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Long-term inducible expression in striatal neurons from helper virus-free HSV-1 vectors that contain the tetracycline-inducible promoter system

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Abstract

Direct gene transfer into neurons in the brain via a virus vector system has potential for both examining neuronal physiology and for developing gene therapy treatments for neurological diseases. Many of these applications require precise control of the levels of recombinant gene expression. The preferred method for controlling the levels of expression is by use of an inducible promoter system, and the tetracycline (tet)-inducible promoter system is the preferred system. Helper virus-free Herpes Simplex Virus (HSV-1) vectors have a number of the advantages, including their large size and efficient gene transfer. Also, we have reported long-term (14 months) expression from HSV-1 vectors that contain a modified neurofilament heavy gene promoter. A number of studies have reported short-term, inducible expression from helper virus-containing HSV-1 vector systems. However, long-term, inducible expression has not been reported using HSV-1 vectors. The goal of this study was to obtain long-term, inducible expression from helper virus-free HSV-1 vectors. We examined two different vector designs for adapting the tet promoter system to HSV-1 vectors. One design was an autoregulatory design; one transcription unit used a tet-regulated promoter to express the tet-regulated transcription factor tet-off, and another transcription unit used a tet-regulated promoter to express the *Lac Z* gene. In the other vector design, one transcription unit used the modified neurofilament heavy gene promoter to express tet-off, and another transcription unit used a tet-regulated promoter to express the *Lac Z* gene. The results showed that both vector designs supported inducible expression in cultured fibroblast or neuronal cell lines and for a short time (4 days) in the rat striatum. Of note, only the vector design that used the modified neurofilament promoter to express tet-off supported long-term (2 months) inducible expression in striatal neurons.

Keywords

Herpes Simplex Virus vector; Tetracycline-inducible promoter system; Long-term expression; Regulated expression; Neuronal-specific expression; Striatal neuron

1. Introduction

Direct gene transfer into neurons in the brain using specific virus vector systems has potential for both elucidating the molecular mechanisms underlying neuronal physiology and for developing gene therapy treatments for specific neurological diseases. Many of these applications require neuronal-specific or neuron subtype-specific expression and precise control of the levels of recombinant gene products. Cell type-specific expression can be

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achieved by using cell type-specific promoters and/or targeted gene transfer to a specific type of neural cell. The preferred method for controlling the levels of recombinant gene expression is by use of an inducible promoter system.

The tetracycline (tet)-inducible promoter system is the preferred promoter system for inducible expression in mammalian cells (Bornkamm et al., 2005; Gossen and Bujard, 1992; Gossen et al., 1995; Urlinger et al., 2000). This system uses a tet-regulated transcription factor, either tet-off or tet-on, to control the activity of a tet-regulated promoter. Tet-regulated promoters contain tet-responsive elements (TRE) upstream of a basal promoter; one of the most commonly used constructs contains seven TRE upstream of a core cytomegalovirus immediate early (CMV IE) promoter (Gossen and Bujard, 1992). Tet-off is a negatively regulated element; the binding of tet to tet-off prevents the tet/tet-off complex from binding to TRE sequences, thereby repressing transcription (Gossen and Bujard, 1992). tet-on is a positively regulated element that acts in the inverse manner; binding of tet to tet-on causes the tet/tet-on complex to bind to TRE sequences and activate transcription (Gossen et al., 1995). The tet promoter system can support inducible expression in the brains of transgenic mice (Furth et al., 1994; Gossen et al., 1995; Shockett et al., 1995; Utomo et al., 1999). This promoter system has been incorporated into specific virus vector systems, including retrovirus vectors (Hofmann et al., 1996; Hwang et al., 1996; Matsuura et al., 2001; Yu et al., 1996), lentivirus vectors (Bahi et al., 2004; Georgievska et al., 2004; Kafri et al., 2000; Koponen et al., 2003; Markusic et al., 2005; Regulier et al., 2002; Vogel et al., 2004), adenoassociated virus (AAV) vectors (Chtarto et al., 2003; Fitzsimons et al., 2001; Folliot et al., 2003; Haberman et al., 1998; Jiang et al., 2004; Wang et al., 2005; Wilsey et al., 2002), adenovirus vectors (Bhattacharjee et al., 2004; Chen et al., 1998; Corti et al., 1999; Harding et al., 1997, 1998; Lee et al., 2005), and helper virus-containing Herpes Simplex Virus (HSV-1) vectors (Fotaki et al., 1997; Ho et al., 1996; Schmeisser et al., 2002) (reviews, Gossen and Bujard, 2002; Mansuy and Bujard, 2000; Toniatti et al., 2004). Due in part to the limited capacity of AAV vectors, retrovirus vectors, and lentivirus vectors, a frequently used vector design is a single transcription unit that uses a tet-regulated promoter to express either tet-on or tet-off, an internal ribosome entry site (ires), followed by a gene of interest (Hofmann et al., 1996). This autoregulatory design may increase the fold inducibility of the system by placing both the transcription factor and the gene of interest under the control of a tet-regulated promoter.

Advantages of HSV-1 vectors include efficient gene transfer and a large size, enabling insertion of multiple genes and genetic regulatory elements to support both cell type-specific and inducible expression of multiple genes. We have reported a large (51 kb) vector (Wang et al., 2000) and large vectors that coexpress 3 or 4 genes (Sun et al., 2004; Wang et al., 2001). Moreover, a 149-kb HSV-1 vector has been described (Wade-Martins et al., 2003). The published studies with HSV-1 vectors containing the tet promoter system used a helper virus and reported only short-term inducible expression in neurons in the brain (Fotaki et al., 1997; Ho et al., 1996; Schmeisser et al., 2002). Gene expression from the helper virus is known to cause side effects, including promoter silencing (Johnson et al., 1992a,b, 1994). Moreover, after two of the studies with the tet promoter system (Fotaki et al., 1997; Ho et al., 1996) were published, it was reported that specific HSV-1 proteins expressed during productive virus growth (specific infected cell proteins (ICP)) interfere with the proper regulation of the tet promoter system (Herrlinger et al., 2000).

We developed a helper virus-free HSV-1 vector system (Fraefel et al., 1996; Sun et al., 1999) that should eliminate the confounding effects of the helper virus (Johnson et al., 1992a,b, 1994), including the effects of specific HSV-1 ICP proteins on the tet-promoter system (Herrlinger et al., 2000), and enable long-term inducible expression from HSV-1 vectors that contain the tet promoter system. Of note, HSV-1 vectors that contain either a tyrosine hydroxylase (TH) promoter or a preproenkephalin promoter support long-term expression

(several months) in specific types of neurons (Jin et al., 1996; Kaplitt et al., 1994; Song et al., 1997). Also, we developed a modified neurofilament heavy gene promoter that supports long-term expression in forebrain neurons from helper virus-free HSV-1 vectors (Sun et al., 2003, 2004, 2005; Zhang et al., 2000). This modified neurofilament promoter contains a neurofilament heavy gene (NFH) promoter, upstream sequences from the TH promoter, and the best characterized mammalian insulator (INS) from the chicken β -globin locus (Zhang et al., 2000). A time course showed that a vector containing this promoter supported expression in similar numbers of striatal neurons between 2 weeks and 6 months after gene transfer (Zhang et al., 2000). In three other studies, vectors containing this promoter supported expression for 7, 8, or 14 months (Sun et al., 2003, 2004, 2005), the longest time points examined. Also, at 6 months after gene transfer, ~12,000 striatal neurons contained recombinant gene products (using 3 injection sites for gene transfer (Sun et al., 2004)).

The goal of this study was to obtain long-term, inducible expression from helper virus-free HSV-1 vectors. We used the tet-off transcription factor because it may support a higher fold induction than tet-on, in the context of a virus vector in the brain. We explored two different vector designs. Both designs contained two transcription units, for flexibility in constructing future vectors to support cell type-specific, inducible, coexpression of multiple genes. One design was an autoregulatory design; one transcription unit used a tet-regulated promoter to express tet-off, and a second transcription unit used a tet-regulated promoter to express the *Lac Z* gene. In the other design, one transcription unit used the modified neurofilament heavy gene promoter to express tet-off, and a second transcription unit used a tet-regulated promoter to express the *Lac Z* gene.

Our results showed that both vector designs supported inducible expression in cultured fibroblast or neuronal cell lines and at a short time (4 days) after gene transfer into the rat striatum. However, only the modified neurofilament promoter vector design supported long-term (2 months) inducible expression in striatal neurons.

2. Results

2.1. HSV-1 vectors that contain the tet-inducible promoter system, packaging into HSV-1 particles, and inducible expression in cultured cells

We evaluated two designs to incorporate the tet promoter system into HSV-1 vectors, an autoregulatory design (Fig. 1A) or a design that expressed tet-off from a modified neurofilament heavy gene promoter that supports long-term expression in forebrain neurons (Fig. 1b). These vectors were packaged into HSV-1 particles using our helper virus-free packaging system. The resulting vector stocks were titered on fibroblast cells; the numbers of infectious vector particles (IVP per milliliter) were determined by X-gal staining at 24 h after transduction of BHK cells. The results (Table 1) showed that each of the four vectors supported similar titers. The titration was performed on BHK fibroblast cells, as the best available assay. These fibroblast cells form a monolayer; in contrast, most neuronal cell lines, including PC12 cells, do not form a monolayer; and the titers obtained on BHK cells are higher than the titers obtained on PC12 cells (Yang et al., 2001; Zhang et al., 2000). Expression from the modified neurofilament promoter (as in pINS-TH-NFHtet-off/TRElac) in fibroblast cells represents ectopic expression; this ectopic expression declines rapidly at longer times after gene transfer (not shown). Next, we determined the titers of vector genomes (VG per milliliter) by isolating DNA from these vector stocks and performing PCR using primers from the *Lac Z* gene (Yang et al., 2001). As a measure of the packaging efficiency, we calculated the ratio of the physical titer to the biological titer (VG/IVP) for each vector stock. The results (Table 1) showed that each of the vectors supported a similar ratio of VG/IVP, and an ~10:1 ratio of VG:IVP is similar to that observed in a previous study that used vectors containing the modified neurofilament promoter (Yang et al., 2001). Thus, HSV-1 vectors that contained the tet-inducible promoter

system were efficiently packaged into HSV-1 particles using each of two different promoters to control expression of tet-off and with the two transcription units in either a sequential or divergent orientation.

We determined the fold induction of β -gal supported by each of these vectors in either fibroblast cells or PC12 cells, a neuronal cell line. Cells maintained under either uninduced conditions (doxy present) or induced conditions (no doxy) were transduced using these vector stocks, 1 day later, protein extracts were prepared, and the levels of β -gal activity were quantified. The results (Table 1) showed that the vectors with the tet-regulated promoter controlling tet-off (autoregulatory design) supported a higher fold-induction (35- to 69-fold) than the vectors with the modified neurofilament promoter controlling tet-off (21- to 26-fold). Each vector design supported a similar fold induction in fibroblast cells or PC12 cells (Table 1; autoregulatory design, 69- or 50-fold induction in BHK or PC12 cells, respectively; modified neurofilament promoter design, 25- or 26-fold induction in BHK or PC12 cells, respectively). Also, the results showed that for the autoregulatory design, the sequential orientation of the transcription units supported a higher fold induction than the divergent orientation (Table 1; 69- or 35-fold for the sequential or divergent orientations, respectively); and for the neurofilament promoter design, the sequential orientation supported a modest increase in the fold induction (25- or 21-fold for the sequential or divergent orientations, respectively). The vectors with the sequential orientation were used in subsequent experiments.

Next, we performed an experiment to determine if expression could be induced from the uninduced condition. Cultures of PC12 cells, under either uninduced or induced conditions, were transduced using the vector with the neurofilament promoter design in the sequential orientation. Twenty-four hours later, specific cultures were harvested, or maintained under their initial condition, or switched from the uninduced to induced condition, and these later cultures were harvested after another 24 or 48 h (48 or 72 h after transduction). The results showed that induced compared to uninduced conditions supported 27- to 30-fold induction between 24 to 72 h after gene transfer (Table 2). Of note, for specific cultures that were transduced using uninduced conditions and then induced at 24 h after gene transfer: Twenty-four hours of induction (cells harvested at 48 h after gene transfer) supported partial (12-fold) induction, and 48 h of induction (cells harvested 72 h after gene transfer) supported full (26-fold) induction (Table 2).

2.2. HSV-1 vectors containing the tet promoter system support long-term, inducible expression in striatal neurons

Vector stocks containing each of the two vector designs, and the sequential orientation of the transcription units, were microinjected into the striatum, and the rats were maintained under either induced or uninduced conditions. The rats were sacrificed 4 days, or 1 or 2 months after gene transfer, and X-gal staining was performed. Control rats, injected with PBS, lacked X-gal-positive striatal cells, but small numbers of cells that lined the brain vasculature contained faint X-gal staining (not shown). Using either vector, rats that were maintained under uninduced conditions and sacrificed at 4 days or 1 or 2 months after gene transfer, contained few X-gal-positive cells (pINS-TH-NFHtet-off/TRElac: 4 days, Fig. 2a; 1 month, Fig. 2d; 2 months, Fig. 2g. pTREtet-off/TRElac: 4 days, Fig. 2j; 1 month, Fig. 2m). In contrast, rats that were maintained under induced conditions, and sacrificed at 4 days after gene transfer, contained significant numbers of X-gal-positive cells proximal to the injection sites (pINS-TH-NFHtet-off/TRElac, Fig. 2b; pTREtet-off/TRElac, Fig. 2k). Of note, significant numbers of X-gal-positive cells were observed in rats that received the vector that expressed tet-off from the modified neurofilament promoter, were maintained under induced conditions, and were sacrificed at either 1 or 2 months after gene transfer (pINS-TH-NFHtet-off/TRElac: 1 month, Fig. 2e; 2 months, Fig. 2h). However, few X-gal-positive cells were observed in rats that

received the vector that expressed tet-off from the tet-regulated promoter, were maintained under induced conditions, and were sacrificed at 1 month after gene transfer (pTREtet-off/TRElac, Fig. 2n), and a 2-month time point was not examined using this vector. High power views showed that many of these X-gal-positive cells contained neuronal morphology, including-positive cell bodies with proximal processes, and distal processes that were not associated with a cell body (pINS-TH-NFHTet-off/TRElac: 4 days, Fig. 2c; 1 month, Fig. 2f; 2 months, Fig. 2i. pTREtet-off/TRElac: 4 days, Fig. 2l; 1 month, Fig. 2o). There were no signs of tissue damage in these sections at any of the time points examined, consistent with previous reports that helper virus-free HSV-1 vectors elicit a minimal immune response, and any small response decays rapidly (Bowers et al., 2003; Olschowka et al., 2003; Zhang and Geller, 2002). {In contrast, helper virus-containing HSV-1 vectors elicit a significant immune response (Wood et al., 1994)}. HSV-1 is known to infect axon terminals and be retrogradely transported to cell bodies (Stevens, 1975), and small numbers of positive cells were observed at distant sites, including the substantia nigra pars compacta and specific cortical areas that contain neurons that project to the striatum (not shown). Subsequent analyses focused on the X-gal-positive striatal cells, the clear majority of the X-gal-positive cells.

The efficiency of gene transfer supported by each vector was quantified using stereological counts of the X-gal-positive cells and the titers of the vector stocks. The efficiency of gene transfer into the brain was quantified as the number of X-gal-positive cells at 4 days after gene transfer (under induced conditions, Table 3) divided by the amount of vector that was injected. The vector using the autoregulatory design supported a 7.9% efficiency of gene transfer {2057 mean X-gal-positive cells at 4 days (Table 3) divided by 2.6×10^4 IVP injected (titer 4.4×10^6 IVP/ml (Table 1), 2 injection sites, 3 μ l injected per site)}. Similarly, the vector using the modified neurofilament promoter design supported an 8.7% efficiency of gene transfer {2274 mean X-gal-positive cells at 4 days (Table 3) divided by 2.6×10^4 IVP injected (titer 4.3×10^6 IVP/ml (Table 1), 2 injection sites, 3 μ l injected per site)}.

The fold induction supported by each vector, at each time point, was quantified by stereological counts of the X-gal-positive cells. The cell counts (Table 3) showed that the vector that used the autoregulatory design supported 34-fold induction at 4 days, but only 5-fold induction at 1 month because few X-gal-positive cells were present under induced conditions at 1 month. Of note, the vector that expressed tet-off from the modified neurofilament promoter supported 46-fold induction at 4 days, 30-fold induction at 1 month, and 32-fold induction at 2 months (Table 3). In the induced condition, the numbers of positive cells declined 7.5 to 10-fold between 4 days and 1 or 2 months (Table 3); by comparison, using a vector that contains only the modified neurofilament promoter, expression declined ~3-fold between 4 days and 2 weeks or 1 month (Zhang et al., 2000). At the longer time points, there were minimal numbers of positive cells in the uninduced condition.

We confirmed that the vector that used the modified neurofilament promoter design targeted expression to neurons. Double staining was performed using antibodies against either *E. coli* β -gal or the neuronal marker, NeuN. Rats that were maintained under uninduced conditions, and were sacrificed at 4 days after gene transfer, contained few faintly-positive β -gal-IR cells, and most of these cells contained NeuN-IR (Figs. 3a–c). Of note, rats that were maintained under induced conditions, and were sacrificed at 4 days, or 1 or 2 months after gene transfer, contained numerous β -gal-IR-positive cells, and most of these cells contained NeuN-IR (4 days, Figs. 3d–f; 1 month, Figs. 3g–i; 2 months, not shown). Rats that did not receive gene transfer lacked β -gal-IR cells but contained NeuN-IR-positive cells (Figs. 3j–l). Cell counts showed that, under induced conditions, this vector supported 85–90% neuronal-specific expression at 4 days, or 1 month, or 2 months after gene transfer (Table 4). Similarly, using the modified neurofilament promoter as the only promoter in the vector (pINS-TH-NFHLac), we observed 89% neuronal-specific expression at 4 days (Zhang et al., 2000), and other vectors

that contain this promoter supported ~90% neuronal-specific expression for up to 14 months (Sun et al., 2004).

We established that the vector that used the modified neurofilament promoter design supported inducible expression at the RNA level. At either 4 days or 1 month after gene transfer, RNA was isolated from rats that had been maintained under either uninduced or induced conditions. *Lac Z* RNA was detected using RT-PCR, and, as an internal control, β -actin RNA was detected in the same assays. The results (Fig. 4) showed significant levels of *Lac Z* RNA only under induced conditions; very low levels of *Lac Z* RNA were observed under uninduced conditions. As controls, RNAs prepared from rats that did not receive gene transfer lacked *Lac Z* RNA but contained β -actin RNA; and assays that omitted the reverse transcriptase lacked both *Lac Z* and β -actin RNAs. To quantify RNA levels, the gel was scanned, and the levels of *Lac Z* RNA in the different samples were compared after normalization using the levels of β -actin RNAs. The results showed that induced versus uninduced conditions supported 29- or 14-fold induction of *Lac Z* RNA at either 4 days or 1 month after gene transfer, respectively.

3. Discussion

We compared two different designs for HSV-1 vectors that contain the tet promoter system, an autoregulatory design or a modified neurofilament promoter design. The packaging efficiencies supported by these vectors, specifically both the biological titers (IVP/ml) and the physical titers (VG/ml), were similar to those supported by a number of other vectors (Fraefel et al., 1996; Sun et al., 1999, 2004, 2005; Wang et al., 1999; Zhang et al., 2000).

In cultured cells, either fibroblast cells or PC12 cells, the autoregulatory design supported higher fold inductions than the modified neurofilament promoter design. Both designs supported similar levels of expression in the induced condition. The higher fold inductions supported by the autoregulatory design appeared to be because the autoregulatory design supported lower levels of “leaky expression”, in the uninduced condition, compared to the modified neurofilament promoter design. These results are consistent with other reports that used the autoregulatory design in virus vectors to increase the fold induction (Shockett et al., 1995). Also, using PC12 cells and the modified neurofilament promoter design, we showed that expression can be induced from the repressed state, and full induction was achieved within 2 days.

The two vectors containing the sequential orientation of the transcription units were examined in the rat striatum. Both vector designs supported an efficiency of gene transfer into the striatum that was similar to the efficiency of gene transfer supported by HSV-1 vectors that contain specific viral promoters (Fraefel et al., 1996; Wang et al., 1999), or particular neuronal-specific or neuronal subtype-specific promoters (Wang et al., 1999), or the modified neurofilament promoter (Zhang et al., 2000). Both vector designs supported inducible expression at 4 days after gene transfer. However, the vector with the autoregulatory design supported minimal expression at 1 month after gene transfer.

The vector design that used the modified neurofilament promoter design supported inducible expression in cultured cells and long-term, inducible expression in striatal neurons. This vector supported ~25-fold induction in cultured fibroblast or neuronal cell lines. In the striatum, this vector supported 14- to 29-fold induction at the RNA level and 30- to 46-fold induction of X-gal-positive cells. As the X-gal assay is not a linear measure of β -gal activity, the fold induction at the protein level may be higher than that measured by counting the X-gal-positive cells. This vector supported expression for 2 months, the longest time examined. This vector supported 85 to 90% neuronal-specific expression, similar to the levels of neuronal-specific expression

supported by the modified neurofilament promoter in other studies (Sun et al., 2004, 2005; Zhang et al., 2000).

At 1 or 2 months after gene transfer, the modified neurofilament promoter design supported relatively modest numbers of X-gal-positive cells, ~200 to 300. In contrast, in a study that used the modified neurofilament promoter, at 6 months after gene transfer, ~12,000 striatal neurons contained recombinant gene products (Sun et al., 2004). There are three critical differences between these studies: First, the current study used only 2 injection sites for gene transfer, whereas the study that obtained long-term expression in 12,000 striatal neurons used 3 injection sites (Sun et al., 2004). Second, the titers (IVP/ml) in this study were ~5-fold lower than the titers in the study that reported long-term expression in ~12,000 striatal neurons (Sun et al., 2004). We have reported procedures (Sun et al., 1999) for obtaining the higher titers required for physiological studies, whereas the titers used here were sufficient for characterizing new vector designs. Third, under induced conditions, the numbers of positive cells declined 7.5- to 10-fold between 4 days and 1 or 2 months; in contrast, using the modified neurofilament promoter alone (pINS-TH-NFHLac), the numbers of positive cells declined only ~3-fold between 4 days and 2 weeks or 1 month (Zhang et al., 2000); thus, the vector using the neurofilament promoter alone supported ~2.5- to 3-fold higher long-term expression than the vector in the present study. Of note, the differences in number of injection sites and titers together represent a 7.5-fold difference in the amount of vector injected into the brain, which accounts for most of the difference in the levels of long-term expression between the current study and the study that reported long-term expression in ~12,000 striatal neurons (Sun et al., 2004).

The levels of long-term expression observed at 1 or 2 months after gene transfer may be maintained for longer periods. Using the modified neurofilament promoter alone, time courses in either the striatum or the hippocampus showed that the numbers of positive cells declined between 4 days and 2 weeks after gene transfer, but the numbers of positive cells at 2 weeks were similar to the numbers of positive cells at 1 or 2 months (hippocampus) or 1, 2, 4, or 6 months (striatum) (Zhang et al., 2000). Additionally, in three other studies, the modified neurofilament promoter supported expression for 7, 8, or 14 months (Sun et al., 2003, 2004, 2005), the longest time points examined. Thus, the vector design that used the modified neurofilament promoter to express tet-off may support inducible expression for times considerably longer than those examined here.

The autoregulatory vector design supported only short-term expression. This autoregulatory design is intended to support high-fold induction by expressing high levels of tet-off under induced conditions (Shockett et al., 1995). In the brain, in the context of HSV-1 vectors, these high levels of a negative regulatory element (essentially a repressor) may block expression. The positively regulated transcription factor tet-on (Gossen et al., 1995) might be better suited for this auto-regulatory design, at least in the context of HSV-1 vectors in the brain.

The initial tet-on transcription factor is leaky in the uninduced condition (Urlinger et al., 2000) and was not used in this study. However, improved versions of tet-on have been reported that support much tighter regulation (Urlinger et al., 2000). Furthermore, the improved tet-on have been coexpressed with a tet repressor (using an ires) to give high-fold inducible, positively regulated expression (Bornkamm et al., 2005; Lamartina et al., 2003). This preferred positive regulatory cassette could be inserted into the modified neurofilament vector design to replace tet-off.

Inducible, long-term, neuronal-specific expression from a HSV-1 vectors may have a number of applications to both gene therapy studies and studies in basic neuronal physiology. The HSV-1 vector design that uses the modified neurofilament promoter to express tet-off and a

tet-regulated promoter to express the *Lac Z* gene can be easily modified to replace the *Lac Z* gene with a gene of choice. HSV-1 vectors have a large capacity, 51-kb and 149-kb vectors have been efficiently packaged (Wade-Martins et al., 2003; Wang et al., 2000), and multiple genes might be placed under inducible control; a cassette with two tet-regulated promoters in a divergent orientation has been reported (Baron et al., 1995). To achieve neuronal subtype specificity with inducible, long-term expression, the modified neurofilament promoter in this vector design might be replaced by a promoter that limits expression to a specific type of neuron; for example, both the TH and the preproenkephalin promoters support long-term expression from HSV-1 vectors (Jin et al., 1996; Kaplitt et al., 1994; Song et al., 1997; Wang et al., 1999).

4. Experimental procedures

4.1. Materials

Restriction endonucleases and DNA modifying enzymes were obtained from New England BioLabs. Dulbecco's modified minimal essential medium (DMEM) fetal bovine serum (FBS), OPTI-MEM I, penicillin/streptomycin, and glutamine were from Invitrogen. Equine serum was obtained from HyClone. Tet-Off Gene Expression Systems, including pTet-Off and pTRE, were obtained from BD Biosciences (Clontech). Reagents for polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR), including primers, Platinum PCR SuperMix, and SuperScript III reverse transcriptase, were obtained from Invitrogen. RNeasy lipid tissue midi kit was obtained from Qiagen. Reagents for experiments with RNA, including RNaseZap, RNaseSecure, SUPER-aseIn, and QuantumRNA β -actin Internal Standard, were obtained from Ambion. Kaighn's modification of Ham's F12 (F-12K) was obtained from the ATCC. G418 was obtained from RPI. 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-Gal), tet, and doxycycline (doxy) were obtained from Sigma. Rabbit anti-*E. coli* β -galactosidase (β -gal) antibody was obtained from ICN, and mouse monoclonal anti-NeuN antibody was obtained from Chemicon. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Ig) G, biotinylated goat anti-mouse IgG, and the avidin-biotinylated peroxidase complex (ABC) reagent were obtained from Vector Laboratories. 2-Nitrophenyl- β -D-galactopyranoside (ONPG), fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, and rhodamine isothiocyanate-conjugated goat anti-mouse IgG were obtained from Sigma.

4.2. Cells

Baby hamster kidney fibroblast (BHK21) cells and 2–2 cells (Smith et al., 1992) were maintained in DMEM supplemented with 10% FBS, penicillin/streptomycin, and 4 mM glutamine (Yang et al., 2001). PC12 cells were grown in F-12K medium supplemented with 15% equine serum, 2.5% FBS, and penicillin/streptomycin. Cells were grown in humidified incubators containing 5% CO₂ at 37 °C. G418 (0.5 mg/ml) was present during the growth of 2–2 cells but was removed before plating the cells for HSV-1 vector packaging.

4.3. Plasmids and vectors

We constructed HSV-1 vectors that contain the tet promoter system, using two different designs (Fig. 1). Both designs contained two transcription units. One transcription unit contained the tet-controlled transcription factor (tet-off or tTA; a 37-kDa protein containing TetR and the VP16 activation domain (Gossen and Bujard, 1992)), and the second transcription unit contained the *Lac Z* gene under the control of a tet-regulated promoter (TRE). One design placed tet-off under the control of a TRE promoter (Fig. 1A), and the second design placed tet-off under the control of a modified neurofilament heavy gene promoter (Fig. 1B; INS-TH-NFH promoter (Zhang et al., 2000)).

HSV-1 vectors containing the tet-inducible promoter system were constructed in several steps. The first step was to place tet-off under the control of the INS-TH-NFH promoter. Tet-off was isolated by PCR (template, pTet-off; primers 5' GCCAAGCTTAAATTCATATGTCTAGATTAG 3' and 5' CATGTCTGGATCCTCGCGGCC 3'), and the PCR products were digested with *HindIII* and *BamHI*. The tet-off PCR fragment was cloned into pBRindi-73linker (Wang et al., 2000, 2001) that had been digested with *HindIII* and *BamHI* to yield the plasmid pBRtet-off. Next, a 7.8-kb fragment containing the INS-TH-NFH promoter was isolated from pINS-TH-NFHIac (Zhang et al., 2000) by a *Sall* complete and *HindIII* partial digestion. This fragment was inserted into pBRtet-off that had been digested with *XhoI* and *HindIII* (5.8 kb) to yield pINS-TH-NFHtet-off. Next, tet-off was placed under the control of a tet-regulated promoter. A 450-bp *XhoI* and *EcoRI* fragment from pTRE and 1-kb *EcoRI* and *BamHI* fragment from pTet-off were inserted into pBRindi-73linker (Wang et al., 2000, 2001) that had been digested with *BamHI* and *XhoI* to yield pTREtet-off. Next, the TRE promoter was inserted upstream of the *Lac Z* gene. A 471-bp *XhoI* and *BamHI* fragment from pTRE and a 4.1-kb *HindIII* and *BamHI* fragment from pINS-NFHIac (Zhang et al., 2000) (includes the *Lac Z* gene, β -globin intron and polyadenylation site) were inserted into pBR-8cutter/linker-I (Wang et al., 2001) that had been digested with *XhoI* and *BamHI* to yield a pBR-TRElac (7.4 kb). The 5.2-kb *PacI* fragment from pBR-TRElac was inserted into pHSVpUC-linker-II (contains HSV-1 vector backbone sequences (Wang et al., 2000, 2001)) that had been digested with *PacI* to yield pTRElac.

Next, vectors containing both tet-off and the *Lac Z* gene were isolated. The 2.2-kb *FseI* fragment from pTREtet-off was inserted into the 10.3-kb *FseI* fragment from pTRElac to yield pTREtet-off/TRElac (Fig. 1a). The 10.3-kb *FseI* fragment from pTRElac was inserted into the 10-kb *FseI* fragment from pINS-TH-NFHtet-off to yield pINS-TH-NFHtet-off/TRElac (Fig. 1b). We isolated two different versions of each vector design that contained the two transcription units in either a divergent or a sequential orientation. DNA sequence analysis confirmed the sequence of the tet-off PCR products and the junctions between the transcription units in these 4 vectors.

4.4. Packaging vectors into HSV-1 particles

Vectors were packaged into HSV-1 particles using the helper virus-free packaging system (Fraefel et al., 1996) and a modified protocol (Sun et al., 1999) that improves the efficiency. Vector stocks were purified and concentrated as described (Lim et al., 1996). Vector stocks were titered by counting the numbers X-gal-positive cells obtained at 1 day after infection of BHK fibroblast cells (infectious vector particles (IVP) per milliliter). The titers of vector genomes were determined by extracting DNA from the vector stocks and quantifying the amounts of vector DNAs using a PCR assay and primers for the *Lac Z* gene (Yang et al., 2001). Wild-type (wt) HSV-1 was not detected (<10 plaque forming units/ml) in any of these vector stocks.

4.5. Expression experiments in cell lines

BHK or PC12 cells (8×10^5 cells/60 mm dish) were incubated in medium that contained or lacked 1 μ g/ml doxy. Cells were grown to ~80% confluent before vector transduction (5×10^4 IVP/dish). Twenty-four hours later, the cells were rinsed with PBS, harvested on ice in PBS, and placed into a 1.5-ml microfuge tube for use in a quantitative β -gal assay. Alternatively, to examine uninduced conditions followed by induction of expression, PC12 cells were plated and transduced as above and harvested at the specific times detailed in the experiment (24, 48, or 72 h after gene transfer). The cells were collected by centrifugation, the cell pellet was resuspended in 1 ml PBS and recentrifuged. The cells were resuspended in 50 μ l of lysis buffer (10 mM Tris HCl pH 7.4, 0.25 M sucrose, 10 mM EDTA), and the cell

suspension was frozen and thawed three times. After a 10-min centrifugation at $18,000 \times g$ at 4°C , the supernatants were collected, the protein concentrations were determined using the BioRad Protein Assay reagent, and β -gal activity was assayed using the ONPG substrate (Hall et al., 1983). For each assay, 25 μl cell supernatant was mixed with 175 μl buffer Z (on ice; 64 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM MgSO_4 , 50 mM β -mercaptoethanol). Each reaction was initiated by addition of 40 μl ONPG (4 mg/ml in buffer Z). After incubation at 30°C for 30 min to 2 h, each reaction stopped by addition of 100 μl 1 M Na_2CO_3 at 4°C . The solutions were analyzed for absorbance (A_{420}) using a spectrophotometer. β -gal activity was defined as 1000 A_{420} /min/mg protein.

4.6. Stereotactic injection of HSV-1 vectors into the brain

All animal procedures were approved by the W. Roxbury VA Hospital IACUC. Male Sprague–Dawley rats (150–175 gm) were used for these experiments. Seven days before the gene transfer, some rats received water containing 2 mg/ml doxy, and this solution was replaced every other day. Vector stocks were delivered by stereotactic injection (2 sites, 3 μl /site) into the striatum (anterior–posterior (AP) +0.8, medial–lateral (ML) +2.5, dorsal–ventral (DV) –5.5; AP +0.8, ML –2.5, DV –5.5). AP is relative to bregma, ML is relative to the sagittal suture, and DV is relative to the bregma-lambda plane (Paxinos and Watson, 1986). Injections were performed using a micropump (model 100, KD Scientific); the 3 μl inoculum was injected over 8 min, and the needle was maintained in place for an additional 5 min before being slowly withdrawn over approximately 5 min. Water containing or lacking doxy was supplied daily after the surgery.

4.7. Immunohistochemistry and X-gal staining

Rats were perfused, and X-gal staining or immunohistochemistry was performed, as described (Zhang et al., 2000). These assays used 25- μm coronal brain sections that contained the striatum, and the sections were cut on a freezing microtome. To localize β -gal to neurons, sections were incubated with both anti- β -gal and anti-NeuN antibodies, and these antibodies were visualized using fluorescein isothiocyanate- or rhodamine isothiocyanate-conjugated secondary antibodies, as described (Zhang et al., 2000).

4.8. Cell counts

Twenty-five- μm coronal sections were prepared from the striatum that contained the area proximal to the injection sites. Every sixth section was analyzed for expression of β -gal, and 3–6 of these sections contained either the X-gal-positive cells or the β -gal-immunoreactivity (IR)-positive cells.

To quantify the numbers of X-gal-positive cells, stereology was performed using the optical dissector method and the StereoInvestigator program (MicroBrightField Inc). With reference to a rat atlas (Paxinos and Watson, 1986) and known landmarks, a contour was drawn around the striatum in each of the sections that contained the X-gal-positive cells. Stereological cell counts were performed under $60\times$ magnification. The counting frame area was $6200\text{ }\mu\text{m}^2$, 120 to 360 sample sites per hemisphere were counted, and the coefficient of error (CE) for each hemisphere was $\leq 10\%$. The statistical significance of the differences in the numbers of X-gal-positive cells were analyzed using Student's unpaired t test.

To quantify neuronal-specific expression, digital images of β -gal-IR-positive cells were obtained under $60\times$ magnification using either a fluorescein or a rhodamine filter, and cell counts were performed. To verify the accuracy of the cell counts, each section was counted at least two times independently, and the two values differed by $<10\%$ for each section.

4.9. RNA analysis

Rats that had received either doxy-containing or regular water were sacrificed at 4 days or 1 or 2 months after gene transfer. The striatum was dissected out of the brains, immediately frozen in dry ice-ethanol, and total RNA was isolated using the Qiagen RNeasy lipid tissue mini-kit. The RT reaction was performed using SuperScript III reverse transcriptase, and PCR SuperMix. QuantumRNA β -actin Internal Standards were added to standardize the amount of total RNA in each reaction. Ten micrograms of RNA was transcribed into cDNA in a 25 μ l reaction that contained a primer from the *Lac Z* gene (5' CACTTCAACATCAACGGTAATCG 3', complementary to nucleotides 2616 to 2638); the reaction was performed at 42 °C for 60 min. 5 μ l of the reaction products was amplified using PCR (one primer was the same primer as for the RT reaction, and the other primer was 5' GTTGATTGAACTGCCTGAACTACC 3', nucleotides 2034 to 2057). These two primers are specific for the *Lac Z* gene and are not homologous to any rat DNA sequences. The conditions for the PCR were determined using the β -actin internal standards to identify the linear range of the reaction. The PCR reaction was performed for 35 cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min) with a final extension time of 10 min at 72 °C. The PCR products were separated on a 1.2% agarose gel. Images of the gels were obtained and analyzed using Kodak Image Station 1000 and associated software. *Lac Z* RNA levels were obtained by comparing the scanned levels of the *Lac Z* RT-PCR products to the scanned levels of the β -actin RT-PCR products, according to the manufacturer's instructions (Ambion).

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References

- Bahi A, Boyer F, Kafri T, Dreyer JL. CD81-induced behavioural changes during chronic cocaine administration: in vivo gene delivery with regulatable lentivirus. *Eur J Neurosci* 2004;19:1621–1633. [PubMed: 15066158]
- Baron U, Freundlieb S, Gossen M, Bujard H. Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res* 1995;23:3605–3606. [PubMed: 7567477]
- Bhattacharjee AK, Ueyama T, Kondoh T, Hayashi S, Abouelfetouh A, Sakai N, Saito N, Kohmura E. In vivo transgene expression using an adenoviral tetracycline-regulated system with neuron-specific enolase promoter. *Biochem Biophys Res Commun* 2004;317:1144–1148. [PubMed: 15094388]
- Bornkamm GW, Berens C, Kuklik-Roos C, Bechet JM, Laux G, Bachl J, Korndorfer M, Schlee M, Holzel M, Malamoussi A, Chapman RD, Nimmerjahn F, Mautner J, Hillen W, Bujard H, Feuillard J. Stringent doxycycline-dependent control of gene activities using an episomal one-vector system. *Nucleic Acids Res* 2005;33:e137. [PubMed: 16147984]
- Bowers WJ, Olschowka JA, Federoff HJ. Immune responses to replication-defective HSV-1 type vectors within the CNS: implications for gene therapy. *Gene Ther* 2003;10:941–945. [PubMed: 12756414]
- Chen J, Bezdek T, Chang J, Kherzai AW, Willingham T, Azzara M, Nisen PD. A glial-specific, repressible, adenovirus vector for brain tumor gene therapy. *Cancer Res* 1998;58:3504–3507. [PubMed: 9721849]
- Chtarto A, Bender HU, Hanemann CO, Kemp T, Lehtonen E, Levivier M, Brotchi J, Velu T, Tenenbaum L. Tetracycline-inducible transgene expression mediated by a single AAV vector. *Gene Ther* 2003;10:84–94. [PubMed: 12525840]
- Corti O, Sanchez-Capelo A, Colin P, Hanoun N, Hamon M, Mallet J. Long-term doxycycline-controlled expression of human tyrosine hydroxylase after direct adenovirus-mediated gene transfer to a rat model of Parkinson's disease. *Proc Natl Acad Sci U S A* 1999;96:12120–12125. [PubMed: 10518586]

- Fitzsimons HL, McKenzie JM, During MJ. Insulators coupled to a minimal bidirectional tet cassette for tight regulation of rAAV-mediated gene transfer in the mammalian brain. *Gene Ther* 2001;8:1675–1681. [PubMed: 11892834]
- Folliot S, Briot D, Conrath H, Provost N, Cherel Y, Moullier P, Rolling F. Sustained tetracycline-regulated transgene expression in vivo in rat retinal ganglion cells using a single type 2 adeno-associated viral vector. *J Gene Med* 2003;5:493–501. [PubMed: 12797114]
- Fotaki ME, Pink JR, Mous J. Tetracycline-responsive gene expression in mouse brain after amplicon-mediated gene transfer. *Gene Ther* 1997;4:901–908. [PubMed: 9349426]
- Fraefel C, Song S, Lim F, Lang P, Yu L, Wang Y, Wild P, Geller AI. Helper virus-free transfer of Herpes Simplex Virus type 1 plasmid vectors into neural cells. *J Virol* 1996;70:7190–7197. [PubMed: 8794366]
- Furth PA, St Onge L, Boger H, Gruss P, Gossen M, Kistner A, Bujard H, Hennighausen L. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc Natl Acad Sci U S A* 1994;91:9302–9306. [PubMed: 7937760]
- Georgievska B, Jakobsson J, Persson E, Ericson C, Kirik D, Lundberg C. Regulated delivery of glial cell line-derived neurotrophic factor into rat striatum, using a tetracycline-dependent lentiviral vector. *Hum Gene Ther* 2004;15:934–944. [PubMed: 15585109]
- Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 1992;89:5547–5551. [PubMed: 1319065]
- Gossen M, Bujard H. Studying gene function in eukaryotes by conditional gene inactivation. *Annu Rev Genet* 2002;36:153–173. [PubMed: 12429690]
- Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. *Science* 1995;268:1766–1769. [PubMed: 7792603]
- Haberman RP, McCown TJ, Samulski RJ. Inducible long-term gene expression in brain with adeno-associated virus gene transfer. *Gene Ther* 1998;5:1604–1611. [PubMed: 10023439]
- Hall CV, Jacob PE, Ringold GM, Lee F. Expression and regulation of *Escherichia coli* lacZ gene fusions in mammalian cells. *J Mol Appl Genet* 1983;2:101–109. [PubMed: 6302193]
- Harding TC, Geddes BJ, Noel JD, Murphy D, Uney JB. Tetracycline-regulated transgene expression in hippocampal neurones following transfection with adenoviral vectors. *J Neurochem* 1997;69:2620–2623. [PubMed: 9375698]
- Harding TC, Geddes BJ, Murphy D, Knight D, Uney JB. Switching transgene expression in the brain using an adenoviral tetracycline-regulatable system. *Nat Biotechnol* 1998;16:553–555. [PubMed: 9624686]
- Herrlinger U, Pechan PA, Jacobs AH, Woiciechowski C, Rainov NG, Fraefel C, Paulus W, Reeves SA. HSV-1 infected cell proteins influence tetracycline-regulated transgene expression. *J Gene Med* 2000;2:379–389. [PubMed: 11045432]
- Ho DY, McLaughlin JR, Sapolsky RM. Inducible gene expression from defective Herpes Simplex Virus vectors using the tetracycline-responsive promoter system. *Mol Brain Res* 1996;41:200–209. [PubMed: 8883953]
- Hofmann A, Nolan GP, Blau HM. Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. *Proc Natl Acad Sci U S A* 1996;93:5185–5190. [PubMed: 8643550]
- Hwang JJ, Scuric Z, Anderson WF. Novel retroviral vector transferring a suicide gene and a selectable marker gene with enhanced gene expression by using a tetracycline-responsive expression system. *J Virol* 1996;70:8138–8141. [PubMed: 8892941]
- Jiang L, Rampalli S, George D, Press C, Bremer EG, O’Gorman MR, Bohn MC. Tight regulation from a single tet-off rAAV vector as demonstrated by flow cytometry and quantitative, real-time PCR. *Gene Ther* 2004;11:1057–1067. [PubMed: 15152187]
- Jin BK, Belloni M, Conti B, Federoff HJ, Starr R, Son JH, Baker H, Joh TH. Prolonged in vivo gene expression driven by a tyrosine hydroxylase promoter in a defective Herpes Simplex Virus amplicon vector. *Hum Gene Ther* 1996;7:2015–2024. [PubMed: 8930662]
- Johnson PA, Miyanochara A, Levine F, Cahill T, Friedmann T. Cytotoxicity of a replication-defective mutant of Herpes Simplex Virus type 1. *J Virol* 1992a;66:2952–2965. [PubMed: 1373198]

- Johnson PA, Yoshida K, Gage FH, Friedmann T. Effects of gene transfer into cultured CNS neurons with a replication-defective Herpes Simplex Virus type 1 vector. *Mol Brain Res* 1992b;12:95–102. [PubMed: 1312210]
- Johnson PA, Wang MJ, Friedmann T. Improved cell survival by the reduction of immediate-early gene expression in replication-defective mutants of Herpes Simplex Virus type 1 but not by mutation of the virion host shutoff function. *J Virol* 1994;68:6347–6362. [PubMed: 8083974]
- Kafri T, van Praag H, Gage FH, Verma IM. Lentiviral vectors: regulated gene expression. *Mol Ther* 2000;1:516–521. [PubMed: 10933976]
- Kaplitt MG, Kwong AD, Kleopoulos SP, Mobbs CV, Rabkin SD, Pfaff DW. Preproenkephalin promoter yields region-specific and long-term expression in adult brain after direct in vivo gene transfer via a defective herpes simplex viral vector. *Proc Natl Acad Sci U S A* 1994;91:8979–8983. [PubMed: 8090756]
- Koponen JK, Kankkonen H, Kannasto J, Wirth T, Hillen W, Bujard H, Yla-Herttuala S. Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2S-M2 shows a tight control of gene expression in vitro and in vivo. *Gene Ther* 2003;10:459–466. [PubMed: 12621450]
- Lamartina S, Silvi L, Roscilli G, Casimiro D, Simon AJ, Davies ME, Shiver JW, Rinaudo CD, Zampaglione I, Fattori E, Colloca S, Gonzalez Paz O, Laufer R, Bujard H, Cortese R, Ciliberto G, Toniatti C. Construction of an rtTA2(s)-m2/tts(kid)-based transcription regulatory switch that displays no basal activity, good inducibility, and high responsiveness to doxycycline in mice and non-human primates. *Mol Ther* 2003;7:271–280. [PubMed: 12597916]
- Lee YB, Cosgrave AS, Glover CP, Bienemann A, Heywood D, Hobson RJ, Uney JB. Increased utility in the CNS of a powerful neuron-specific tetracycline-regulatable adenoviral system developed using a post-transcriptional enhancer. *J Gene Med* 2005;7:576–583. [PubMed: 15580589]
- Lim F, Hartley D, Starr P, Lang P, Song S, Yu L, Wang Y, Geller AI. Generation of high-titer defective HSV-1 vectors using an IE 2 deletion mutant and quantitative study of expression in cultured cortical cells. *Biotechniques* 1996;20:460–469. [PubMed: 8679207]
- Mansuy IM, Bujard H. Tetracycline-regulated gene expression in the brain. *Curr Opin Neurobiol* 2000;10:593–596. [PubMed: 11084322]
- Markusic D, Oude-Elferink R, Das AT, Berkhout B, Seppen J. Comparison of single regulated lentiviral vectors with rtTA expression driven by an autoregulatory loop or a constitutive promoter. *Nucleic Acids Res* 2005;33:e63. [PubMed: 15809225]
- Matsuura N, Lie DC, Hoshimaru M, Asahi M, Hojo M, Ishizaki R, Hashimoto N, Noji S, Ohuchi H, Yoshioka H, Gage FH. Sonic hedgehog facilitates dopamine differentiation in the presence of a mesencephalic glial cell line. *J Neurosci* 2001;21:4326–4335. [PubMed: 11404418]
- Olschowka JA, Bowers WJ, Hurley SD, Mastrangelo MA, Federoff HJ. Helper-free HSV-1 amplicons elicit a markedly less robust innate immune response in the CNS. *Mol Ther* 2003;7:218–227. [PubMed: 12597910]
- Paxinos, G., Watson, C., 1986. *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Sidney.
- Regulier E, Pereira de Almeida L, Sommer B, Aebischer P, Deglon N. Dose-dependent neuroprotective effect of ciliary neurotrophic factor delivered via tetracycline-regulated lentiviral vectors in the quinolinic acid rat model of Huntington's disease. *Hum Gene Ther* 2002;13:1981–1990. [PubMed: 12427308]
- Schmeisser F, Donohue M, Weir JP. Tetracycline-regulated gene expression in replication-incompetent Herpes Simplex Virus vectors. *Hum Gene Ther* 2002;13:2113–2124. [PubMed: 12542843]
- Shockett P, Difilippantonio M, Hellman N, Schatz DG. A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice. *Proc Natl Acad Sci U S A* 1995;92:6522–6526. [PubMed: 7604026]
- Smith IL, Hardwicke MA, Sandri-Goldin RM. Evidence that the Herpes Simplex Virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. *Virology* 1992;186:74–86. [PubMed: 1309283]
- Song S, Wang Y, Bak SY, Lang P, Ullrey D, Neve RL, O'Malley KL, Geller AI. An HSV-1 vector containing the rat tyrosine hydroxylase promoter enhances both long-term and cell type-specific expression in the midbrain. *J Neurochem* 1997;68:1792–1803. [PubMed: 9109503]

- Stevens JG. Latent Herpes Simplex Virus and the nervous system. *Curr Top Microbiol Immunol* 1975;70:31–50. [PubMed: 169103]
- Sun M, Zhang GR, Yang T, Yu L, Geller AI. Improved titers for helper virus-free Herpes Simplex Virus type 1 plasmid vectors by optimization of the packaging protocol and addition of noninfectious Herpes Simplex Virus-related particles (previral DNA replication enveloped particles) to the packaging procedure. *Hum Gene Ther* 1999;10:2005–2011. [PubMed: 10466634]
- Sun M, Zhang G, Kong L, Holmes C, Wang X, Zhang W, Goldstein DS, Geller AI. Correction of a rat model of Parkinson's disease by coexpression of tyrosine hydroxylase and aromatic amino acid decarboxylase from a helper virus-free Herpes Simplex Virus type 1 vector. *Hum Gene Ther* 2003;14:415–424. [PubMed: 12691607]
- Sun M, Kong L, Wang X, Holmes C, Gao Q, Zhang W, Pfeilschifter J, Goldstein DS, Geller AI. Coexpression of tyrosine hydroxylase, GTP cyclohydrolase I, aromatic amino acid decarboxylase, and vesicular monoamine transporter-2 from a helper virus-free HSV-1 vector supports high-level, long-term biochemical and behavioral correction of a rat model of Parkinson's disease. *Hum Gene Ther* 2004;15:1177–1196. [PubMed: 15684695]
- Sun M, Kong L, Wang X, Lu X, Gao Q, Geller AI. Comparison of protection of nigrostriatal neurons by expression of GDNF, BDNF, or both neurotrophic factors. *Brain Res* 2005;1052:119–129. [PubMed: 16018990]
- Toniatti C, Bujard H, Cortese R, Ciliberto G. Gene therapy progress and prospects: transcription regulatory systems. *Gene Ther* 2004;11:649–657. [PubMed: 14985790]
- Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* 2000;97:7963–7968. [PubMed: 10859354]
- Utomo AR, Nikitin AY, Lee WH. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat Biotechnol* 1999;17:1091–1096. [PubMed: 10545915]
- Vogel R, Amar L, Thi AD, Saillour P, Mallet J. A single lentivirus vector mediates doxycycline-regulated expression of transgenes in the brain. *Hum Gene Ther* 2004;15:157–165. [PubMed: 14975188]
- Wade-Martins R, Saeki Y, Chiocci EA. Infectious delivery of a 135-kb LDLR genomic locus leads to regulated complementation of low-density lipoprotein receptor deficiency in human cells. *Mol Ther* 2003;7:604–612. [PubMed: 12718903]
- Wang Y, Yu L, Geller AI. Diverse stabilities of expression in the rat brain from different cellular promoters in a helper virus-free Herpes Simplex Virus type 1 vector system. *Hum Gene Ther* 1999;10:1763–1771. [PubMed: 10446916]
- Wang X, Zhang G, Yang T, Zhang W, Geller AI. Fifty-one kilobase HSV-1 plasmid vector can be packaged using a helper virus-free system and supports expression in the rat brain. *BioTechniques* 2000;27:102–106. [PubMed: 10649778]
- Wang X, Zhang G, Sun M, Geller AI. General strategy for constructing large HSV-1 plasmid vectors that co-express multiple genes. *BioTechniques* 2001;31:204–212. [PubMed: 11464513]
- Wang JJ, Niu DB, Zhang T, Wang K, Xue B, Wang XM. A tetracycline-regulatable adeno-associated virus vector for double-gene transfer. *Neurosci Lett* 2005;378:106–110. [PubMed: 15774267]
- Wilsey J, Zolotukhin S, Prima V, Shek EW, Matheny MK, Scarpace PJ. Hypothalamic delivery of doxycycline-inducible leptin gene allows for reversible transgene expression and physiological responses. *Gene Ther* 2002;9:1492–1499. [PubMed: 12407421]
- Wood MJ, Byrnes AP, Pfaff DW, Rabkin SD, Charlton HM. Inflammatory effects of gene transfer into the CNS with defective HSV-1 vectors. *Gene Ther* 1994;1:283–291. [PubMed: 7584093]
- Yang T, Zhang G, Zhang W, Sun M, Wang X, Geller AI. Enhanced reporter gene expression in the rat brain from helper virus-free HSV-1 vectors packaged in the presence of specific mutated HSV-1 proteins that affect the virion. *Mol Brain Res* 2001;90:1–16. [PubMed: 11376851]
- Yu JS, Sena-Esteves M, Paulus W, Breakefield XO, Reeves SA. Retroviral delivery and tetracycline-dependent expression of IL-1 β -converting enzyme (ICE) in a rat glioma model provides controlled induction of apoptotic death in tumor cells. *Cancer Res* 1996;56:5423–5427. [PubMed: 8968096]
- Zhang W, Geller A. In Chinese: comparison of the immune response to gene transfer into the rat brain with helper or helper virus-free HSV-1 vectors. *Huaxi Yide Daxue Xuebao* 2002;33:175–178.

Zhang G, Wang X, Yang T, Sun M, Zhang W, Wang Y, Geller AI. A tyrosine hydroxylase-neurofilament chimeric promoter enhances long-term expression in rat forebrain neurons from helper virus-free HSV-1 vectors. *Mol Brain Res* 2000;84:17–31. [PubMed: 11113528]

Fig. 1.

Schematic diagrams of pTREtet-off/TRElac (a) or pINS-TH-NFHetet-off/TRElac (b) with the sequential orientation of the transcription units. An HSV-1 origin of DNA replication (*ori_S*, small circle) and a HSV-1 *a* sequence (contains the packaging site, vertical line segment) support DNA replication and packaging into HSV-1 particles, respectively. The vectors contain two transcription units: the first transcription unit contains either the TRE promoter (a) or the INS-TH-NFH promoter (b) (gray segment), tet-off (black segment), and the second intron and polyadenylation site from the mouse β -globin gene (triangle and poly A, respectively). The second transcription unit contains the tet-regulated promoter (TRE promoter, gray segment), the *E. coli* Lac Z gene (black segment), and the second intron and polyadenylation site from the mouse β -globin gene. A cassette of 3 polyadenylation sites (tri A, horizontal line segment) was placed 3' to the fragment that contains the HSV-1 immediate early (IE) 4/5 promoter (cross hatched segment) to reduce any effects this promoter might have on recombinant gene expression. Sequences from pBR322 (clear segment) were included to support propagation of the vector in *E. coli*.

Fig. 2.

X-gal-positive striatal cells from rats sacrificed at 4 days to 2 months after gene transfer with HSV-1 vectors containing the tet promoter system. Rats received water containing or lacking doxy. (a–i) pINS-TH-NFHtet-off/TRElac. (a–c) Rats sacrificed at 4 days after gene transfer; (a) rat received doxy, low power view, and (b and c) rat did not receive doxy, low or high power views, respectively. The arrows in the high power view (c) indicate X-gal-positive cell bodies. (d–f) Rats sacrificed at 1 month after gene transfer; (d) rat received doxy, low power view, and (e and f) rat did not receive doxy, low or high power views, respectively. (g–i) Rats sacrificed at 2 months after gene transfer; (g) rat received doxy, low power view, and (h and i) rat did not receive doxy, low or high power views, respectively. (j–l) pTREtet-off/TRElac. (j–l) Rats sacrificed at 4 days after gene transfer; (j) rat received doxy, low power view, and (k and l) rat did not receive doxy, low or high power views, respectively. (m–o) Rats sacrificed at 1 month after gene transfer; (m) rat received doxy, low power view, and (n and o) rat did not receive doxy, low or high power views, respectively. Scale bars (a, b, d, e, g, h, j, k, m, n) 100 μ m; (c, f, i, l, o) 20 μ m.

Fig. 3.

β -gal-IR-positive striatal cells that also contain NeuN-IR from rats sacrificed at 4 days or 1 month after gene transfer with pINS-TH-NFHtet-off/TRElac. β -gal-IR was detected using a rabbit anti-*E. coli* β -gal antibody and was visualized using a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. In the same sections, NeuN-IR was detected using a mouse monoclonal anti-NeuN and was visualized using a rhodamine isothiocyanate-conjugated goat anti-mouse IgG. NeuN is a neuronal marker found in the nucleus. (a–c) The rat was sacrificed at 4 days after gene transfer, and the rat received doxy; (A) β -gal-IR, (b) NeuN-IR, (c) merged. Few β -gal-IR cells were observed, and most of these cells also contained NeuN-IR (arrows). (d–f) The rat was sacrificed at 4 days after gene transfer, and the rat did not receive doxy; (d) β -gal-IR, (e) NeuN-IR, (f) merged. Numerous β -gal-IR cells were observed, and most of these cells also contained NeuN-IR. (g–i) The rat was sacrificed at 1 month after gene transfer, and the rat did not receive doxy; (g) β -gal-IR, (h) NeuN-IR, (i) merged. (j–l) A rat that did not receive gene transfer; (j) β -gal-IR, (k) NeuN-IR, (l) bright field. Scale bar: 20 μ m.

Fig. 4.

Long-term, inducible expression of *Lac Z* RNA in the striatum of rats sacrificed at either 4 days or 1 month after receiving pINS-TH-NFHTet-off/TRElac. The rats received water containing or lacking doxy. Total RNA was isolated from striata, reverse transcription was performed using primers from the *Lac Z* gene and the β -actin gene, and the reaction products were amplified by PCR using the same primers as for RT and a second primer from each gene. The *Lac Z* primers are specific for this gene and do not recognize any rat genes. The RT-PCR products were displayed on an agarose gel. The predicted size of the *Lac Z* RT-PCR products is 605 bp, and the predicted size of the β -actin RT-PCR products is 294 bp. Lanes: – or +, rats received water lacking or containing doxy, respectively. Lanes: no vector, the rats did not receive gene transfer. Lanes: no RT, the rats received both gene transfer and doxy, but the RT was omitted from the reverse transcription reaction. Lane: plasmid DNA, pINS-TH-NFHTet-off/TRElac plasmid DNA isolated from *E. coli*.

HSV-1 vectors that contain the tet-inducible promoter system supported regulated expression in cultured BHK fibroblast cells or PC 12 cells at 1 day after gene transfer

Table 1

Vector	Orientation ^a	Purified titers ^b			β-gal activity ^c			
		VG/ml	IVP/ml	VG/IVP	BHK cells		PC12 cells	
					Doxy ^d	Fold induction	Doxy ^d	Fold induction
					+	–	+	–
pTREtet-off/TRElac	Sequential	5.6 × 10 ⁷	4.4 × 10 ⁶	13	3.5	240	6.2	307
pTREtet-off/TRElac	Divergent	5.1 × 10 ⁷	4.3 × 10 ⁶	12	2.6	90	35	50
pINS-TH-NFHTet-off/TRElac	Sequential	4.5 × 10 ⁷	4.3 × 10 ⁶	11	9.8	240	9.1	235
PINS-TH-NFHTET-OFF/TRElac	Divergent	4.8 × 10 ⁷	3.7 × 10 ⁶	13	12	240	21	26

^aThe two transcription units in the vector were in either a sequential or divergent orientation relative to each other.

^bThe titers of each vector stock following purification and concentration. VG/ml is vector genomes per milliliter. IVP per milliliter is infectious vector particles/ml.

^cβ-gal activity was measured using the ONPG substrate as 1000 A420/min/mg protein. Each condition was repeated 3 or 4 times, and the average values are shown.

^dDoxy, doxycycline.

Table 2

Using pINS-TH-NFHtet-off/TRElac, β -gal can be induced from the uninduced condition, in PC 12 cells

Period of Doxy (h) ^a	Culture harvested (h)	β -gal activity ^b	Fold induction
0–24	24	7.2	
None	24	214	30
0–48	48	7.0	
None	48	186	27
0–24	48	82	12
0–72	72	6.8	
None	72	190	28
0–24	72	178	26

Cells were plated at –24 h, and cultures were transduced at 0 h.

^aDoxy, doxycycline.

^b β -gal activity was measured using the ONPG substrate as 1000 A420/min/mg protein. Each condition was repeated 3 times, and the average values are shown.

Table 3
Long-term inducible expression in cells in the rat striatum from HSV-1 vectors that contain the tet-inducible promoter system

Vector	Average X-gal positive cells/striatum					
	4 days		1 month		2 months	
	Doxy ^a		Fold induction		Doxy ^a	
	+	-	+	-	+	-
pTREtet-off/TRElac	61 ± 22	2057 ± 323	34	5 ± 5	26 ± 24	5
pINS-TH-NFHTet-off/TRElac	49 ± 15	2274 ± 353	46	10 ± 8	303 ± 115	30
					7 ± 5	223 ± 33
						32

The titers of the vector stocks are in Table 1. Gene transfer used 2 injection sites in each striatum (see Experimental procedure for stereotactic coordinates), and 3 µl of vector stock was injected at each site. Three striata were analyzed for each condition and time point. The means ± SDs are shown.

^aDoxy, doxycycline.

Table 4

Long-term, neuronal-specific expression in the rat striatum from pINS-TH-NFHtet-off/TRElac

Time after gene transfer	Total β -gal-IR positive cells	β -gal-IR and NeuN-IR positive cells	% Costained cells
4 days	343 $\pm$ 46	310 $\pm$ 48	90
1 month	82 $\pm$ 14	70 $\pm$ 18	85
2 months	52 $\pm$ 14	47 $\pm$ 14	88

β -gal-IR was detected using a rabbit anti-*E. coli* β -gal antibody that was visualized with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG, and NeuN-IR was detected in the same sections using a mouse monoclonal anti-NeuN antibody that was visualized with a rhodamine isothiocyanate-conjugated goat anti-mouse IgG. The rats were maintained under induced conditions (no doxy); 3 striata were analyzed for each time point. The means $\pm$ SDs data are shown.