97**
	pAC1 -dox	3.45 ± 0.72	0.72 ± 0.26
	pAC1 +dox	6.10 ± 1.87*	3.56 ± 0.53**
Rat Schwann cells	pHUB1 -dox	10.01 ± 1.60	2.80 ± 0.72
	pHUB1 +dox	16.62 ± 3.23*	16.04 ± 2.50**
	pAC1 -dox	6.83 ± 2.00	2.29 ± 2.34
	pAC1 +dox	9.53 ± 2.08*	9.22 ± 4.33**
293T cells	pHUB1 -dox	69.18 ± 2.01	1.42 ± 0.06
	pHUB1 +dox	82.06 ± 1.69*	2.26 ± 0.49*
	pAC1 -dox	51.89 ± 24.28	0.34 ± 0.13
	pAC1 +dox	72.05 ± 23.12*	1.28 ± 0.22**

HeLa cells and rat Schwann cells (RSC) were transfected with the indicated constructs pHUB1 and pAC1 using 1 µg DNA and 3 µl FuGENE transfection reagent. Cells were cultured in the absence or presence of 1 µg/ml doxycycline and analyzed in a FACStar - analyser/sorter 96 h posttransfection. 293T cells were calcium-phosphate transfected with 2 µg of pHUB1 and pAC1. Cells were cultured with and without 1 µg/ml doxycycline and FACS analyzed 48 h posttransfection. The percentage of fluorescent cells as well as the product of the percentage of fluorescent cells and the mean fluorescence are given. The values shown are means and standard deviations of separate transfections. HeLa and RSC, *n*=8; 293T/pHUB1, *n*=5; 293T/pAC1, *n*=8. Differences between treated (+dox) and untreated (-dox) cultures were statistically significant (\*, *P*<0.05; \*\*, *P*<0.005 for all cell lines for both plasmids).

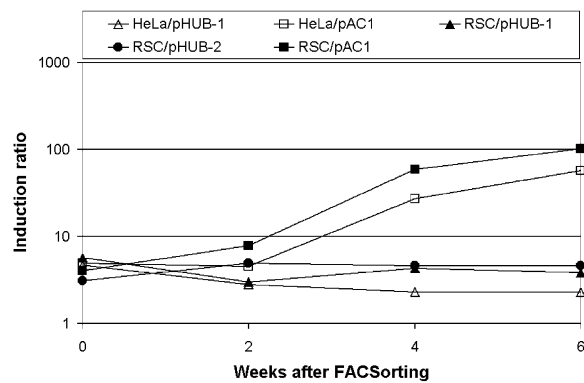


**Figure 3** Quantitative analysis of doxycycline-responsive EGFP expression after transient transfection into 293T cells. 293T cells were transfected with pAC1 or pHUB1. Cells were cultured in the presence or absence of dox (1 µg/ml) for 48 h. Cells were lysed and equivalent amounts of proteins were analyzed by fluorimetry. The fluorescence of mock-transfected cells (3.9 ± 1.0) was subtracted for all samples. Hatched boxes, +dox; filled boxes, -dox. Bars represent means and standard deviations from separate transfections: pHUB1 (*n*=5); pAC1 (*n*=3). AU, arbitrary units. Fold induction representing the ratio between the fluorescence of dox-treated cells versus untreated cells is shown. Differences between treated (+dox) and untreated (-dox) cultures were statistically highly significant (*P*<0.005).

constantly uninduced culture was obtained 3 days after dox removal (Figure 5).

#### Comparison between autoregulatory *ptet<sub>bid</sub>* and constitutive CMV promoters after infection

In order to avoid the variability of stable clones that might result from different sites of integration or a



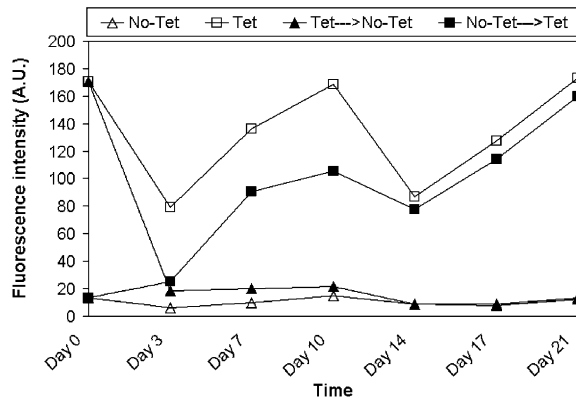
**Figure 4** Long-term doxycycline-responsive EGFP expression after FACS sorting of HeLa and rat Schwann cells transfected with regulatable AAV vectors. HeLa (empty symbols) and RSC (filled symbols) were lipofected with pHUB1 (triangles), pAC1 (squares) or pHUB2 (circles) and cultured in the presence or absence of dox (1 µg/ml). Four days post-transfection, cells were FACS-sorted on the basis of GFP fluorescence and further cultured in the presence or in the absence of dox (1 µg/ml). Levels of gene expression were expressed as the percentage of fluorescent cells multiplied by the mean fluorescence. Induction ratio represents the ratio of these values in the induced (+dox) and the non-induced (-dox) samples. Data are from one out of eight separate experiments.

different number of integrated copies, CMV and *ptet<sub>bid</sub>* promoters were further compared using recombinant virus infections.

U87-MG cells were infected with rAAV-pCMV-EGFP or rAAV-*ptet<sub>bid</sub>*-EGFP at a multiplicity of 100 IU/cell. Two to 21 days postinfection, EGFP-positive cells were visualized in live cultures using an inverted fluorescence microscope. Fluorescent cells started to be detectable in dox-treated cultures 4 days postinfection. In contrast, no fluorescent cells were observed in the non-treated cultures until 21 days postinfection (data not shown).

To determine whether a more sensitive method could allow one to evidence residual GFP expression in non-induced cells, the cultures were fixed at 21 days

postinfection, mounted and photographed using increasing exposure times (Figure 6a-c). The fluorescence intensity of individual cells was evaluated as the mean optical density after conversion of the RGB signal into 255 gray levels. The mean fluorescence of 20 individual



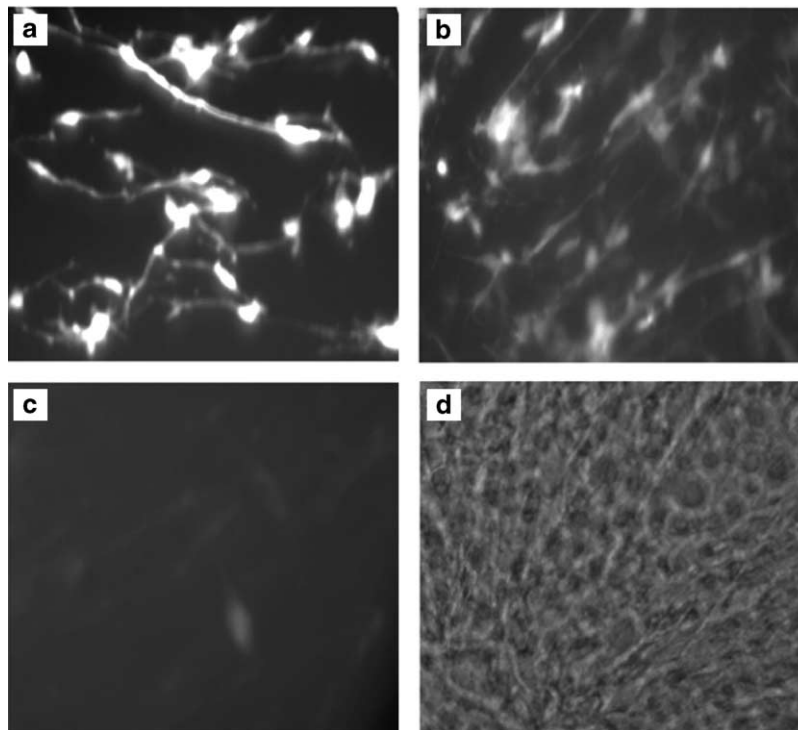
**Figure 5** Kinetics of induction and extinction of EGFP gene expression. Cells were grown either in the presence or absence of dox (1  $\mu\text{g}/\text{ml}$ ) for 21 days. At day 0, cells that were grown in the presence of dox were washed with PBS and further grown in dox-free medium (filled triangles). Cells grown in the absence of dox were also washed with PBS and then grown in the medium containing dox (filled squares) cells were passaged at day. Control cells were grown either continuously in the absence of dox (empty triangles) or continuously in the presence of dox (empty squares). Fluorescence intensity in cell lysates was measured by fluorimetry and is expressed in arbitrary units (AU). Data are from one representative experiment out of three.

cells was compared for induced and non-induced rAAV-ptet<sub>bidi</sub>-EGFP-infected cultures as well as for rAAV-pCMV-EGFP-infected cultures (Table 2). A linear relationship between time of exposure and mean optical density was demonstrated using extracts from 293T EGFP-transfected cells (data not shown). The background fluorescence, measured in non-infected cultures, was subtracted from all values. A 34-fold increase of fluorescence intensity in induced *versus* non-induced cultures was obtained (Table 2).

The CMV promoter resulted in a five-fold lower expression level than the fully induced ptet<sub>bidi</sub> (Table 2).

#### Inducibility of EGFP gene expression driven by rAAV-ptet<sub>bidi</sub>-EGFP in the rat brain

Two microliters of a viral suspension with a titer of  $5.0 \times 10^8$  IU/ml were injected in the globus pallidus. Two animals were given dox-supplemented food and three were fed normally. Animals were perfused 1 month postinjection and 50  $\mu\text{m}$  brain sections were analyzed by fluorescence microscopy in order to detect native GFP fluorescence. In the dox-treated animals, efficient and widespread transduction of the globus pallidus was observed (Figure 6a and c and Table 3, animals 1 and 2). In contrast, in one of the untreated animals, only a few cells harboring a low level of fluorescence were detected (Figure 6b and d and Table 3, animal 3). In the two other untreated animals, no cell harboring detectable green fluorescence was observed and hence the number of transduced cells was considered to be zero (Table 3, animals 4 and 5). The position of the needle tract in these animals confirmed that the recombinant virus was delivered into the globus pallidus (data not shown).



**Figure 6** Comparison of EGFP gene expression driven by CMV and ptet<sub>bidi</sub> promoters in rAAV-infected U87-MG cells. U87-MG cells were infected with rAAV-ptet<sub>bidi</sub>-EGFP (a, c, d) or rAAV-pCMV-EGFP (b) at a multiplicity of 100 IU/cell in the presence (a, b) or absence (c, d) of dox. After 21 days, cultures were fixed with 4% paraformaldehyde and examined under fluorescence (a-c) (d) bright field microscopy.

**Table 2** Quantification of the fluorescence of rAAV-ptet<sub>bidr</sub>-EGFP- and rAAV-pCMV-EGFP-infected U87-MG human astrocytoma cells

Exposure time(ms)	racyclinerAAV-ptet <sub>bidr</sub> -EGFP/(-dox) (A)	rAAV-ptet <sub>bidr</sub> -EGFP/(+dox) (B)	rAAV-pCMV-EGFP (C)	Ratio B:A	Ratio B:C
1256	–	0.075 ± 0.043	0.017 ± 0.007	–	4.41
2060	–	0.164 ± 0.056	0.040 ± 0.014	–	4.15
3063	0.011 ± 0.004	0.386 ± 0.043	0.078 ± 0.043	34.16	6.86

Cells were infected with rAAV-ptet<sub>bidr</sub>-EGFP (A, B) or rAAV-pCMV-EGFP (C) at a multiplicity of 100 I.U. per cell in the presence (B, C) or in the absence (A) of dox. After 21 days, cultures were fixed and analyzed by quantitative microscopy. Images acquired using different times of exposure were converted to grey levels and optical densities of individual cells were measured. The values shown are means and standard deviations of 20 randomly selected cells. Statistical analysis (Student test) showed that at exposure times of 2060 msec and 1256 msec, the fluorescence of untreated (–dox) rAAV-ptet<sub>bidr</sub>-EGFP-infected cells (A) was not significantly different from the background. In contrast, at 3063 msec, A was significantly different from the background ( $P < 0.05$ ). Differences between treated (+dox) and untreated (–dox) cultures were highly significant ( $P < 0.005$ ).

**Table 3** Quantitative analysis of rAAV-ptet<sub>bidr</sub>-EGFP-mediated transduction in the rat globus pallidus

Animal	Treatment	Number of fluorescent cells per animal <sup>a</sup>	Cumulated optical densities of GFP-positive cells per section <sup>b</sup>
1	Dox	670	18.0 ± 5.5
2	Dox	565	22.1 ± 7.1
3	–	100	0.73 ± 0.37
4	–	ND	NA
5	–	ND	NA

Recombinant AAV-ptet<sub>bidr</sub>-EGFP was injected in the globus pallidus. Rats were either fed with Dox diet (6.0 g/kg) or untreated. One month postsurgery, rats were perfused and 50 µm brain sections were analyzed by fluorescence microscopy.

<sup>a</sup>The number of cells harbouring detectable native gfp fluorescence was counted on every fifth section and multiplied by 5.

<sup>b</sup>After converting images to grey scale, integrated optical densities of individual cells were measured using the Scion image analysis software. Data shown are the sum of the optical densities of all the detectable cells on one section. Data shown are means and standard deviations of 3 different representative sections in the vicinity of the delivery site. ND, not detectable; NA, not applicable

The number of GFP-positive cells per animal was 20-fold higher in the dox-treated animals (Table 3, animals 1 and 2) as compared to untreated animals (Table 3, animals 3–5). In addition, the fluorescence intensity of the transduced cells varied from one cell to the other and was generally higher in the dox-treated animals (Figure 7). Therefore, to obtain a reliable comparison between the two groups of animals, the integrated optical densities of individual cells were quantified. The total fluorescence per section was expressed as the cumulated optical densities of all the detectable cells. The mean total fluorescence of three representative sections close to the virus delivery site was used as a relative measure of GFP expression (Table 3). Using this method, an 80-fold induction ratio was obtained.

## Discussion

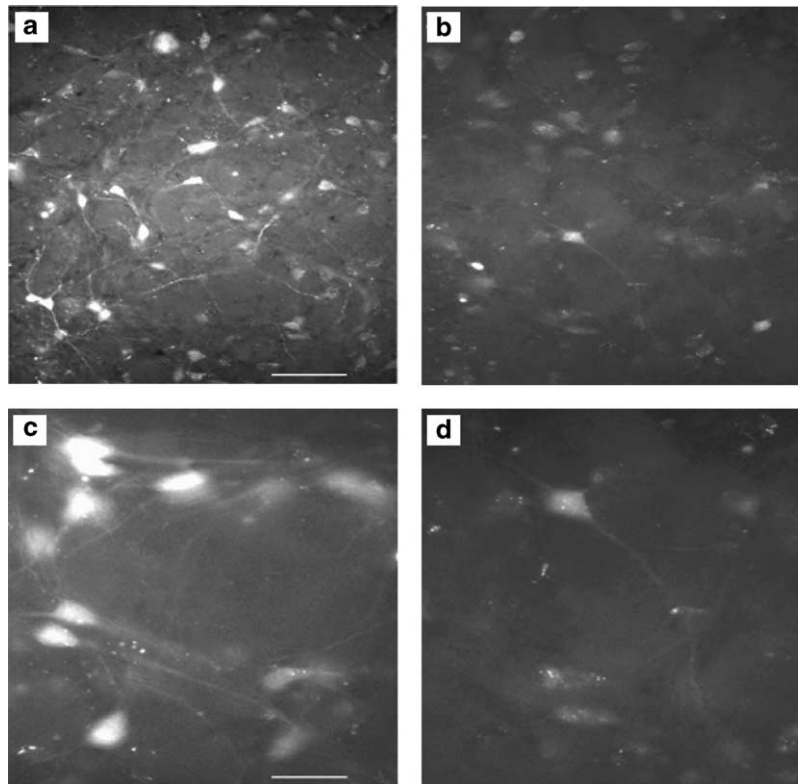
We describe a single tet-inducible AAV vector in which transcription of both the rtTA and the transgene is initiated from a bidirectional tet-responsive promoter and terminated at bidirectional SV40 polyadenylation sites flanking both ITRs. The EGFP reporter gene was used to monitor gene expression because it allows quantitative measurements of gene expression by different methods (FACS, fluorimetry or quantitative microscopy). Our autoregulatory vector exhibits a low basal expression and an induced expression superior to that obtained using the CMV promoter. Similar levels of induction going up to two orders of magnitude were obtained in different cell types, after either transfection

or infection as well as *in vivo* after stereotaxic injection in the rat brain.

The basal level of EGFP expression in the uninduced state was higher than the background fluorescence. This residual transgene expression is likely to prevent clinical applications in situations in which toxic genes are used (eg, for cancer gene therapy). However, in many potential clinical applications, such as the transfer of a neurotrophic gene in neurodegenerative disorders, the transgene product is already endogenously produced at low levels in the targeted tissue and is overexpressed through gene transfer in order to obtain a therapeutic effect. For example, in a rat model of Parkinson's disease, a therapeutic level of GDNF delivered by an AAV vector was less than 10-fold higher than the endogenous GDNF level measured in AAV-lacZ-injected animals.<sup>40</sup> In this case, a low residual transgene expression in the uninduced state will probably not preclude safe use of the vector.

The autoregulatory vector described here, using bidirectional promoter and polyadenylation sequences, has a relatively small size allowing one to incorporate inserts of up to 1.3 kb. For example, it allows one to incorporate the tet silencer<sup>20</sup> under the control of either of the ITRs in order to tighten regulation, while still accepting inserts of up to ~700 bp. Shorter polyA sites could potentially be used to further enlarge the cloning capacity.

Similar constructs, but using the complete CMV promoter or the Schwann-cell-specific P0 promoter to drive expression of rtTA, showed a high basal expression



**Figure 7** Inducibility of EGFP gene expression driven by rAAV-ptet<sub>bid</sub>-EGFP in the rat globus pallidus. A viral suspension containing  $10^6$  IU of rAAV-ptet<sub>bid</sub>-EGFP in 2  $\mu$ l was injected in the rat brain (coordinates: AP = -1.0; L = +2.5; V = -5.5 according to Paxinos and Watson<sup>46</sup>). Animals were with dox-supplemented (a, c) or unsupplemented (b, d) food. One month postsurgery, 50  $\mu$ m vibratome brain sections were examined under fluorescence microscopy. Images were acquired using a constant exposure time (456 ms). Bars: 50  $\mu$ m (a, b) and 100  $\mu$ m (c, d).

resulting in modest induction factors (lower than five-fold), suggesting that the presence of enhancer sites prevents the establishment of efficient regulation. Direct tet-independent activation of the TRE is likely to occur in these constructs due to the close proximity of the CMV or P0 enhancer sites which are known to be active in both orientations. Due to such kind of interference, tight regulation combining transactivator/promoter systems and tissue-specific promoters is likely to be feasible only using two separate constructs.<sup>41</sup>

The vector showed a slow inducibility probably reflecting the need to accumulate a sufficient amount of tet-activated transactivator to stimulate transcription from the TRE. Indeed, at the time of addition of the antibiotics, only a low amount of transactivator resulting from the leakiness of the TRE is present.

After removal of the antibiotic, the rate of extinction of gene expression mostly depends on the half-life of the antibiotic and on deactivation of rtTA. With the progressive decrease of antibiotic concentration, the operator sites are freed and rtTA concentration drops, further accelerating the extinction of gene expression. The ability to observe extinction of transgene expression furthermore depends on the stability of the transgene product and will thus need to be reevaluated with relevant therapeutic genes.

Toxic effects of the tet transactivator due to transcriptional squelching by subdomains of VP16 have been described.<sup>12</sup> However, active subfragments of VP16 having reduced interactions with cellular transcription factors and no reduction of induced transcription level<sup>42</sup>

can be advantageously substituted for the complete VP16 transactivation domain in autoregulatory AAV vectors.<sup>23</sup>

Residual transgene expression in autoregulatory tet systems contained in AAV vectors could result from (i) basal activity of the minimal CMV promoter which might furthermore be stimulated at distance by enhancer sequences present in the AAV ITRs,<sup>19</sup> (ii) transcription of the transgene initiated at the promoter present in the ITRs<sup>22</sup> and (iii) non-specific binding of the rtTA in the absence of antibiotics.

Insulator sequences that eliminate inappropriate interaction between promoters and enhancers have been used to isolate the tet-cassette from the ITRs.<sup>23</sup> Efficient insulation in episomal DNA has been recently demonstrated in adenoviral vectors.<sup>44</sup> However, insulators do not contain signals for transcription termination.<sup>22</sup> Therefore, transcription initiated from the ITRs is not likely to be affected by the presence of these sequences.

The use of bidirectional polyadenylation sites placed between AAV ITRs and the transcription cassettes in the construct described here prevents tetracycline-independent expression of the transgene from the promoter sequence contained in the ITRs.<sup>19</sup> Whether enhancer sequences potentially present in the ITRs activate TRE at distance still remains to be determined.

In conclusion, the autoregulatory tet-inducible (tetON) AAV vector described here represents a substantial improvement over a constitutive expression of the transactivator because it allows a tighter extinction of the transgene and higher levels of transactivator and consequently of transgene product after induction.

Furthermore, as compared to previously described autoregulatory tet-repressible (tetOFF) AAV vectors,<sup>15,23</sup> the tet-inducible vector prevents chronic antibiotic administration in the uninduced state.

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