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Isolation of an enhancer from the rat tyrosine hydroxylase promoter that supports long-term, neuronal-specific expression from a neurofilament promoter, in a helper virus-free HSV-1 vector system

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Abstract

Direct gene transfer into neurons, using a virus vector, has been used to study neuronal physiology and learning, and has potential for supporting gene therapy treatments for specific neurological diseases. Many of these applications require high-level, long-term recombinant gene expression, in forebrain neurons. We previously showed that addition of upstream sequences from the rat tyrosine hydroxylase (TH) promoter to a neurofilament heavy gene (NF-H) promoter supports long-term expression in forebrain neurons, from helper virus-free Herpes Simplex Virus (HSV-1) vectors. This element in the TH promoter satisfied the definition of an enhancer; it displayed activity at a distance from the basal promoter, and in both orientations. This enhancer supported physiological studies that required long-term expression; a modified neurofilament promoter, containing an insulator upstream of the TH-NFH promoter, supported expression in ~11,400 striatal neurons at 6 months after gene transfer, and expression for 7, 8, or 14 months, the longest times tested. In contrast, the NF-H promoter alone does not support long-term expression, indicating that the critical sequences are in the 6.3 kb fragment of the TH promoter. In this study, we performed a deletion analysis to identify the critical sequences in the TH promoter that support long-term expression. We localized these critical sequences to an ~320 bp fragment, and two subfragments of ~100 bp each. Vectors that contained each of these small fragments supported levels of long-term, neuronal-specific expression that were similar to the levels supported by a vector that contained the initial 6.3 kb fragment of the TH promoter. These small fragments of the TH promoter may benefit construction of vectors for physiological studies, and may support studies on the mechanism by which this enhancer supports long-term expression.

Keywords

herpes simplex virus vector; long-term expression; tyrosine hydroxylase promoter; neurofilament heavy gene promoter; enhancer; insulator; striatal neuron

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1. Introduction

Gene transfer into neurons in the central nervous system has potential for both understanding neuronal physiology and for supporting gene therapy of specific neurological disorders. Virus vector systems that are being developed include Herpes Simplex Virus (HSV-1) plasmid vectors (amplicons) [8], recombinant HSV-1 vectors [4], adenovirus vectors [16], adeno-associated virus vectors [14], and lentivirus vectors [24]. Advantages of HSV-1 plasmid vectors include high-efficiency gene transfer and a large capacity, supporting coexpression of multiple genes from complex genetic regulatory elements that support both cell type-specific and inducible expression. We have reported vectors that coexpress 3 or 4 genes [38,44], and a large (51 kb) vector [45]. Additionally, a 149 kb HSV-1 vector has been described [42]. Of note, to reduce the side effects associated with gene transfer using helper virus-containing HSV-1 vectors, we developed a helper virus-free packaging system for HSV-1 plasmid vectors [5]. Due to these attractive properties, many scientists have modified neuronal physiology by expressing numerous genes from these vectors. However, a significant issue is the lack of mechanistic knowledge of promoter function in these vectors, a prerequisite for the rational design of promoters that support long-term expression.

Although a number of viral or neuronal-specific promoters do not support long-term expression (reviewed in [50]), specific promoters have been identified that support long-term expression from HSV-1 vectors. Examples of neuronal-specific promoters that do not support long-term expression include the neurofilament heavy gene (NF-H) promoter or a voltage-gated sodium channel promoter [46]. In contrast, five neuronal subtype-specific promoters support significant levels of long-term expression from HSV-1 plasmid vectors [11,13,27,35,46]. The preproenkephalin (ENK) promoter supports expression for 2 months in two brain areas that contain enkephalinergic neurons (amygdala and ventromedial hypothalamus, helper virus system [13]). The phosphate-activated glutaminase or vesicular glutamate transporter-1 promoters support glutamatergic neuron-specific expression; the glutamic acid decarboxylase promoter supports GABAergic neuron-specific expression; and these promoters support expression for 2 months (helper virus-free system [27]). Large, 6.8 kb or a 9 kb fragments, of the tyrosine hydroxylase (TH) promoter support expression for at least 2 months in midbrain dopaminergic neurons (helper virus system [11,35]; helper virus-free system [46]). In contrast, a small, 766 bp fragment, of the TH promoter does not support long-term expression (helper virus-free system [46]). These results suggested that upstream sequences in the TH promoter can enhance expression.

We used upstream sequences from the TH promoter to construct a chimeric promoter that supports long-term expression in forebrain neurons [50]. We fused upstream sequences from the TH promoter [2] to the 5' end of a NF-H promoter [33]. The TH-NFH promoter supported expression for 1 month in either perirhinal or postrhinal cortices, 2 months in the hippocampus, and 6 months in the striatum, (the longest time points examined; helper virus-free system [50]). Time courses showed that the levels of long-term expression declined initially, and then were stable: The numbers of positive cells at 2 weeks after gene transfer were 15 to 20 % of that observed at 4 days. Of note, the numbers of positive cells were similar between 2 weeks to 2 months in the hippocampus or between 2 weeks to 6 months in the striatum [50]. Interestingly, the element in the TH promoter that enhanced expression satisfied the definition of an enhancer [26]: The NF-H promoter fragment was not small, ~600 bp, and the TH promoter fragment was large, 6.3 kb (-0.6 to -6.8 kb); thus, the critical element in the TH promoter was located ~600 bp to 6.8 kb from the basal NF-H promoter/transcription start site. Both orientations of the TH promoter fragment enhanced expression, and this enhancement appeared to require specific sequences in the TH promoter fragment because substitution of a similar sized fragment from *E. coli* bacteriophage λ did not enhance expression [50]. In summary, the

TH promoter fragment contains an orientation-independent element that can act at a distance from the basal promoter/transcription start site, the definition of an enhancer [26].

Further improvements in long-term expression were obtained by adding the best characterized mammalian insulator (INS), from the chicken β -globin locus [50], to the TH-NFH promoter [50]. At 2 weeks to 1 month after gene transfer, the INS-TH-NFH promoter supported 30 to 40 % of the numbers of cells observed at 4 days; in contrast, at 2 weeks, the TH-NFH promoter supported 15 to 20 % of the number of cells observed at 4 days [50]. Vectors that contained the INS-TH-NFH promoter supported expression for 7, 8, or 14 months [38-40], the longest time points examined. Of note, at 6 months after gene transfer, ~11,400 striatal neurons contained recombinant gene products (using 3 injection sites for gene transfer, [38]). Furthermore, use of the INS-TH-NFH promoter to express a tetracycline-regulated transcription factor resulted in inducible, long-term (2 months) expression in striatal neurons [7].

As the INS-TH-NFH promoter supports significant levels of long-term expression, it will be informative to elucidate the mechanism(s) by which this promoter supports long-term expression. The TH promoter fragment is the critical element for long-term expression in the INS-TH-NFH promoter. The TH promoter fragment is large, ~6.3 kb. Thus, we undertook a deletion analysis to identify the critical sequences in the TH promoter fragment for supporting long-term expression. Our results identified two ~100 bp fragments, within an ~320 bp fragment, that, following fusion to the NF-H promoter, supported long-term expression in striatal neurons.

2. Results

2.1. Identification of a 1.2 kb fragment from the TH promoter that supports long-term expression from the NFH promoter, in striatal neurons

pTH-NFHlac (Fig. 1A) contains a 6.3 kb fragment of the TH promoter [50], and we replaced this TH promoter fragment with smaller fragments from the TH promoter. The strategy of this deletion analysis is diagrammed in Fig. 1B. The first set of deletions, isolated using PCR, divided the 6.3 kb fragment into six ~1.2 kb fragments; each fragment contained ~1 kb of unique sequence and ~100 bp of overlap at each end with the adjacent fragment. The use of ~100 bp overlaps at the ends of each fragment was intended to ensure that an essential element located near an end was not interrupted, and thus overlooked. These six fragments were labeled f1 through f6, and the corresponding vectors were labeled pTHf1-NFHlac through pTHf6-NFHlac; the nucleotides of the TH promoter fragment in each vector are listed in Table 1. These nucleotides are relative to the 5' end of the 6.3 kb fragment; these nucleotide numbers (n) can be converted to distance from either the TH promoter start site ($= -6,842 + n$) or distance from the NFH promoter start site in each vector ($= -6,957 + n$).

These six vectors, and two control vectors, were packaged into HSV-1 particles using our helper virus-free packaging system. pTH-NFHlac was the positive control, and pNFHlac was the negative control; pNFHlac does not support long-term expression in the brain [21,22,43, 46,50]. The resulting vector stocks were titered on Baby Hamster Kidney (BHK) fibroblast cells; the numbers of infectious vector particles (IVP/ml) were determined by 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-gal) staining at 24 hours after transduction of BHK cells. As the best available assay, the titering was performed on BHK fibroblast cells. These fibroblast cells form a monolayer; in contrast, PC12 cells, and most neuronal cell lines, do not form a monolayer; and the titers obtained on BHK cells are higher than the titers obtained on PC12 cells [47,50]. Expression from these modified neurofilament promoters in fibroblast cells represents ectopic expression; this ectopic expression declines rapidly at longer times after gene transfer (not shown). The titers ranged from 1.5 to 4×10^6 IVP/ml; specific vector stocks

were diluted, as indicated, to a titer of 1.5×10^6 (Table 1). Next, we determined the titers of vector genomes (VG/ml) by isolating DNA from these vector stocks and performing PCR using primers from the Lac Z gene [47]. As a measure of the packaging efficiency, for each vector stock, we determined the ratio of physical titer to biological titer (VG/IVP). The results (Table 1) showed that each of these vector stocks contained a similar ratio of VG/IVP, and an ~10:1 ratio of VG:IVP is similar to that observed in a number of other studies with vectors that contained either the NF-H promoter or specific modified neurofilament promoters [47]. Thus, HSV-1 vectors that contained specific ~1.2 kb fragments of the TH promoter were efficiently packaged into HSV-1 particles, with titers similar to those obtained using either pTH-NFHlac or pNFHlac.

Each of these vector stocks was microinjected into the striatum, the rats were sacrificed at 4 days, or 1 or 2 months after gene transfer, and staining for β -galactosidase (β -gal)-immunoreactive (IR) positive cells was performed using the avidin-biotinylated peroxidase complex (ABC) reagent and the horse radish peroxidase (HRP) reaction. Control rats that received PBS lacked β -gal-IR positive striatal cells (not shown). Using each of the vectors, rats sacrificed at 4 days after gene transfer contained numerous β -gal-IR cells proximal to the needle tracks (pTHf1-NFHlac, Fig. 2A; pTHf4-NFHlac, Fig. 2C), and high power views revealed β -gal-IR cells with neuronal morphology (pNFHlac, Fig. 2E; pTH-NFHlac, Fig. 2G; pTHf1-NFHlac, Fig. 2I; pTHf4-NFHlac, Fig. 2K; pTHf5-NFHlac, Fig. 2M). In contrast, at 1 month (not shown) or 2 months after gene transfer, significant numbers of β -gal-IR cells were observed using only specific vectors. Low power views showed no β -gal-IR cells proximal to a needle track using pTHf1-NFHlac (Fig. 2B), but numerous β -gal-IR cells using pTHf4-NFHlac (Fig. 2D). High power views showed that the positive control, pTH-NFHlac (Fig. 2H), or pTHf4-NFHlac (Fig. 2L) supported large number of β -gal-IR cells; pTHf5-NFHlac supported limited numbers of β -gal-IR cells (Fig. 2N); and using the negative control, pNFHlac (Fig. 2F), or pTHf1-NFHlac (Fig. 2J), pTHf2-NFHlac, pTHf3-NFHlac, or pTHf6-NFHlac (not shown), no β -gal-IR cells were observed.

Stereological methods were used to quantify the numbers of β -gal-IR positive striatal cells supported by each vector, in rats sacrificed at 4 days, or 1 or 2 months after gene transfer (Table 1). The efficiency of gene transfer was quantified as the ratio of β -gal-IR cells at 4 days divided by the biological titer (IVP) of vector that was injected into the striatum. To determine if there were any differences in the efficiency of gene transfer between the different vectors, we compared the efficiency of gene transfer supported by each of vector to that supported by pTH-NFHlac. Also, this ratio is designed to correct for any underestimation of the titers due to using fibroblast cells in the titering. The results (Table 1) showed that the efficiency of gene transfer supported by each vector was similar to that supported by pTH-NFHlac.

The level of long-term expression supported by each vector was quantified using the cell counts at 1 or 2 months after gene transfer (Table 1). Using the negative control, pNFHlac, no β -gal-IR cells were observed at 1 or 2 months, similar to results from previous studies [21,43,46, 50]. Of note, pTHf4-NFHlac supported similar numbers of β -gal-IR cells as the positive control, pTH-NFHlac (Table 1). pTHf5-NFHlac supported lower numbers of β -gal-IR cells than the positive control. Using each of the other 4 vectors (pTHf1-NFHlac, pTHf2-NFHlac, pTHf3-NFHlac, pTHf6-NFHlac), few, or no, positive cells were observed at 1 month, and no positive cells were observed at 2 months.

The numbers of positive cells were modest, ~100 to 130 at 1 or 2 months after gene transfer (Table 1). The experimental design used here differed in two critical aspects from our study that observed expression in ~11,400 cells at 6 months [38]; only one injection site, instead of three injection sites, was used, and the titers (IVP/ml) were ~10-fold lower (many of the vector stocks were diluted to yield a similar titer for each vector stock). The ~30-fold lower amount

of vector injected into the striatum accounts for most of the difference in the numbers of positive cells between the current study (Table 1) and the ~11,400 positive cells observed in an earlier study ([38]; see discussion).

We confirmed that the neurofilament promoter in these vectors targeted expression to neurons. Using sections from rats sacrificed at 4 days or 1 or 2 months after gene transfer, double staining was performed using antibodies against either *E. coli* β -gal or a neuronal marker, NeuN. Photomicrographs showed that rats sacrificed at 2 months after receiving either the positive control, pTH-NFHLac (Fig. 3A-C), or pTHf4-NFHLac (Fig. 3D-F), contained numerous β -gal-IR cells that also contained NeuN-IR; in contrast, a rat that received pTHf1-NFHLac (Fig. 3G-I) lacked β -gal-IR cells but contained NeuN-IR cells. Cell counts (Table 2) showed that at 4 days after gene transfer, all the vectors supported similar levels of neuronal-specific expression (~70 to 90 %). Vectors that supported long-term expression (pTH-NFHLac or pTHf4-NFHLac) continued to support neuronal-specific expression at 1 or 2 months (Table 2). In contrast, vectors that supported little (pTHf5-NFHLac) or no (pTHf2-NFHLac or pTHf3-NFHLac) long-term expression supported only low levels of neuronal-specific expression at 1 or 2 months.

In summary, these results showed that pTHf4-NFHLac supports long-term, neuronal-specific expression; pTHf5-NFHLac supports low levels of long-term expression with reduced neuronal specificity; and the other four vectors do not support long-term expression (Tables 1 and 2).

2.2. Identification of an ~320 bp fragment from the TH promoter that supports long-term expression from the NFH promoter, in striatal neurons

To further localize the critical sequence from the TH promoter fragment, we examined a second set of deletions. We constructed 3 vectors that contained sequences from THf4 (pTHf4-1-NFHLac, pTHf4-2-NFHLac, pTHf4-3-NFHLac); each vector contained an ~250-300 bp fragment of the TH promoter. Also, we constructed a vector that contained the ~120 bp overlap (ov) fragment present in both THf4 and THf5 (pTHf4-ov5-NFHLac). Additionally, we constructed 2 vectors that contained sequences from THf5 (pTHf5-1-NFHLac, pTHf5-2-NFHLac). The boundaries of the TH promoter fragment in each vector are presented in Table 3.

These 6 vectors were analyzed using the same strategy and assays as used to analyze the first set of deletions. These six vectors were efficiently packaged into HSV-1 particles (Table 3); the biological (IVP/ml) or physical (VG/ml) titers of each of these six vectors were similar to those obtained using the three control vectors (pTH-NFHLac, pNFHLac, or pTHf4-NFHLac). These six vectors, and the three control vectors, were injected into the striatum, the rats were sacrificed 4 days or 1 or 2 months later, and β -gal-IR cells were detected. Using each vector, rats sacrificed at 4 days contained numerous β -gal-IR cells proximal to the needle tracks (pTHf4-2-NFHLac, Fig. 4A), and high power views showed β -gal-IR cells with neuronal morphology (pNFHLac, Fig. 4C; pTHf4-NFHLac, Fig. 4E; pTHf4-2-NFHLac, Fig. 4G; pTHf4-3-NFHLac, Fig. 4I). Of note, at 1 month (not shown) or 2 months after gene transfer, significant numbers of β -gal-IR cells were observed using only pTHf4-2-NFHLac or either positive control (pTH-NFHLac or pTHf4-NFHLac). A low power view showed β -gal-IR cells proximal to the needle track using pTHf4-2-NFHLac (Fig. 4B). High power views showed that using the negative control, pNFHLac (Fig. 4D), no β -gal-IR cells were observed. In contrast, the positive control (pTHf4-NFHLac, Fig. 4F) or pTHf4-2-NFHLac (Fig. 4H) supported large number of β -gal-IR cells; pTHf4-ov5-NFHLac supported limited numbers of β -gal-IR cells (not shown); and using pTHf4-3-NFHLac (Fig. 4J), pTHf4-1-NFHLac, pTHf5-1-NFHLac, or pTHf5-2-NFHLac (not shown), no β -gal-IR cells were observed.

We used stereology to quantify the numbers of β -gal-IR striatal cells supported by each vector, at each time point (Table 3). The cell counts at 4 days, in combination with the titers, showed

that the efficiency of gene transfer supported by each vector was similar to that supported by the positive control, pTH-NFHLac. Of note, at 1 or 2 months, pTHf4-2-NFHLac supported similar numbers of β -gal-IR cells as the positive controls, pTH-NFHLac or pTHf4-NFHLac (Table 3). pTHf4-ov5-NFHLac supported lower numbers of β -gal-IR cells than the positive controls (Table 3). Using the negative control (pNFHLac), or each of the other 4 vectors (pTHf4-1-NFHLac, pTHf4-3-NFHLac, pTHf5-1-NFHLac, pTHf5-2-NFHLac), few, or no, positive cells were observed at 1 month, and no positive cells were observed at 2 months.

We confirmed that the neurofilament promoter in these vectors targeted expression to neurons, by costaining using antibodies against either β -gal or NeuN. Photomicrographs showed that a rat sacrificed at 2 months after receiving pTHf4-2-NFHLac (Fig. 5A-C) contained numerous β -gal-IR cells that also contained NeuN-IR, and a rat that received pTHf4-ov5-NFHLac (Fig. 5D-F) contained some β -gal-IR cells that also contained NeuN-IR and some β -gal-IR cells that lacked NeuN-IR. Cell counts (Table 4) showed that at 4 days after gene transfer, all the vectors supported similar levels of neuronal-specific expression (~70- to 85 %). Vectors that supported long-term expression (pTHf4-NFHLac or pTHf4-2-NFHLac) continued to support neuronal-specific expression at 1 or 2 months (Table 4). In contrast, vectors that supported little (pTHf4-ov5-NFHLac) or no (pTHf4-1-NFHLac or pTHf4-3-NFHLac) long-term expression supported only low levels of neuronal-specific expression at 1 or 2 months.

In summary, these results showed that pTHf4-2-NFHLac supports long-term, neuronal-specific expression; pTHf4-ov5-NFHLac supports low levels of long-term expression with reduced neuronal specificity; and the other four vectors do not support long-term expression (Tables 3 and 4).

2.3. Identification of two ~100 bp fragment from the TH promoter that support long-term expression from the NFH promoter, in striatal neurons

To further localize the critical sequence from the TH promoter fragment, we examined a third set of deletions. We constructed five vectors that contained sequences from THf4-2 (pTHf4-2-A-NFHLac through pTHf4-2-E-NFHLac); each vector contained an ~70 to 100 bp fragment of the TH promoter, with ~40 bp overlaps between adjacent fragments to ensure that no essential elements were overlooked. The boundaries of the TH promoter fragment in each vector are presented in Table 5.

This third set of vectors was analyzed using the strategy and assays detailed above. These five vectors were efficiently packaged into HSV-1 particles (Table 5); the biological (IVP/ml) or physical (VG/ml) titers of each vector were similar to those obtained using the control vectors (pTH-NFHLac, pNFHLac, or pTHf4-2-NFHLac). These experimental and control vectors were injected into the striatum, the rats were sacrificed 4 days or 1 or 2 months later, and β -gal-IR cells were detected. Using each vector, rats sacrificed at 4 days contained numerous β -gal-IR cells proximal to the needle tracks (pTHf4-2-A-NFHLac, Fig. 6A), and high power views showed β -gal-IR cells with neuronal morphology (pTHf4-2-A-NFHLac, Fig. 6C; pTHf4-2-B-NFHLac, Fig. 6E; pTHf4-2-C-NFHLac, Fig. 6G). In contrast, at 1 month (not shown) or 2 months after gene transfer, significant numbers of β -gal-IR cells were observed using only pTHf4-2-A-NFHLac or pTHf4-2-C-NFHLac, or either positive control. A low power view showed β -gal-IR cells proximal to the needle track using pTHf4-2-A-NFHLac (Fig. 6B). Of note, high power views showed that pTHf4-2-A-NFHLac (Fig. 6D) or pTHf4-2-C-NFHLac (Fig. 6H) supported large number of β -gal-IR cells. In contrast, using pTHf4-2-B-NFHLac (Fig. 6F), or pTHf4-2-D-NFHLac or pTHf4-2-E-NFHLac (not shown), no β -gal-IR cells were observed.

We quantified the numbers of β -gal-IR striatal cells supported by each vector, at each time point, using stereology (Table 5). The cell counts at 4 days, in combination with the titers, showed that the efficiency of gene transfer supported by each vector was similar to that

supported by the positive control, pTH-NFHlac. Of note, at 1 or 2 months, pTHf4-2-A-NFHlac or pTHf4-2-C-NFHlac supported similar numbers of β -gal-IR cells as the positive controls, pTH-NFHlac or pTHf4-2-NFHlac (Table 5). In contrast, using each of the other 3 vectors (pTHf4-2-B-NFHlac, pTHf4-2-D-NFHlac, or pTHf4-2-E-NFHlac), or the negative control (pNFHlac), no positive cells were observed at either 1 or 2 months.

We confirmed that the neurofilament promoter in these vectors targeted expression to neurons, by costaining using antibodies against either β -gal or NeuN. Photomicrographs showed at 2 months pTHf4-2-A-NFHlac (Fig. 7A-C) or pTHf4-2-C-NFHlac (Fig. 7G-I) supported numerous β -gal-IR cells that also contained NeuN-IR. In contrast, pTHf4-2-B-NFHlac (Fig. 7D-F) lacked β -gal-IR cells. Cell counts (Table 6) showed that at each time point, pTHf4-2-A-NFHlac, pTHf4-2-C-NFHlac, or the positive control (pTHf4-2-NFHlac), supported similar levels of neuronal-specific expression (~70 to 85 %).

2.4. The stabilities of long-term expression supported by these vectors

We compared the stabilities of long-term expression supported by these vectors by evaluating the numbers of β -gal-IR cells at 1 or 2 months divided by the number of β -gal-IR cells at 4 days. This ratio is intended to be independent of any variability in either the titering or the gene transfer process because this ratio uses the number of β -gal-IR cells at 4 days as the initial value. For control vectors that were examined in more than one set of deletions, the values across the sets were averaged. The results (Fig. 8) showed that pTH-NFHlac, pTHf4-NFHlac, pTHf4-2-NFHlac, pTHf4-2-A-NFHlac, or pTHf4-2-C-NFHlac each supported a similar stability of long-term expression, ~20 to 30 %, at either 1 or 2 months. In contrast, pTHf5-NFHlac or pTHf4-5ov-NFHlac supported a lower level of long-term expression, <20 % at 1 month and ~10 % at 2 months. The negative control, pNFHlac, did not support long-term expression.

3. Discussion

3.1. Two ~100 bp fragment from the TH promoter support long-term expression from the NFH promoter, in striatal neurons

At 1 or 2 months after gene transfer, modest numbers of β -gal-IR cells, ~100 to 250, were observed, using the vectors that supported long-term expression (pTH-NFHlac, pTHf4-NFHlac, pTHf4-2-NFHlac, pTHf4-2-A-NFHlac, or pTHf4-2-C-NFHlac). In contrast, in a study that used the INS-TH-NFH promoter, at 6 months after gene transfer, ~11,400 striatal neurons contained recombinant gene products [38]. Two differences between these studies account for most of the differences in the numbers of positive cells: First, the current study used only one injection site for gene transfer, whereas the study that obtained long-term expression in ~11,400 striatal neurons used three injection sites [38]. Second, in the current study, most of the vector stocks were diluted to match the lowest titer in a specific set of vector stocks; thus, the titers (IVP/ml) in this study were ~10-fold lower than the titers in the study that reported long-term expression in ~11,400 striatal neurons [38]. Together, these two issues account for an ~30-fold difference in the numbers of positive cells, most of the difference in the numbers of positive cells between these two studies. We have described procedures [41] to obtain the higher titers required for physiological studies; the titers of the current vector stocks were sufficient to test specific vectors for supporting long-term expression. Additionally, in the study that observed long-term expression in ~11,400 striatal neurons, the vector contained the INS-TH-NFH promoter [38]; addition of the INS to the initial TH-NFH promoter improved long-term expression [50]; analogously, addition of the INS to the smaller TH-NFH promoters developed here may further improve long-term expression.

THf4-2-A and THf4-2-C each supported long-term (2 months) expression when fused to the 5' end of the NF-H promoter. Consistent with this result, larger fragments that contained these two fragments (THf4-2, THf4) also supported long-term expression. The levels of long-term expression supported by THf4-2-A or THf4-2-C were similar to that supported by the initial fragment (TH -0.6 to -6.8 kb). In this study, the initial 6.3 kb TH promoter fragment, and specific smaller fragments derived from the initial fragment, supported 20 to 30 % of the number of cells at 1 or 2 months compared to 4 days, as assayed by β -gal-IR. In our earlier study that developed the TH-NFH promoter, at 2 weeks we observed 15 to 20 % of the number of cells compared to 4 days, as assayed by X-gal staining [50]. The β -gal-IR assay used here lacks the low level of background staining obtained using X-gal, which may explain the modestly higher stabilities of long-term expression reported here compared to the earlier study [50].

THf4-2-A and THf4-2-C localize the critical elements for long-term expression to short sequences. THf4-2-A is 84 bp, and contains 44 bp of unique sequence; the remaining sequence is also contained in THf4-2-B, which did not support long-term expression. THf4-2-C is 104 bp, and contains 24 bp of unique sequence, the remaining sequence is also contained in either THf4-2-B or THf4-2-D, which did not support long-term expression. It seems plausible that the element(s) that support long-term expression are located in the unique sequence of either THf4-2-A or THf4-2-C, although a specific element could extend into a repeated sequence. DNA sequence homology analyzes did not reveal any element present in both THf4-2-A and THf4-2-C, and sequences in either THf4-2-A and THf4-2-C were not repeated elsewhere in the initial 6.8 kb fragment of the TH promoter. Of note, THf4-2-A and THf4-2-C each contained putative binding sites for multiple transcription elements (determined using the Transfac data base).

It appears likely that these two ~100 bp fragments (THf4-2-A or THf4-2-C) each contain an enhancer. The improved long-term expression is not a sequence-independent spacing effect because insertion of a 5.6 fragment from *E. coli* bacteriophage λ at the 5' end of the NF-H promoter did not enhance expression [50]. Furthermore, localization of the critical element(s) in the initial 6.3 kb TH promoter fragment to two small ~100 bp fragments suggests that specific genetic regulatory elements support long-term expression. As the NF-H fragment is ~600 bp, each of these two ~100 bp fragments could act at a distance from the basal promoter/transcription start site. Both orientations of the initial 6.3 kb TH promoter fragment supported long-term expression [50]; however, these two ~100 bp fragments were not tested in the reverse orientation. Nonetheless, these two fragments account for the vast majority of the long-term expression activity of the initial 6.3 kb TH promoter fragment; consequently, as the initial fragment has activity in both orientations, it seems plausible that these smaller fragments will also have activity in both orientations. Thus, the data are consistent with the definition of an enhancer, an orientation-independent element that can act at a distance from the basal promoter/transcription start site [26].

The THf4-ov5 fragment supported low levels of long-term expression. At 2 months, the THf4-ov5 fragment supported approximately one sixth the number of positive cells as the initial TH promoter fragment, or either THf4-2-A or THf4-2-C. Also, the THf4-ov5 fragment did not support neuronal-specific expression (35 to 40 %); in contrast, the initial TH promoter fragment, or either THf4-2-A or THf4-2-C, supported neuronal-specific expression (70 to 80 %). Because of these differences in the expression supported by THf4-ov5 compared to the initial TH promoter fragment, it seems unlikely that THf4-ov5 contains important elements for supporting long-term expression in forebrain neurons.

3.2. Comparisons to previous analyses of the TH promoter

Specific genetic elements that support short-term changes in the activity of the TH promoter have been identified; these elements include CREB, AP1, POU, AP2, or E2A/MyoD. Specific binding sites for these elements in the TH promoter have been shown to be functional; for example, either CREB or AP1 can regulate the activity of the TH promoter [6,9,10,15,17,18, 48]. However, the binding sites for these transcription factors are located proximal to the transcription start site in the TH promoter, and are not present in the initial upstream fragment of the TH promoter (-0.6 to -6.8 kb) that supports long-term expression from the NF-H promoter.

Studies in transgenic mice have shown that large fragments of the TH promoter, up to 9 kb, are required for catecholaminergic neuron-specific expression [1,12,23,30]. Of note, transgenic mice containing 0.15 or 2.4 kb fragments of the rat TH promoter [23], or 0.2 or 2.5 kb fragments of the human TH promoter [30], did not support catecholaminergic neuron-specific expression. Transgenic mouse founder analysis suggested that different regions of the TH promoter are required for catecholaminergic neuron-specific expression in the locus coeruleus, hypothalamus, brainstem, or midbrain [20]. However, large (several kb), and overlapping, fragments were examined in this study [20], confounding specific sequence comparisons. Similar to the results in transgenic mice, HSV-1 vectors that contain 6.8 or 9.0 kb fragments of the TH promoter supported catecholaminergic neuron-specific expression [11,35,46]. Based on sequence comparisons between the TH promoters of different species, five short sequences in the human TH promoter were identified as conserved sequences [29]. However, a lentivirus vector that contained a basal TH promoter and these 5 sequences did not support catecholaminergic neuron-specific expression [29]. The locations of these 5 sequences in the human TH promoter place them outside of THf4-2-A or THf4-2-C, which are derived from the rat TH promoter. In summary, previous studies on the TH promoter, which focused on identifying elements that support catecholaminergic neuron-specific expression, do not inform our isolation of two sequences in the TH promoter that enhance long-term expression from a heterologous promoter, the NF-H promoter.

3.3. The NF-H promoter, passively regulated by the absence of the neuronal silencer element, can be activated by heterologous enhancers

A number of neuronal-specific promoters, including the NF-H promoter, contain the neuronal silencer (REST) element [3,31,32]. These negatively regulated promoters are passively activated in neurons in the absence of the neuronal silencer, and are actively repressed in nonneuronal cells (by the silencer). It is possible that these cellular promoters are particularly vulnerable to the mechanisms that shut off expression from almost all HSV-1 promoters as the virus enters the latent state [28,36,37]. Conversely, as these negatively regulated cellular promoters lack enhancers, addition of an enhancer may support significant increases in long-term expression. We have shown that specific short sequences from the TH promoter, and an ~1 kb upstream sequence from the ENK promoter [43], can enhance long-term expression from the NF-H promoter. It remains to be determined if these sequences from the TH or ENK promoters can enhance long-term expression from other promoters that contain the neuronal silencer, or specific promoters that are regulated by a different mechanism.

3.4. Future directions

THf4-2, THf4-2-A, or THf4-2-C may be used to improve the INS-TH-NFH promoter. The current INS-TH-NFH promoter has supported expression for 7, 8, or 14 months [38-40]; coexpression of up to 4 genes from two transcription units (using ires) [38]; inducible, long-term expression in neurons [7]; and physiological studies, on topics as diverse as gene therapy for Parkinson's disease or enhancing visual learning [38-40,49]. The current INS-TH-NFH promoter contains an ~6.3 kb fragment of the TH promoter, and smaller versions of the INS-

TH-NFH promoter will be easier to use in vector constructions, may support higher levels of long-term expression, and may support coexpression of larger numbers of genes.

Specific transcription factor binding sites in THf4-2-A or THf4-2-C, and the cognate transcription factors, may be identified using standard of molecular biology techniques, such as gel shifts or yeast 1 or 2 hybrid screens. Mechanistic information about how specific elements in the TH promoter enhance long-term expression may guide rational vector design to further improve long-term expression.

4. Materials and Methods

4.1 Materials

DNA modifying enzymes and restriction endonucleases were obtained from New England BioLabs. OPTI-MEM I, penicillin/streptomycin, glutamine, Dulbecco's modified minimal essential medium (DMEM), and fetal bovine serum (FBS) were from Invitrogen. Reagents for PCR, including primers and Platinum PCR SuperMix, were obtained from Invitrogen. G418 was obtained from RPI. X-Gal was obtained from Sigma. Rabbit anti-*E. coli* 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 ABC reagent were obtained from Vector Laboratories. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Ig)G, and rhodamine isothiocyanate-conjugated goat anti-mouse IgG were obtained from Sigma.

4.2. Cells

Cells were grown in humidified incubators containing 5 % CO₂ at 37 °C. BHK21 cells [47] or 2-2 cells [34] were grown in DMEM supplemented with 10 % FBS, 4 mM glutamine, and penicillin/streptomycin. G418 (0.5 mg/ml), present during the growth of 2-2 cells, was removed before plating cells for vector packaging.

4.3. Vectors

pTH-NFHIac has been described [50], and is diagrammed in Fig. 1A. pTH-NFHIac contains a 6.3 kb BamH I fragment from the rat TH promoter (-0.5 kb to -6.8 kb [2]) inserted at the 5' end (BamH I site) of a 0.6 kb fragment of the mouse NFH promoter (from plasmid pH-615 [33]). The TH promoter fragment is flanked by BamH I sites, and these sites were used to excise this fragment and insert smaller fragments from the TH promoter. To attempt to reduce interactions between the vector backbone and the transcription unit, the HSV-1 IE 4/5 promoter in the HSV-1 ori_s fragment was followed by a cassette of three SV40 early region polyadenylation sites [46].

We constructed HSV-1 vectors that placed specific fragments of the TH promoter 5' to the NFH promoter. We used PCR to isolate specific fragments of the TH promoter (Fig. 1B; PCR primers and nucleotide positions are listed below). The PCR products were digested with the appropriate combination of BamH I, Bcl I, or Bgl II; each of these enzymes yields the same overhang (5' GATC 3'). pTH-NFHIac was digested with BamH I, the large fragment was isolated (Fig. 1A), and the PCR products were inserted into this fragment. Candidate vectors with the correct orientation of the TH promoter fragment were identified by PCR using the 5' (sense) primer from each TH promoter fragment (listed below) and a primer from the NFH promoter (5' GTGTTCCCAGATACTACACGCGGC 3'; antisense to nucleotides 97 to 121). Candidate vectors were also analyzed by digestion with BamH I, or Spe I, or Pst I. For the second and third sets of deletions, the TH promoter fragments were also analyzed by DNA sequencing (performed by the core facility at Brigham and Women's Hospital).

The template for all the PCR reactions was pTH-NFHLac. The nucleotide numbers listed for the PCR primers use the 5' end of the 6.3 kb BamH I fragment from TH promoter fragment as nucleotide 1. TH promoter fragment f1: 5' GCGCATGTCTGGATCCTCCTGCCTCTGC 3' (nucleotides 1 to 18, contains BamH I site), and 5' GCGAGATCTCCAACCTGTGCACCAGTGAGTCACG 3' (antisense to nucleotides 1,208 to 1,232, contains Bgl II site). f2: 5' CGCGGATCCGGGAACTGAATGTCACTCTTATTGC 3' (nucleotides 1,124 to 1,148, contains BamH I site), and 5' GCGAGATCTCTCAACCTAGGCTCTAGGCCC 3' (antisense to nucleotides 2,322 to 2,342, contains Bgl II site). f3: 5' CGCGGATCCGGGCTGCCTGGAAGATACTCTGG 3' (nucleotides 2,228 to 2,250, contains BamH I site), and 5' GGGAGATCTGGGCACTATGGAAACCTATGTGC 3' (antisense to nucleotides 3,428 to 3,450, contains Bgl II site). f4: 5' CGCGGATCCGCTTCTGCGTGTCTATGGTAGAAGG 3' (nucleotides 3,344 to 3,368, contains BamH I site), and 5' CGCTGATCACAACCTGCTGGCCCAGACTCTTGC 3' (antisense to nucleotides 4,541 to 4,563, contains Bcl I site). f5: 5' CGCGGATCCGCACAATGTAGTCAGCCAGCTCCG 3' (nucleotides 4,432 to 4,455, contains BamH I site), and 5' TGGCATGATCACTGAAGTCTCACTGC 3' (antisense to nucleotides 5,555 to 5,580, contains Bcl I site). f6: 5' GCGAGATCTCTGACTCAGCATTTATCCTGCTCC 3' (nucleotides 5,387 to 5,410, contains Bgl II site), and 5' GTGCAGTGTTCAGATACTACACG 3' (antisense to nucleotides 102 to 126 in the NFH promoter; the PCR products contain a BamH I site). f4-1: 5' CGCGGATCCAAAGCTATGGGGTAGATCTG 3' (nucleotides 3,451 to 3,470, contains BamH I site), and 5' GCGAGATCTTTTACACCCCTGTAATGCCT 3' (antisense to nucleotides 3,751 to 3,770, contains Bgl II site). f4-2: 5' CGCGGATCCGGGCTCAGGAAGGAGGGAG 3' (nucleotides 3,771 to 3,789, contains BamH I site), and 5' GCGAGATCTCTGTCTTCAAGGAAGACAGC 3' (antisense to nucleotides 4,071 to 4,090, contains Bgl II site). f4-3: 5' CGCGGATCCAAGTGTGGGAGCTGAGGAC 3' (nucleotides 4,091 to 4,110, contains BamH I site), and 5' GCGAGATCTCAAGATCTAAGGCATCTGTGG 3' (antisense to nucleotides 4,411 to 4,431, contains Bgl II site). f4-ov5: 5' CGCGGATCCGCACAATGTAGTCAGCCAG 3' (nucleotides 4,431 to 4,450, contains BamH I site), and 5' GCGAGATCTCAACTGCTGGCCCAGAC 3' (antisense to nucleotides 4,547 to 4,563, contains Bgl II site). f5-1: 5' CGCGGATCCTCTCCAGGAGGGTCC 3' (nucleotides 4,564 to 4,579, contains BamH I site), and 5' GCGAGATCTGAAGGCAGAGCTGGCTGCACC 3' (antisense to nucleotides 4,990 to 5,010, contains Bgl II site). f5-2: 5' CGCGGATCCACGGTGGGAATGTCTCTATG 3' (nucleotides 5,011 to 5,030, contains BamH I site), and 5' GCGAGATCTTACAAAAGGAAGTCTCCCTAACC 3' (antisense to nucleotides 5,364 to 5,386, contains Bgl II site). f4-2-A: 5' CGCGGATCCGGGCTCAGGAAGGAGGGAG 3' (nucleotides 3,771 to 3,789, contains BamH I site), and 5' GGGAGATCTCAAGGCCAGTGCAGTGGGCC 3' (antisense to nucleotides 3,835 to 3,854, contains Bgl II site). f4-2-B: 5' CGCGGATCCCTGAGCCCAGAGCAGGCAA 3' (nucleotides 3,815 to 3,834, contains BamH I site), and 5' GGGAGATCTAGAGCCTAGAGGAGTGATT 3' (antisense to nucleotides 3,900 to 3,918, contains Bgl II site). f4-2-C: 5' CGCGGATCCAAGTCTAGGGGATGCTTC 3' (nucleotides 3,879 to 3,898, contains BamH I site), and 5' GGGAGATCTTCTGGTTGGTTCGAAGTGAG 3' (antisense to nucleotides 3,963 to 3,982, contains Bgl II site). f4-2-D: 5' CGCGGATCCTGTCCAGAGAGCCTTCAAA 3' (nucleotides 3,943 to 3,961, contains BamH I site), and 5' GGGAGATCTGCTGCAGCTGCTAAATCTC 3' (antisense to nucleotides 4,026 to 4,046, contains Bgl II site). f4-2-E: 5' CGCGGATCCCCGCGTGTGCCTCTTCAAA 3' (nucleotides 4,007 to 4,025, contains BamH I site), and 5' GGGAGATCTCTGTCTTCAAGGAAGACAG 3' (antisense to nucleotides 4,072 to 4,090, contains Bgl II site).

4.4. Packaging vectors into HSV-1 particles

Vectors were packaged into HSV-1 particles using the helper virus-free packaging system [5] and a modified protocol [41] that improves the efficiency. Vector stocks were purified and concentrated as described [19]. Vector stocks were titered by counting the numbers X-gal positive cells obtained at 1 day after infection of BHK fibroblast cells (IVP/ml). The titers of vector genomes were determined by extracting DNA from the vector stocks and quantifying the amounts of vector DNAs using PCR and primers for the Lac Z gene [47]. Wild-type (wt) HSV-1 was not detected (<10 plaque forming units/ml) in any of these vector stocks.

4.5. Stereotactic injection of vector stocks into the striatum

All animal procedures were approved by the W. Roxbury VA Hospital IACUC. Male Sprague Dawley rats (150-175 gm) were used for these experiments. Vector stocks were delivered by stereotactic injection (2 sites, 1 site in each hemisphere, 3 μ l/site) into the striatum (anterior-posterior (AP) +0.8, medial-lateral (ML) $\pm$ 2.5, dorsal-ventral (DV) -5.5). AP is relative to bregma, ML is relative to the sagittal suture, and DV is relative to the bregma-lambda plane [25]. Injections were performed using a micropump (model 100, KD Scientific); the 3 μ l inoculum was injected over 8 minutes, and the needle was maintained in place for an additional 5 minutes before being slowly withdrawn over approximately 5 minutes.

4.6. Immunohistochemistry

Rats were perfused, and immunohistochemistry assays were performed, as described [50]. These assays used 25 μ m coronal brain sections that contained the striatum, and the sections were cut on a freezing microtome. To quantify the numbers of cells that contained β -gal, β -gal-IR was detected using a rabbit anti- β -gal antibody that was visualized using a biotin-conjugated goat anti-rabbit IgG, the ABC reagent, and the HRP reaction. To localize β -gal to neurons, sections were incubated with both rabbit anti- β -gal and mouse monoclonal anti-NeuN antibodies, and these antibodies were visualized using fluorescein isothiocyanate- or rhodamine isothiocyanate-conjugated secondary antibodies, as described [50].

4.7. Cell counts

Twenty-five μ m coronal sections that contained the area proximal to each injection site were prepared from the striatum. Every 6th section was analyzed for β -gal-IR, and 2-5 of these sections contained the β -gal-IR cells.

To quantify the numbers of β -gal-IR cells, stereology was performed using the optical dissector method and the StereoInvestigator program (MicroBrightField Inc). With reference to a rat atlas [25] and known landmarks, a contour was drawn around the striatum in each of the sections that contained the β -gal-IR cells. Stereological cell counts were performed under 60 $\times$ magnification. The counting frame area was 6200 μ m², 120 to 360 sample sites per hemisphere were counted, and the coefficient of error (CE) for each hemisphere was $\leq$ 10 %.

To quantify neuronal-specific expression, digital images of β -gal-IR 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.

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Fig. 1.

Schematic diagrams of pTH-NFHLac (A) or the deletion strategy used to identify sequences within the 6.3 kb TH promoter fragment that support long-term expression (B). (A) pTH-NFHLac [50]. A HSV-1 origin of DNA replication (ori_S, small circle) and the HSV-1 **a** sequence (contains the packaging site, diagonal line segment) support replication of the vector and subsequent packaging into HSV-1 particles, respectively. The transcription unit contains a 5' upstream fragment from TH promoter (gray segment) followed by the NFH promoter (black segment), the *Lac Z* gene (cross-hatched segment), the second intron from the mouse α -globin gene (triangle), and the SV40 polyadenylation signal (SV40 poly A, vertical line segment). A cassette of 3 polyadenylation sites (tri A, horizontal line segment) was placed 5' to the TH

promoter fragment to reduce any effects on expression from the fragment that contains the HSV-1 immediate early (IE) 4/5 promoter (clear segment). Sequences from pBR322 (clear segment) enable propagation of the vector in *E. coli*. The TH promoter fragment is flanked by BamHI (B) sites, which were used to insert specific TH promoter fragments. (B) A 6.3 kb BamHI fragment, from -0.5 to -6.8 kb in the TH promoter, supports long-term expression in pTH-NFHLac [50]. The three lines show schematic diagrams of the TH promoter fragments tested for long-term expression in each of the three sets of deletions; the nucleotide numbers of the ends of each fragment are listed in Tables, 1, 3, or 5, respectively. Although the fragments are diagrammed as sequential, adjacent fragments were designed to overlap at their ends to ensure that essential elements were not interrupted. Four fragments that supported long-term expression are indicated with bold rectangles, and the name and size of each of fragment is on the left. Two fragments that supported low levels of long-term expression are each indicated by a rectangle within a rectangle.

Fig. 2.

Low and high power views of β -gal-IR striatal cells from rats sacrificed at 4 days or 2 months after gene transfer with vectors containing the first set of deletions of the TH promoter fragment, or control vectors. β -gal-IR was detected using an anti- β -gal antibody that was visualized using a biotin-conjugated secondary antibody, the ABC reagent, and the HRP reaction. The bp of the TH promoter fragments in these vectors are listed in Table 1. (A-D) Low power views: (A and B) pTHf1-NFHLac, rat sacrificed at (A) 4 days or (B) 2 months; many β -gal-IR cells are observed proximal to the needle track at 4 days, but not at 2 months. (C and D) pTHf4-NFHLac, rat sacrificed at (C) 4 days or (D) 2 months; many β -gal-IR cells are located proximal to the needle tracks at either 4 days or 2 months. (E-N) High power views:

(E and F) pNFHlac, the negative control vector, rat sacrificed at (E) 4 days or (F) 2 months; many β -gal-IR cells are observed at 4 days, but not at 2 months. (G and H) pTH-NFHlac, the positive control vector, rat sacrificed at (G) 4 days or (H) 2 months; many β -gal-IR cells are observed at 4 days or 2 months. (I and J) pTHf1-NFHlac, a vector with a TH promoter fragment that does not support long-term expression, rat sacrificed at (I) 4 days or (J) 2 months. (K and L) pTHf4-NFHlac, a vector with a TH promoter fragment that supports long-term expression, rat sacrificed at (K) 4 days or (L) 2 months; many β -gal-IR cells are observed at 4 days or 2 months. (M and N) pTHf5-NFHlac, a vector with a TH promoter fragment that supports limited long-term expression, rat sacrificed at (M) 4 days or (N) 2 months; many β -gal-IR cells are observed at 4 days, and a few β -gal-IR cells are present at 2 months. Scale bars: (A-D) 400 μ m; (E-N) 30 μ m.

Fig. 3. β -gal-IR positive striatal cells that contain NeuN-IR from rats sacrificed at 2 months after gene transfer with vectors containing the first set of deletions of the TH promoter fragment, or a control vector. β -gal-IR was detected using anti-*E. coli* β -gal that was visualized using a fluorescein isothiocyanate-conjugated secondary antibody, and NeuN, a neuronal marker found in the nucleus, was detected using anti-NeuN that was visualized using a rhodamine isothiocyanate-conjugated secondary antibody. (A-C) pTH-NFHLac; (A) β -gal-IR, (B) NeuN-IR, (C) merged. Many β -gal-IR cells were observed, and most of these cells contained NeuN-IR (arrowheads). (D-F) pTHf4-NFHLac; (D) β -gal-IR, (E) NeuN-IR, (F) merged. (G-I) pTHf1-NFHLac; (G) β -gal-IR, (H) NeuN-IR, (I) brightfield. Scale bar: 30 μ m.

Fig. 4.

Low and high power views of β -gal-IR striatal cells from rats sacrificed at 4 days or 2 months after gene transfer with vectors containing the second set of deletions of the TH promoter fragment, or control vectors. The bp of the TH promoter fragments in these vectors are listed in Table 3. (A and B) Low power views of pTHf4-2-NFHLac, rat sacrificed at (A) 4 days or (B) 2 months. Many β -gal-IR cells are observed proximal to the needle track at either 4 days or 2 months. (C-J) High power views: (C and D) pNFHLac, the negative control vector, rat sacrificed at (C) 4 days or (D) 2 months. (E and F) pTHf4-NFHLac, the positive control vector, rat sacrificed at (E) 4 days or (F) 2 months. (G and H) pTHf4-2-NFHLac, a vector with a TH promoter fragment that supports long-term expression, rat sacrificed at (G) 4 days or (H) 2

months. (I and J) pTHf4-3-NFHLac, a vector with a TH promoter fragment that does not support long-term expression, rat sacrificed at (I) 4 days or (J) 2 months. Scale bars: (A and B) 400 μm ; (C-J) 30 μm .

Fig. 5.

β-gal-IR positive striatal cells that contain NeuN-IR from rats sacrificed at 2 months after gene transfer with vectors containing the second set of deletions of the TH promoter fragment. (A-C) pTHf4-2-NFHLac; (A) β-gal-IR, (B) NeuN-IR, (C) merged. Many β-gal-IR cells were observed, and most of these cells contained NeuN-IR (arrowheads). (D-F) pTHf4-ov5-NFHLac; (D) β-gal-IR, (E) NeuN-IR, (F) merged. Some β-gal-IR cells contained NeuN-IR (arrowheads), and some β-gal-IR cells lacked NeuN-IR (arrows). Scale bar: 30 μm.

Fig. 6.

Low and high power views of β -gal-IR striatal cells from rats sacrificed at 4 days or 2 months after gene transfer with vectors containing the third set of deletions of the TH promoter fragment, or control vectors. The bp of the TH promoter fragments in these vectors are listed in Table 5. (A and B) Low power views of pTHf4-2-A-NFHLac, rat sacrificed at (A) 4 days or (B) 2 months. Many β -gal-IR cells are observed proximal to the needle track at either 4 days or 2 months. (C-H) High power views: (C and D) pTHf4-2-A-NFHLac, a vector with a TH promoter fragment that supports long-term expression, rat sacrificed at (C) 4 days or (D) 2 months. (E and F) pTHf4-2-B-NFHLac, a vector with a TH promoter fragment that does not support long-term expression, rat sacrificed at (E) 4 days or (F) 2 months. (G and H) pTHf4-2-

C-NFHLac, a vector with a TH promoter fragment that supports long-term expression, rat sacrificed at (G) 4 days or (H) 2 months. Scale bars: (A and B) 400 μm ; (C-H) 30 μm .

Fig. 7.

β-gal-IR positive striatal cells that contain NeuN-IR from rats sacrificed at 2 months after gene transfer with vectors containing the third set of deletions of the TH promoter fragment. (A-C) pTHf4-2-A-NFHLac; (A) β-gal-IR, (B) NeuN-IR, (C) merged. Many β-gal-IR cells were observed, and most of these cells contained NeuN-IR (arrowheads). (D-F) pTHf4-2-B-NFHLac; (D) β-gal-IR, (E) NeuN-IR, (F) merged. (G-I) pTHf4-2-C-NFHLac; (G) β-gal-IR, (H) NeuN-IR, (I) merged. Scale bar: 30 μm.

Fig. 8.

The stabilities of long-term expression supported by vectors containing specific fragments of the TH promoter, and control vectors. For each vector and time point, the % of β -gal-IR cells at 4 days is shown (the average number of β -gal-IR cells at 1 month or 2 months after gene transfer divided by the average number of β -gal-IR cells at 4 days ($\times 100$); calculated using the data in Tables 1, 3, and 5). pTH-NFHIac and pNFH-lac were tested in all three sets of deletions; pTHf4-NFHIac and pTHf4-2-NFHIac were each tested in two specific sets of deletions; and the averages of the multiple experiments were used for each of these vectors and time points.

The titers and long-term expression supported by HSV-1 vectors containing the first set of deletions of the TH promoter fragment

TABLE 1

Vector	TH promoter fragment	Purified titers ^a			Average β-gal-IR cells per hemisphere			Relative efficiency of gene transfer ^b
		VG/ml	IVP/ml	VG/IVP	Time after gene transfer			
					4 days	1 month	2 months	
pTH-NFHLac	1-6342	1.6×10 ⁷	1.5×10 ⁶	11	607±82	131±47	125±23	1.0
pNFHLac	--	2.0×10 ⁷	1.5×10 ⁶	13	584±118	0±0	0±0	1.0
pTH1-NFHLac	1-1,232	1.6×10 ⁷	1.5×10 ⁶	11	436±42	0±0	0±0	0.7
pTH2-NFHLac	1,124-2,342	1.5×10 ⁷	1.5×10 ⁶	10	342±84	18±14	0±0	0.6
pTH3-NFHLac	2,228-3,450	1.5×10 ⁷	1.5×10 ⁶	10	426±103	12±8	0±0	0.7
pTH4-NFHLac	3,344-4,563	1.6×10 ⁷	1.5×10 ⁶	11	578±119	114±34	107±31	1.0
pTH5-NFHLac	4,432-5,580	1.4×10 ⁷	1.5×10 ⁶	9.3	560±177	52±23	38±15	0.9
pTH6-NFHLac	5,387-6,342	1.4×10 ⁷	1.5×10 ⁶	9.2	614±199	0±0	0±0	1.0

Three rats were used for each vector and time point, and HSV-1 vectors were injected into the striatum in each hemisphere (2 injections per rat); thus, 6 hemispheres were analyzed for each condition. The means±SDs are shown.

^aThe titers of each vector stock after purification and concentration. VG/ml is vector genomes/ml; IVP/ml is infectious vector particles/ml.

^bThe efficiency of gene transfer is the number of positive cells at 4 days/the amount of vector injected. The relative efficiency of gene transfer is the efficiency of gene transfer with a specific condition/the efficiency of gene transfer with pTH-NFHLac.

Table 2
The numbers of β -gal-IR cells that costain for NeuN-IR from rats sacrificed at 4 days to 2 months after receiving HSV-1 vectors containing the first set of deletions of the TH promoter fragment

Vector	Time after gene transfer				2 months			
	4 days		1 month		Total β -gal-IR cells		β -gal-IR & NeuN-IR cells	
pTH-NFHIac	Total β -gal-IR cells	218	β -gal-IR & NeuN-IR cells	192	% costained	88	Total β -gal-IR cells	120
pTH1-NFHIac				134		74		89
pTH2-NFHIac				126		77		8
pTH3-NFHIac				122		69		5
pTH4-NFHIac				162		86		96
pTH5-NFHIac				146		76		19
pTH6-NFHIac				156		77		24*
								78
								33
								77

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. These brains were sectioned into 6 sets of serial sections, and one set was assayed.

* Vectors that supported little or no long-term expression supported few β -gal-IR cells to score at 1 or 2 months after gene transfer

TABLE 3
The titers and long-term expression supported by HSV-1 vectors containing the second set of deletions of the TH promoter fragment

Vector	TH promoter fragment	Purified titers ^a			Average β-gal-IR cells per hemisphere			Relative efficiency of gene transfer ^b
		VG/ml	IVP/ml	VG/IVP	Time after gene transfer			
					4 days	1 month	2 months	
pTH-NFHIac	1-6342	2.2×10 ⁷	2.0×10 ⁶	11	729±175	216±64	189±64	1.0
pNFHIac	--	2.4×10 ⁷	1.9×10 ⁶	13	684±148	0±0	0±0	0.9
pTHI4-NFHIac	3,344-4,563	2.2×10 ⁷	1.9×10 ⁶	12	637±104	176±44	198±119	0.9
pTHI4-1-NFHIac	3,451-3,770	2.0×10 ⁷	2.0×10 ⁶	10	716±112	22±18	0±0	1.0
pTHI4-2-NFHIac	3,771-4,090	1.9×10 ⁷	1.8×10 ⁶	11	626±112	190±116	188±48	0.8
pTHI4-3-NFHIac	4,091-4,431	2.0×10 ⁷	1.7×10 ⁶	12	627±123	24±14	0±0	0.8
pTHI4-ov5-NFHIac	4,431-4,563	1.7×10 ⁷	1.6×10 ⁶	11	624±97	112±35	30±23	0.9
pTHI5-1-NFHIac	4,564-5,010	1.5×10 ⁷	1.5×10 ⁶	10	480±34	12±20	0±0	0.7
pTHI5-2-NFHIac	5,011-5,386	1.7×10 ⁷	1.9×10 ⁶	11	630±76	0±0	0±0	0.9

Three rats were used for each vector and time point, and HSV-1 vectors were injected into the striatum in each hemisphere (2 injections per rat); thus, 6 hemispheres were analyzed for each condition. The means±SDs are shown.

^aThe titers of each vector stock after purification and concentration. VG/ml is vector genomes/ml; IVP/ml is infectious vector particles/ml.

^bThe efficiency of gene transfer is the number of positive cells at 4 days/the amount of vector injected. The relative efficiency of gene transfer is the efficiency of gene transfer with a specific condition/the efficiency of gene transfer with pTH-NFHIac.

Table 4
The numbers of β -gal-IR cells that costain for NeuN-IR from rats sacrificed at 4 days to 2 months after receiving HSV-1 vectors containing the second set of deletions of the TH promoter fragment

Vector	Time after gene transfer					
	4 days		1 month		2 months	
	Total β -gal-IR cells	β -gal-IR & NeuN-IR cells	% costained	Total β -gal-IR cells	β -gal-IR & NeuN-IR cells	% costained
pTHf4-NFHIac	89	67	75	124	88	71
pTHf4-1-NFHIac	112	79	71	22*	8	36
pTHf4-2-NFHIac	76	54	71	132	98	74
pTHf4-3-NFHIac	212	176	83	24*	6	25
pTHf4-ov5-NFHIac	120	92	77	76*	32	42
					35*	34

β -gal-IR was detected using a rabbit anti- 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. These brains were sectioned into 6 sets of serial sections, and one set was assayed.

* Vectors that supported little or no long-term expression supported few β -gal-IR cells to score at 1 or 2 months after gene transfer

The titers and long-term expression supported by HSV-1 vectors containing the third set of deletions of the TH promoter fragment

Vector	TH promoter fragment	Purified titers ^a			Average β-gal-IR cells per hemisphere			Relative efficiency of gene transfer ^b
		VG/ml	IVP/ml	VG/IVP	Time after gene transfer			
					4 days	1 month	2 months	
pTH-NFHLac	1-6342	2.2×10 ⁷	2.0×10 ⁶	11	777±176	243±75	230±84	1.0
pNFHLac	--	2.4×10 ⁷	1.9×10 ⁶	13	708±127	0±0	0±0	0.8
pTHf4-2-NFHLac	3,771-4,090	1.9×10 ⁷	1.8×10 ⁶	11	731±170	226±96	195±42	0.9
pTHf4-2-A-NFHLac	3,771-3,854	2.8×10 ⁷	2.4×10 ⁶	12	802±176	190±30	152±50	1.0
pTHf4-2-B-NFHLac	3,815-3,918	1.6×10 ⁷	1.6×10 ⁶	10	630±90	0±0	0±0	0.9
pTHf4-2-C-NFHLac	3,879-3,982	2.0×10 ⁷	1.8×10 ⁶	11	716±37	222±37	184±57	0.9
pTHf4-2-D-NFHLac	3,943-4,046	2.2×10 ⁷	1.9×10 ⁶	12	705±72	0±0	0±0	0.9
pTHf4-2-E-NFHLac	4,007-4,090	2.0×10 ⁷	1.8×10 ⁶	11	687±140	0±0	0±0	0.7

Three rats were used for each vector and time point, and HSV-1 vectors were injected into the striatum in each hemisphere (2 injections per rat); thus, 6 hemispheres were analyzed for each condition. The means±SDs are shown.

^aThe titers of each vector stock after purification and concentration. VG/ml is vector genomes/ml; IVP/ml is infectious vector particles/ml.

^bThe efficiency of gene transfer is the number of positive cells at 4 days/the amount of vector injected. The relative efficiency of gene transfer is the efficiency of gene transfer with a specific condition/the efficiency of gene transfer with pTH-NFHLac.

The numbers of β -gal-IR cells that costain for NeuN-IR from rats sacrificed at 4 days to 2 months after receiving HSV-1 vectors containing the third set of deletions of the TH promoter fragment

Table 6

Vector	Time after gene transfer					
	4 days		1 month		2 months	
	Total β -gal-IR cells	β -gal-IR & NeuN-IR cells	% costained	Total β -gal-IR cells	β -gal-IR & NeuN-IR cells	% costained
pTHf4-2-NFHIac	145	119	82	282	212	75
pTHf4-2-A-NFHIac	120	87	73	222	160	72
pTHf4-2-C-NFHIac	138	112	81	180	136	77
				138	98	71
				168	124	74
				186	126	68

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.