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Neuroscience Letters 378 (2005) 106–110
Neuroscience Letters
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A tetracycline-regulatable adeno-associated virus vector for double-gene transfer

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Received 4 November 2004; received in revised form 7 December 2004; accepted 7 December 2004

Abstract

An increasing demand for polycistronic vectors that express multiple genes simultaneously has arisen in recent years to obtain an efficient gene therapy. Armed with the knowledge that the expression of transgene in mammalian cells often requires tight control, we constructed in this study a tetracycline-regulated double-gene adeno-associated virus (AAV) vector carrying green and red fluorescent protein genes and expressed it in PC12 cells. When detected by fluorescence microscope and fluorescence-activated cell sorting, gene expression was induced by 44–66-fold and could be reversibly controlled by doxycycline. This double-gene AAV vector may be useful for regulated expression of two genes or a marker to monitor transgene expression.

Keywords: Tetracycline-regulatable; Adeno-associated virus; PC12 cells; Green fluorescent protein

Adeno-associated virus (AAV) vectors are excellent candidates as tools for gene therapy of neurological diseases. They are able to transduce dividing and non-dividing cells, and they provide long-term gene expression that is required to treat chronic diseases such as Parkinson's disease (PD). Furthermore, AAV vectors have a good safety profile since they elicit only low titer and transient neutralizing antibodies, and no inflammation is found when administered in the brain [15]. In addition, AAV is a defective virus that integrates into the human chromosome 19q13.3 [17], yet produces no known pathology.

Traditionally, most vectors for gene therapy were monocistronic. An increasing demand for more complex polycistronic vectors has arisen in recent years to obtain complex gene therapy effects [3]. For example, current gene therapy models for PD have focused on two treatment strategies: the replacement of biosynthetic enzymes such as tyrosine hydroxylase (TH) for dopamine synthesis and the addition of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) for protection and restoration of dopaminergic neurons [10]. Co-transfected with a vector expressing BDNF and a retroviral vector expressing TH, the normal neonatal rat astrocytes were implanted into the striatum of PD rats induced by 6-hydroxydopamine, and a synergistic therapeutic effect was obtained [16]. If these two therapeutic genes are to be placed into a single vector, a double-gene vector is needed.

The tetracycline (tet)-regulatable system is an ideal system for tightly regulated gene expression mediated by recombinant AAV (rAAV). There is no known interaction with the endogenous materials because the system is entirely prokaryotic. And the small-molecule inducer-doxycycline (Dox, an analog of tetracycline) can be used at doses far lower than those used clinically as an antibiotic [4]. Since rAAV has a cassette limit of approximately 5 kb, the tet responsive cassette shows great promise for use with rAAV, as it is relatively small compared with other regulated systems and may allow for rAAV-mediated transfer of larger transgenes or double transgenes within a single vector. Two types of systems, tet-off and tet-on, were used. The tet-off system is based upon

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doi:10.1016/j.neulet.2004.12.039

the interaction between the tetracycline transactivator (tTA) and the tetracycline-responsive element (TRE). The former consists of the prokaryotic tetracycline repressor fused to the activator domain of the herpes simplex virus VP16 protein. The latter consists of seven copies of the prokaryotic tetracycline operator site fused to a minimal cytomegalovirus promoter [6]. In the presence of tetracycline, tTA loses its ability to bind TRE and the expression is shut off.

In this study, we constructed a double-gene AAV vector containing a bidirectional promoter for tet-off transgene expression. Using fluorescence microscope and fluorescence-activated cell sorting (FACS) methods, we found that this vector could function in PC12 cell line, suggesting that this vector would be useful for double-gene gene transfer and tet-off transgene expression.

First of all, we wanted to construct three vectors. The pBI-EGFP plasmid (Clontech, USA) containing the bicistronic promoter and the pBluescrip II KS(+) (Stratagene, USA) were digested by *Pst*I and *Eco*RV, and then ligated to generate an intermediate plasmid pKS-BI. The pKS-BI plasmid and the pAV53 plasmid (a kind gift from Dr. Depei Liu) [2] were digested by *Pst*I and partially digested by *Sal*I, and then ligated to generate another intermediate plasmid pAV-bi. The *Bam*HI and *Hin*dIII sites were erased from the pSNAV plasmid (AGTC Gene Technology, China) containing inverted terminal repeats (ITR) of AAV and neomycin-resistance expression cassettes, to create the intermediate plasmid pSNAVB̂Ĥ. Both pSNAVB̂Ĥ and pAV-bi were digested by *Xho*I and partially digested by *Sal*I to generate a tet-off double-gene AAV vector (Fig. 1A).

Both pAV53 plasmid and the pcDNA2.1-GFP plasmid (GIBCO, USA), which contains green fluorescent protein (GFP), were digested by *Pst*I and partially digested by *Xba*I to generate an intermediate plasmid pAV53-GFP. We designed a couple of primers: 5′-CCGctcgag-GTCGCCACCATGGTGCGC-3′, 5′-CCGctcgagAGATAC-ATTGATGAGTTTGGA-3′, which had an *Xho*I site. With these primers we amplified red fluorescent protein (RFP) and SV40 polyA from the pDsRed1-N1 plasmid (Clontech, USA) by PCR method. The fragment of PCR product and the plasmid pSVbi(+) were digested by *Xho*I and then ligated to generate an intermediate plasmid pSVbi-RFP. Both pSVbi-RFP and pAV53-GFP plasmids were digested by *Bgl*II and *Eco*RV and then ligated to create the reporter plasmid pSVbi-RFP-GFP (Fig. 1B).

After digestion by *Eco*RI and *Bam*HI, the pcDNA3.1(−) plasmid (Invitrogene, USA) and the pUHD15.1 plasmid (Clontech, USA) were then ligated to generate an intermediate plasmid pcDNA3.1(−)-tTA. The pcDNA3.1(−)-tTA plasmid and the pcDNA3.1(+)/Hygro(+) plasmid (Gibco BRL, USA) containing hygromycine resistance gene were digested by *Nhe*I and *Bam*HI, and then ligated to generate the inducer plasmid pcDNA3.1/Hygro(+)-tTA (Fig. 1C).

The PC12 cell line was purchased from American Type Culture Collection (Bethesda, USA). The cells were maintained in a humidified 5% CO_2 incubator in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, USA) supplemented with 10% horse serum and 5% fetal calf serum (HyClone, USA) at the dishes (Nunclon, Denmark) coated with Collagen type I (Sigma, USA). Cells were split every 3 days using standard techniques.

PC12 cells were transfected with LipofectAMINE 2000 transfection reagent using the modified protocol as previously described [12]. Briefly, 1 day before transfection, 2–8 × 10^5 cells in 2 ml of growth medium without antibiotics were placed at a 35-mm dish. Then 1.0 μg pcDNA3.1/Hygro(+)-tTA and 4.0 μg pSVbi-RFP-GFP DNA was added to 250 μl serum-free DMEM in a polypropylene tube. In a second tube, 10 μl LipofectAMINE 2000 was added to 250 μl serum-free DMEM and mixed. Contents of the tubes were combined within 5 min and incubated for 20 min at room temperature. The entire 0.5 ml mixture was added to the cells. Twenty-four hours after transfection, the cells were trypsinized and diluted in a ratio of 1:5 into complete medium containing geneticin (500 μg/ml) and hygromycin (250 μg/ml). Neomycin–hygromycin resistant clones were isolated 2 weeks later [7].

Transfected PC12 cells were analyzed in a FACStar analyzer/sorter (Becton Dickinson). Cells were sorted on the basis of GFP/RFP fluorescence 2 weeks after transfection. Kanamycin (100 mg/ml) was added 1 day before and after sorting to avoid infection during FACS sorting. The sorted

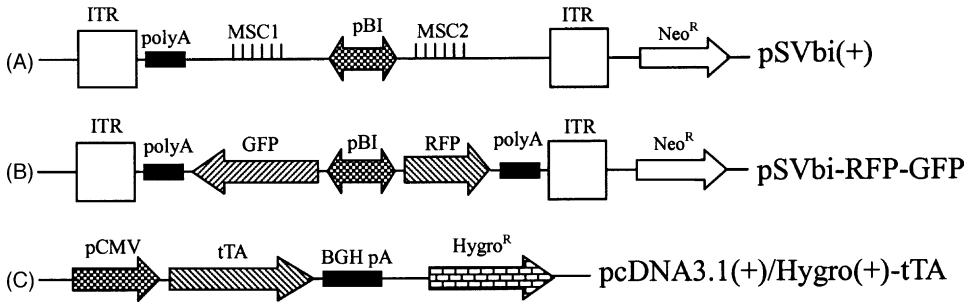

Fig. 1. Schematic diagram of vectors used in this study. ITR, inverted terminal repeat; polyA, SV40 fragment containing the early and late polyadenylation signals; pBI, bidirectional tet-responsive promoter; MSC, multiple cloning site; GFP, green fluorescent protein gene; RFP, red fluorescent protein gene; pCMV, early cytomegalovirus promoter; tTA, tetracycline-controlled transactivator; BGH pA, bovine growth hormone polyadenylation signal; Neo®, neomycin resistance gene; Hygro®, hygromycin resistance gene.

cells were cultured as usual and split when confluent. Dox-treated cells were reanalyzed in FACStar [1].

We cultured FACS-sorted PC12 cells with the density of 1×10^5 ml^{-1}. Cells in each group were treated with different concentrations of Dox (0.0001–100.0 ng/ml) or without Dox (the control) for the first 5 days. Then the cells were washed twice with phosphate-buffered saline (PBS), detached with trypsin, and given two additional washes with PBS while in suspension. Cells were then plated onto new tissue culture dishes and given an extra wash with PBS 10 h later and then fed with fresh media [13]. After treatment of Dox, we used fluorescence microscope and FACS to detect the fluorescence intensity of RFP/GFP.

The live cells were detected under fluorescence microscope (Leica, Germany) equipped with FITC/TRITC filters coupled with a SPOT camera (Diagnostic Instruments, USA). Images were obtained as tiff files with the SPOT Advanced software (Diagnostic Instruments, USA). The fluorescence intensity was quantified with the Metamorph software (Universal Imaging, USA). The fluorescence intensity of individual cells was evaluated as the mean optical density after conversion of the RGB signal into 0–255 gray levels, then the quantitative assay was done. The background fluorescence, measured in non-infected cultures, was subtracted from all values.

In the single tet-regulatable AAV vector (Fig. 1A), transcription of both insert genes is initiated from a bidirectional tet-responsive promoter. To determine if this vector could function, we inserted the reporter genes RFP and GFP into the multiple cloning sites of this vector and constructed a reporter

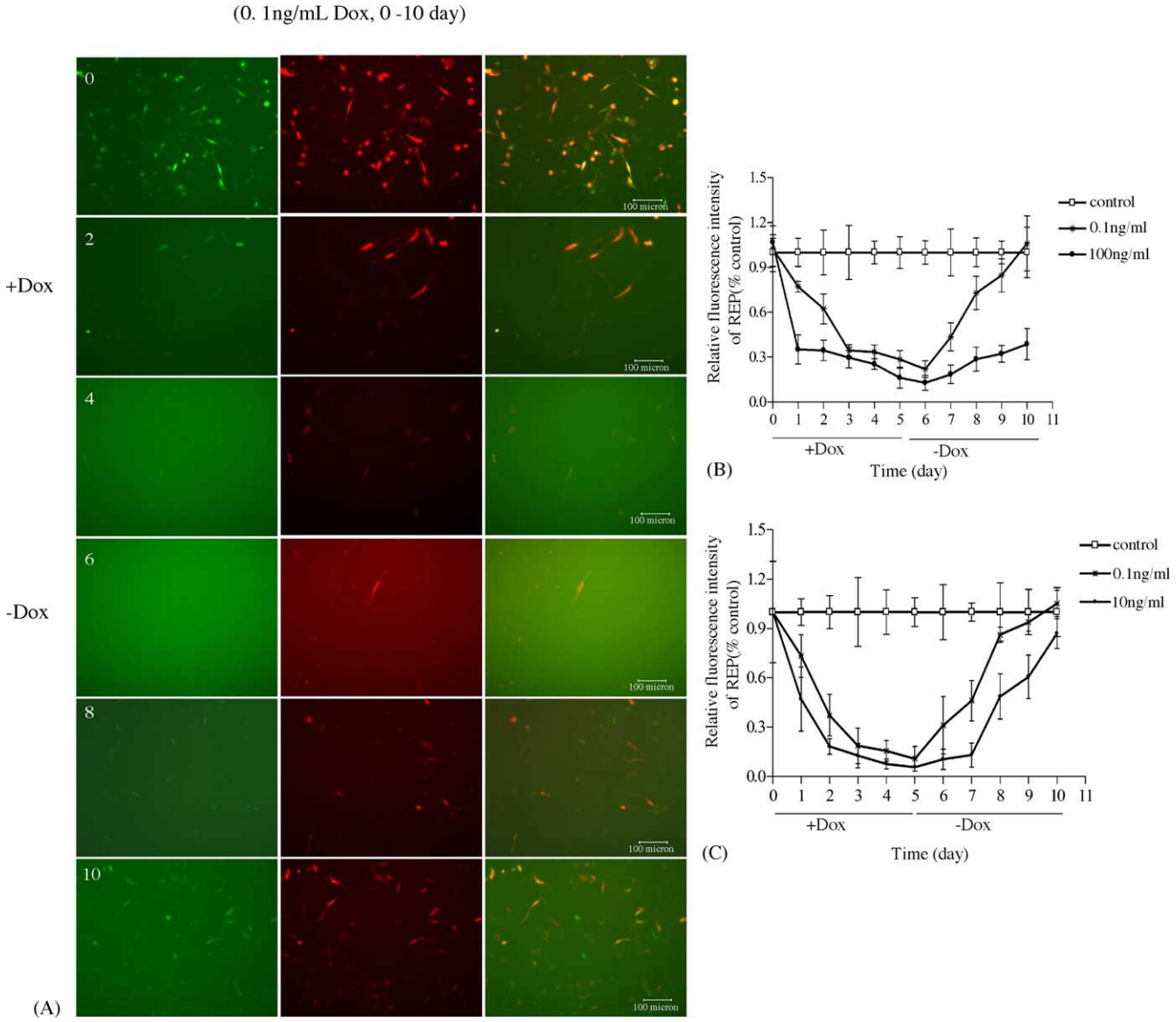

Fig. 2. Dox-regulated expression of RFP and GFP by Fluorescence Microscope. From the first to the fifth day there was an addition of Dox into the medium. On the fifth day, the cells were washed by PBS and then cultured in the absence of Dox. (A) This showed Dox-regulated expression of RFP and GFP under 0.1 ng/ml Dox. The fluorescence intensity ratio of RFP (B) and GFP (C) was calculated. Each value represents the mean ± S.E.M. of three samples.

plasmid pSVbi-RFP-GFP (Fig. 1B). The transcription of RFP and GFP is driven by pBI, a bi-directional tet-responsive promoter, upon it's binding to tTA. Thus, we constructed an inducer plasmid pcDNA3.1/Hygro(+)-tTA (Fig. 1C) containing CMV promoter and tTA gene. The constitutive expression of tTA could drive pBI to promote the transcription of RFP and GFP.

We co-transfected PC12 cells with the pSVbi-RFP-GFP plasmid harboring geneticin-resistance gene and the pcDNA3.1-tTA plasmid harboring the hygromycin-resistance gene, two weeks after geneticin–hygromycin selection, RFP/GFP-positive cells were FACS-sorted and further cultured in the absence of Dox. A stable clone, named PC12-RFP-GFP, with high expression of RFP/GFP was obtained and retained fluorescence for over 20 passages with no decrease in intensity. In the presence of Dox, the fluorescence intensity of RFP (Fig. 2B) and GFP (Fig. 2C) decreased gradually. Three- and six-fold induction were obtained for RFP under 0.1 and 10.0 ng/ml Dox, respectively, and 9- and 18-fold for GFP under 0.1 and 10.0 ng/ml Dox, respectively, on the fifth day. After withdrawal of Dox, the fluorescence intensity of RFP and GFP recovered gradually, and reached the same level of the control 5 days later (Fig. 2A).

Fluorescence microscopic observation of transfected cells cultured in the presence and in the absence of Dox revealed an important regulation of transgene expression (Fig. 2B and C). FACS can detect the percentage of positive cells and the mean fluorescence. We used FACS to further confirm the Dox-regulated expression of RFP/GFP. The expression of RFP and GFP was down-regulated by Dox 96 h after addition of different concentrations of Dox (Fig. 3A). When the percentage of RFP/GFP-positive cells was multiplied by the mean fluorescence, a statistically significant factor of induction was obtained in cells even for the group treated with 0.0001 ng/ml Dox. On the fifth day, 2-, 54- and 66-fold induction of RFP (Fig. 3B) was obtained for 0.0001, 1.0 and 10.0 ng/ml group respectively, and 3-, 35- and 44-fold induction of GFP (Fig. 3C) was obtained for 0.0001, 1.0 and

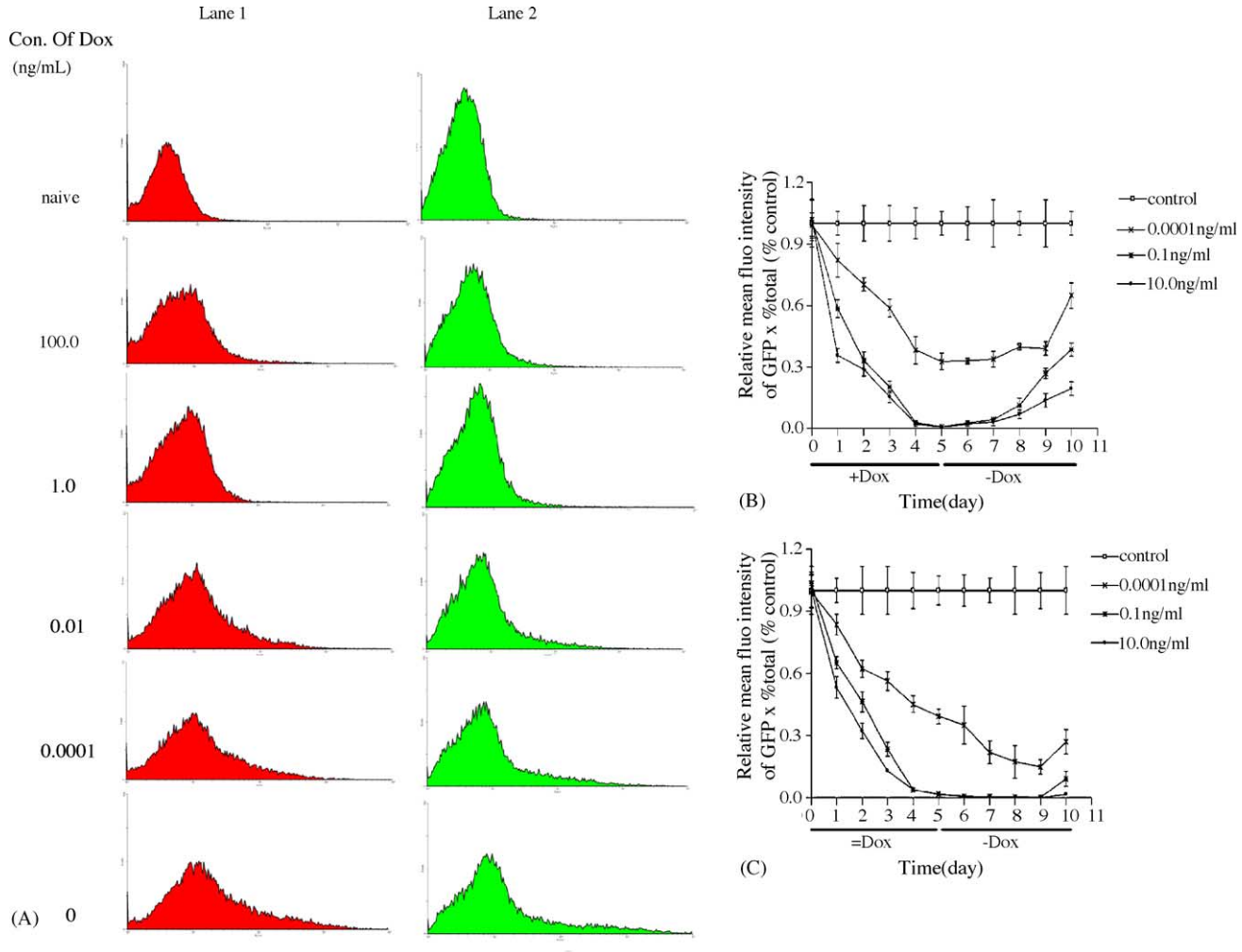

Fig. 3. Dox-regulated expression of RFP and GFP by FACS. From the first to the fifth day there was a Dox addition into the medium. (A) This showed Dox-regulated expression of RFP (lane 1) and GFP (lane 2) 96 h after addition Dox. On the fifth day, the cells were washed by PBS and then cultured in the medium without Dox. The ration of arithmetic product of the mean fluorescence and percentage of positive cells was calculated for RFP (B) and GFP (C). Each value represents the means ± S.E.M. of three samples.

10.0 ng/ml group, respectively. After withdrawal of Dox, the fluorescence intensity of RFP and GFP recovered gradually.

It has been reported that AAV ITRs contain promoter/enhancer elements [5,8] that can: (i) initiate transcription of an mRNA of the insert genes and (ii) activate at distance the minimal CMV promoter contained in bidirectional promoter pBI. In order to reduce the interference of the AAV ITR on tet-dependent transcription, the two transcription cassettes were placed in opposite directions starting from the middle of the vector and terminated by SV40 polyadenylation sequences [9]. It remains to be determined whether enhancer sequences potentially present in the ITR can activate TRE at distance.

The vector has a relatively small size, allowing one to incorporate inserts of up to 4.0 kb. For example, it allows one to incorporate the tTA gene under the control of the ITR to tighten regulation, while still accommodating inserts of up to 2.0 kb bp [1]. Shorter polyA sites such as bovine growth hormone polyA could be used to further enlarge the cloning capacity.

The RFP/GFP reporter genes were used to monitor gene expression because they allow quantitative measurement of gene expression by simple methods such as FACS or quantitative microscopy [11]. Induction up to two orders of magnitude was obtained in PC12 cells after transfection, and the expression of RFP/GFP was reversibly controlled by Dox. FACS has the higher sensitivity to detect all RFP/GFP-expressing cells including those with a level of fluorescence too low to be observed by microscopy. Therefore, a lower concentration (0.0001 ng/ml) of Dox could result in significant induction by FACS.

But high-level expression of tTA in both the on and off states may lead to transcriptional squelching, which occurs when the VP16 component of the transactivator titrates out essential components of the transcriptional machinery. And the VP16 region of the tTA is postulated to have toxic cellular effects [6]. But the potential transcriptional 'squelching' and the cytotoxicity can be reduced when the weak minimal thymidine kinase promoter is used in place of the minimal CMV promoter of tTA [14].

Our vector, which can deliver long-term and controllable gene expression, may have useful applications in the field of gene transfer for neurological disorders such as Parkinson's disease that may require expression of several therapeutic gene products at the same time. The feature that the expression of transgene can be reversibly controlled is important for preventing toxic or side effects of over-expressed transgene.

Acknowledgments

This study was supported by grants from the National Basic Research Program of China (G1999054008), the High Tech Research and Development (863) Programme of China (2001AA217161), and National Natural Science Foundation of China (30170304).

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