A tetracycline-regulatable adeno-associated virus vector for double-gene transfer

Jian-jun Wang a, Dong-bin Niu a, Ting Zhang a, Kun Wang a, Bing Xue a, Xiao-min Wang a.b,∗

a Neuroscience Research Institute, Peking University, Beijing 100083, PR China
b Department of Physiology, Capital University of Medical Sciences, Beijing 100054, PR China

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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.

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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
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 AA V 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 pBluescript II KS(+) (Stratagene, USA) were digested by PstI and EcoRV, 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 PstI and partially digested by SsII, and then ligated to generate another intermediate plasmid pAV-bi. The BamHI and HindIII sites were erased from the pSNNAV plasmid (AIGTC Gene Technology, China) containing inverted terminal repeats (ITR) of AA V and neomycin-resistance expression cassettes, to create the intermediate plasmid pSNAVBi. Both pSNAVBi and pAV-bi were digested by XhoI and partially digested by SsII to generate a tet-off double-gene AA V vector (Fig. 1A).

Both pAV53 plasmid and the pcDNA2.1-GFP plasmid (GIBCO, USA), which had an XhoI site. With XhoI and then ligated to generate an intermediate plasmid pAV53-GFP. We designed a couple of primers: 5′-GGTACCTGAGTCCGCACCATGTGGCTGCCG-3′, 5′-CCGCTAGAGATATCTTTGTTGGA-3′, which had an XhoI 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 SV40 polyA was digested by BglII and EcoRV and then ligated to create the reporter plasmid pSVbi-RFP-GFP (Fig. 1B).

After digestion by EcoRI and BamHI, the pcDNA3.1(−) plasmid (Invitrogen, USA) and the pUHD151. plasmid (Clontech, USA) were then digested to generate an intermediate plasmid pcDNA3.1(−)-tTA. The pcDNA3.1(−)-tTA plasmid and the pcDNA3.1(+)-Hygro(+/-) plasmid ( Gibco BRL, USA) containing hygromycin resistance gene were digested by XhoI and BamHI, 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% CO2 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 polystyrene 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 generic (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; NosR, neomycin resistance gene; HygroR, hygromycin resistance gene.](image)
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 $1 \times 10^5$ 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 vector.

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 its 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

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**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.
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References