Published in final edited form as:

Brain Res. 2011 September 30; 1415: 109–118. doi:10.1016/j.brainres.2011.07.061.

A 16 bp upstream sequence from the rat tyrosine hydroxylase promoter supports long-term expression from a neurofilament promoter, in a helper virus-free HSV-1 vector system

Guo-rong Zhang, Hua Zhao, Xu Li, Soumya Awasthi, and Alfred I. Geller*

Department of Neurology, West Roxbury VA Hospital/Harvard Medical School, W. Roxbury, MA 02132

Abstract

Helper virus-free Herpes Simplex Virus vector-mediated gene transfer has supported studies on neuronal physiology, and may support specific gene therapies. Long-term, neuron-specific expression is required for many of these applications. A neurofilament heavy gene (NFH) promoter does not support long-term expression. We previously developed a promoter that supports long-term expression by fusing 6.3 kb of upstream sequences from the rat tyrosine hydroxylase (TH) promoter to a NFH promoter, and this promoter has supported physiological studies. The TH promoter fragment contains an enhancer, as it has activity in both orientations and at a distance from the basal promoter. Identifying this enhancer may support further improvements in long-term expression. A previous deletion analysis identified two ~100 bp fragments that each support long-term expression, and are contained within an ~320 bp fragment located ~3 kb from the TH promoter transcription start site. As this analysis used overlapping fragments, the two ~100 bp fragments contained 44 or 23 bp of unique sequence. Here, we used mutagenesis to identify a short sequence that supports long-term expression. We studied a 42 bp sequence, centered on the 23 bp unique sequence. Analysis of the wt sequence, and five mutations containing clustered changes that spanned the sequence, identified two adjacent mutations that do not support long-term expression, that together defined a 16 bp maximum essential sequence. This 16 bp sequence contains a putative E2F-1/DP-1 transcription factor binding site, and this transcription factor is expressed in many brain areas.

Keywords

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

1. Introduction

Direct gene transfer into neurons in the brain is useful for both understanding neuronal physiology and for supporting gene therapies for specific neurological disorders. Advantages of Herpes Simplex Virus (HSV-1) plasmid vectors (amplicons) include high-

© 2011 Elsevier B.V. All rights reserved.

*Corresponding Author: Dr. Alfred Geller, Research Building 3, West Roxbury VA Hospital/Harvard Medical School, 1400 VFW Parkway, West Roxbury, MA 02132; 857-203-5104; fax, 857-203-5563; alfred_geller@hms.harvard.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

efficiency gene transfer and a large capacity, supporting coexpression of multiple genes (Fraefel et al., 1996; Geller and Breakefield, 1988). Many applications of HSV-1 vectors to neuronal physiology or gene therapy require long-term, neuron-specific expression. We developed a chimeric promoter that fuses upstream sequences from the rat tyrosine hydroxylase (TH) promoter to a mouse neurofilament promoter (TH-NFH promoter). After gene transfer into a number of different forebrain areas, this promoter supports greater than 90 % neuron-specific expression, and significant levels of long-term expression (Sun et al., 2004; Zhang et al., 2000; Zhang et al., 2005; Zhang et al., 2009). However, the levels of long-term expression might be improved by identifying the specific elements that support long-term expression. Further, these elements could potentially support long-term expression from other promoters in HSV-1 vectors.

Although several neuron-specific or viral promoters support only short-term expression from HSV-1 vectors (reviewed in (Zhang et al., 2000)), a number of promoters have been identified that support long-term expression. Neuron-specific promoters that support only short-term expression include the voltage-gated sodium channel promoter and the neurofilament heavy gene (NFH) promoter (Wang et al., 1999). In contrast, a number of neuron type-specific promoters support significant levels of long-term expression from HSV-1 plasmid vectors (Jin et al., 1996; Kaplitt et al., 1994; Rasmussen et al., 2006; Song et al., 1997; Wang et al., 1999). The preproenkephalin (ENK) promoter supports expression for 2 months in the amygdala and ventromedial hypothalamus, brain areas that contain enkephalinergic neurons (Kaplitt et al., 1994). The glutamic acid decarboxylase (GAD) promoter supports GABAergic-specific expression, the phosphate-activated glutaminase (PAG) promoter supports glutamatergic-specific expression; the vesicular glutamate transporter-1 (VGLUT1) promoter supports expression in VGLUT1-containing glutamatergic neurons; and specific fragments of the VGLUT1 promoter support expression in predominately different subtypes of glutamatergic-neurons (Rasmussen et al., 2006; Zhang and Geller, 2010; Zhang et al., 2011). The GAD, PAG, and VGLUT1 promoters support expression for 2 months. Of note, relatively large fragments of the rat TH promoter (6.8 or 9 kb) support expression for 2 months in midbrain dopaminergic neurons (Jin et al., 1996; Song et al., 1997; Wang et al., 1999). In contrast, a relatively small fragment of this promoter (766 bp) supports only short-term expression (Wang et al., 1999). Together, these results indicated that upstream sequences in the TH promoter support long-term expression, and such upstream elements are typically enhancers, rather than basal promoter elements.

We constructed a chimeric promoter that supports long-term expression in forebrain neurons (Zhang et al., 2000) by fusing upstream sequences from the TH promoter (Brown et al., 1987) to the 5' end of a NFH promoter (Schwartz et al., 1994). The TH-NFH promoter supported expression for 1 month in either postrhinal or perirhinal cortex, for 2 months in the hippocampus, and 6 months in the striatum, the longest times tested (Zhang et al., 2000). Kinetic studies revealed that expression levels initially declined, and then were stable: By 2 weeks after gene transfer, the numbers of positive cells had declined to 15–20 % of that observed at 4 days. But, importantly, at 2 months in the hippocampus, or at 6 months in the striatum, the numbers of positive cells were similar to those observed at 2 weeks in each area (Zhang et al., 2000). Of note, the elements in the TH promoter that enhance long-term expression satisfy the definition of an enhancer (Ptashne, 1988): Both orientations of the TH promoter fragment enhanced expression, and this enhancement is not a sequence-independent spacing effect because a similar sized fragment from *E. coli* bacteriophage λ did not enhance expression from the NFH promoter (Zhang et al., 2000). Further, the element can act at a distance; the NFH promoter is ~600 bp, and the TH promoter fragment is 6.3 kb (–0.6 to –6.8 kb); thus, the critical element in the TH promoter is located at least ~600 bp from the NFH promoter transcription start site. Moreover, we obtained higher levels of long-term expression by adding the chicken β -globin locus insulator (INS), a well known

mammalian INS, to the TH-NFH promoter (Zhang et al., 2000). The INS-TH-NFH promoter supports both gene therapy studies (Sun et al., 2004; Sun et al., 2005; Zhang et al., 2009) and studies on visual learning (Zhang et al., 2005; Zhang et al., 2010).

Because the TH enhancer fragment is a key component of the INS-TH-NFH promoter, defining the critical enhancer elements that support long-term expression is indicated. As the TH promoter fragment is large, ~6.3 kb, we performed a deletion analysis to identify critical subfragments that support long-term expression (Gao et al., 2007). Three sequential rounds of deletion analyses identified two ~100 bp fragments; following fusion of each fragment to the NFH promoter, each fragment supported long-term expression in striatal neurons. These two ~100 bp fragments are proximal; they are contained within an ~320 bp fragment located ~3 kb upstream from the TH promoter transcription start site (Gao et al., 2007). Because this analysis examined overlapping fragments, these two ~100 bp fragments contain either 44 or 23 bp of unique sequence (Gao et al., 2007).

Here, we used mutagenesis to identify a short sequence that supports long-term expression. We analyzed a 42 bp sequence, containing the 23 bp of unique sequence with flanking sequence on each end. We isolated five mutations containing clustered changes that spanned the sequence. The wt sequence and each mutation were fused to the NFH promoter in a HSV-1 vector. The wt sequence, and three of the mutations, each supported long-term expression in the striatum. Two adjacent mutations did not support long-term expression, and together defined a 16 bp maximum essential sequence for long-term expression. This 16 bp sequence contains a putative E2F-1/DP-1 transcription factor binding site, and this transcription factor is expressed in many brain areas.

2. Results

2.1. Identification of a 16 bp upstream sequence from the TH promoter that supports long-term expression from the NFH promoter, in striatal neurons

Here, we undertook to identify the critical enhancer sequence in the ~100 bp fragment that contains 23 bp of unique sequence (Gao et al., 2007). Because sequences flanking the 23 bp of unique sequence could be part of the enhancer, we began with a 42 bp sequence that contains the 23 bp unique sequence and added either 9 or 10 bp to the 5' or 3' end, respectively (Fig. 1). We inserted this 42 bp sequence at the 5' end of the NFH promoter in a HSV-1 vector (Fig. 2; vector pTHoligo-wt-NFHlac). Further, we isolated five mutations in different parts of this sequence; each mutation changed 3 to 7 bp, and together the mutations spanned the 42 bp sequence (Fig. 1). Each mutation was inserted into a HSV-1 vector (Fig. 2; pTHoligo-Mut-1-NFHlac to pTHoligo-Mut-5-NFHlac).

These six vectors were packaged into HSV-1 particles using our helper virus-free packaging system. 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. Specific vector stocks were diluted as indicated to achieve a concentration of 1×10^6 IVP/ml for each vector stock. We previously used a PCR assay to titer the number of vector genomes, and we found that vectors containing only the NFH promoter, the entire 6.3 kb TH enhancer fragment, or specific subfragments of the TH fragment, were all efficiently packaged into HSV-1 particles with similar efficiencies, and with packaging efficiencies similar to other vectors (Gao et al., 2007; Zhang et al., 2000). Thus, we did not repeat the assay for vector genomes in this study.

Each of these six vector stocks was microinjected into the striatum, the rats were sacrificed at 2 months after gene transfer, and β -galactosidase (β -gal)-immunoreactive (IR) positive

cells were visualized using the avidin-biotinylated peroxidase complex (ABC) reagent and the horse radish peroxidase (HRP) reaction. We chose a 2 month survival time as representative of long-term expression: Previous studies showed that most of the decrease in numbers of expressing cells occurs between 4 days and 2 weeks after gene transfer (Gao et al., 2007; Wang et al., 1999; Zhang et al., 2000); a time course showed the numbers of expressing cells is similar at 2 weeks and 1, 2, 4, or 6 months (Zhang et al., 2000); and similar numbers of expressing cells are present at 14 months (Sun et al., 2004). In a rat sacrificed at 2 months after receiving the positive control vector, pTHoligo-wt-NFHlac, a low power view showed numerous β -gal-IR cells proximal to the needle track (Fig. 3A), and a high power view showed that many of these cells contain neuronal morphology, namely relatively large cell bodies at some distance from the needle track (Fig. 3B). Neuronal identity was confirmed by costaining for a neuronal marker, as detailed below in section 2.2. The clear ellipsoid-like regions in these panels, and other specific panels in Fig. 3, are characteristic of striatal morphology; for example, such clear regions are also found in the Paxinos and Watson atlas with either an acetylcholinesterase enzyme assay or Cresyl violet staining (Paxinos and Watson, 1986). Of note, three of the mutations; specifically pTHoligo-Mut-1-NFHlac, pTHoligo-Mut-2-NFHlac, and pTHoligo-Mut-5-NFHlac; supported similar patterns of β -gal-IR cells as the wt vector pTHoligo-wt-NFHlac: Under low power, each of these three vectors supported numerous β -gal-IR cells proximal to the needle track (pTHoligo-Mut-1-NFHlac, Fig. 3C; pTHoligo-Mut-2-NFHlac, Fig. 3E; and pTHoligo-Mut-5-NFHlac, Fig. 3K). Further, high power views revealed β -gal-IR cells with neuronal morphology, specifically positive cells at some distance from the needle track with relatively large cell bodies, similar to pTHoligo-wt-NFHlac (pTHoligo-Mut-1-NFHlac, Fig. 3D; pTHoligo-Mut-2-NFHlac, Fig. 3F; and pTHoligo-Mut-5-NFHlac, Fig. 3L). In contrast, the other two mutations; specifically pTHoligo-Mut-3-NFHlac and pTHoligo-Mut-4-NFHlac; appeared to support fewer β -gal-IR cells than the wt vector pTHoligo-wt-NFHlac (cell counts are presented in the next paragraph): Under low power, each of these two vectors supported few β -gal-IR cells (pTHoligo-Mut-3-NFHlac, Fig. 3G; and pTHoligo-Mut-4-NFHlac, Fig. 3I). Further, high power views revealed that the few β -gal-IR cells lacked neuronal morphology; these cells were on the needle track with small cell bodies, possibly representing blood cells (pTHoligo-Mut-3-NFHlac, Fig. 3H; and pTHoligo-Mut-4-NFHlac, Fig. 3J). As a control, using sections from a rat that received pTHoligo-wt-NFHlac, omission of the primary antibody resulted in no β -gal-IR cells (Fig. 3M and N). Further, control rats that received a vector that expresses TH and aromatic amino acid decarboxylase (pINS-TH-NFHth/ires/aadc (Sun et al., 2003)) lacked β -gal-IR positive cells (Fig. 3O and P).

The level of long-term expression supported by each vector was quantified by counting the cells in every fourth section (Table 1). pTHoligo-wt-NFHlac supported 298 ± 36 (mean $\pm$ s.e.m.) cells per hemisphere, and the vectors containing each of the three mutations that supported significant levels of expression (pTHoligo-Mut-1-NFHlac, pTHoligo-Mut-2-NFHlac, and pTHoligo-Mut-5-NFHlac) supported similar numbers of β -gal-IR cells. In contrast, the other two vectors (pTHoligo-Mut-3-NFHlac and pTHoligo-Mut-4-NFHlac) supported less than one-third of the β -gal-IR cells as supported by pTHoligo-wt-NFHlac. {Because all the positive cells were counted, including those on the needle track that lacked neuronal morphology, pTHoligo-Mut-3-NFHlac and pTHoligo-Mut-4-NFHlac may support expression in fewer cells than reported in these cell counts.} Statistical tests confirmed these observations: A one-way ANOVA showed a significant effect of vector (<0.001). Pairwise comparisons (Student-Neumann-Keuls test) confirmed that pTHoligo-wt-NFHlac, pTHoligo-Mut-1-NFHlac, pTHoligo-Mut-2-NFHlac, and pTHoligo-Mut-5-NFHlac all supported similar levels of expression (all $p > 0.05$). Importantly, pTHoligo-Mut-3-NFHlac and pTHoligo-Mut-4-NFHlac each supported fewer cells than each of the other four vectors (all $p < 0.005$). Lastly, pTHoligo-Mut-3-NFHlac and pTHoligo-Mut-4-NFHlac supported

expression in similar numbers of cells ($p>0.05$). In summary, Mut-3 and Mut-4 span critical enhancer sequences for long-term expression; and, in contrast, Mut-1, Mut-2, and Mut-5 do not span critical sequences for long-term expression.

The numbers of positive cells were modest, ~250 to 300 at 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 (Sun et al., 2004); only one injection site, instead of three injection sites, was used, and the titers (IVP/ml) were ~10 to 20-fold lower (six vector stocks were used here, and specific stocks were diluted to yield a titer of 1×10^6 IVP/ml for each vector stock). The ~30- to 60-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 (Sun et al., 2004).

Nonetheless, the levels of long-term expression supported by pTHoligo-wt-NFHLac were similar to, or modestly higher than, the levels of long-term expression supported in sixteen previously published experiments that tested different vectors containing specific TH enhancer fragments (Table 2). These sixteen different experiments are from five different studies (Gao et al., 2007; Sun et al., 2004; Zhang et al., 2000; Zhang et al., 2009; Zhang et al., 2010). In these sixteen experiments, the entire 6.3 kb TH upstream fragment was tested four times, and specific smaller fragments containing the TH enhancer were also tested. The levels of long-term expression were evaluated as the number of cells at 2 months after gene transfer (or the closest time point to 2 months in a specific experiment) divided by the IVP injected. The results show that pTHoligo-wt-NFHLac supports similar, or modestly higher, levels of long-term expression than those supported by different vectors containing the entire 6.3 kb TH enhancer fragment, or smaller fragments that contain the TH enhancer (Table 2). Thus, these results strongly suggest that the 42 bp TH enhancer in pTHoligo-wt-NFHLac contains full activity for supporting long-term expression, as compared to the entire 6.3 kb TH enhancer fragment.

2.2. pTHoligo-wt-NFHLac supported neuron-specific expression

We confirmed that the NFH promoter in pTHoligo-wt-NFHLac targeted expression to neurons. We used sections from a rat that was sacrificed at 2 months after receiving pTHoligo-wt-NFHLac, and performed immunofluorescent costaining using antibodies against *E. coli* β -gal or a neuronal marker, NeuN. Photomicrographs revealed that most of the β -gal-IR cells also contained NeuN-IR (Fig. 4A–C). As a control for the assay, omission of the primary antibodies resulted in no IR (Fig. 4D–F). Cell counts showed that pTHoligo-wt-NFHLac supported 97 % neuron-specific expression (143 β -gal-IR cells were scored, and 138 of these cells also contained NeuN-IR).

2.3. The 16 bp maximum critical sequence contains an E2F-1/DP-1 binding site; the 6.3 kb upstream TH fragment contains only one putative E2F-1/DP-1 binding site, and other specific promoters that support long-term expression contain putative E2F-1/DP-1 binding sites

The maximum critical sequence is 16 bp (Fig. 1). Together, Mut-3 and Mut-4 span 14 bp; there are 2 bp between Mut-3 and Mut-2; and Mut-4 and Mut-5 have adjacent ends. This 16 bp maximum critical sequence is 5' CTTCCAGCCTGTACGC 3'. The initial deletion analysis (Gao et al., 2007) identified an ~100 bp fragment that supported long-term expression, and due to overlap with adjacent fragments that did not support long-term expression, this fragment contained 23 bp of unique sequence. The 16 bp maximum critical sequence identified here contains 15 bp of the 23 bp of unique sequence previously

identified, and the last (3') bp of this 16 bp sequence is also contained in a fragment previously found not to support long-term expression (Gao et al., 2007).

We used the Genomatix software to determine that this 16 bp sequence contains one putative binding site for each of two known transcription factors. The 16 bp sequence contains one putative binding site for a member of the ETS1 factor family. However, the initial 6.3 kb TH upstream promoter fragment contains several tens of putative binding sites for members of the ETS1 family. Moreover, the members of the ETS1 family are not expressed at high levels in the CNS, according to both the Genomatix site and an examination of the Allen Brain Atlas. Thus, it appears unlikely that this putative ETS1 family binding site plays an important role in supporting long-term expression. Most importantly, the 16 bp sequence contains one putative binding site for the E2F-1/DP-1 transcription factor (Tao et al., 1997). The putative binding site is on the antisense strand; in the Genomatix software, the core similarity is 0.78 and the matrix similarity is 0.82. Mut-3 mutated the core of the binding site; Mut-4 is located just outside the binding site, but canonical binding sites and specific binding sites often differ. The initial 6.3 kb TH upstream promoter fragment (Zhang et al., 2000) contains only one putative binding site for E2F-1/DP-1, namely the site in this 16 bp fragment. Moreover, an examination of the Allen Brain Atlas revealed that E2F-1 is expressed at high levels throughout the brain; specifically, on a relative scale of 1 to 100, E2F-1 is present at ~100 in all the brain areas reported. Further, DP-1 is expressed at mid-range levels in all the brain areas reported.

Genomatix software-based searches revealed that putative E2F-1/DP-1 transcription factor binding sites are present in two other promoters that support long-term expression, and are absent from a promoter that supports only short-term expression. A 10 kb fragment of the glutamic acid decarboxylase promoter contains three putative E2F-1/DP-1 transcription factor binding sites; in a HSV-1 vector, this promoter supports long-term (2 months) GABAergic-specific expression (Rasmussen et al., 2007). Also, an 11 kb fragment of the vesicular glutamate transporter-1 (VGLUT1) promoter contains two putative E2F-1/DP-1 transcription factor binding sites; in a HSV-1 vector, this promoter supports long-term (2 months) VGLUT1-specific expression (Rasmussen et al., 2007; Zhang and Geller, 2010). In contrast, the 0.6 kb fragment of the NFH promoter lacks putative E2F-1/DP-1 transcription factor binding sites; and, in a HSV-1 vector, this promoter does not support long-term expression (Wang et al., 1999).

Genomatix software-based searches revealed that putative E2F-1/DP-1 transcription factor binding sites are absent, or present, in specific herpesvirus immediate early (IE) promoters, and these promoters support minimal levels of long-term expression. The cytomegalovirus IE promoter and the HSV-1 IE 4/5 promoter both lack a putative E2F-1/DP-1 transcription factor binding sites. The HSV-1 IE 1 promoter lacks putative E2F-1/DP-1 transcription factor binding sites, but there is one putative E2F-1/DP-1 transcription factor binding site in the middle of the IE 1 transcribed region, which is closer to the LAT promoter(s), which support long-term expression (Dobson et al., 1995; Lachmann and Efsthathiou, 1997), than to the IE 1 promoter. The IE 3 promoter contains one putative E2F-1/DP-1 transcription factor binding site, and there is one putative E2F-1/DP-1 transcription factor binding site near the 3' end of the IE 2 coding region. In HSV-1 vectors, the IE 4/5 and CMV IE promoters support very low levels of long-term expression; at 1–6 months after gene transfer, we observed expression in ≤ 1 % of the number of cells as observed at 4 days (During et al., 1994; Fraefel et al., 1996; Wang et al., 1999; Zhang et al., 2000). Similarly, in transgenic mice, specific herpesvirus IE promoters support expression in very limited populations of neurons in the brain (Fritschy et al., 1996; Loiacono et al., 2002; Loiacono et al., 2003; Mitchell, 1995; Taus and Mitchell, 2001). Also, fusion of the 6.3 kb TH upstream promoter fragment to the 5' end of the CMV IE promoter does not increase the levels of long-term

expression (Zhang and Geller, 2010). Thus, as shown in many reports, the VP16 transcription factor complex is the primary transcriptional regulator for herpesvirus IE promoters (Roizman and Sears, 1993), and E2F-1/DP-1 has at most a secondary role in regulating these promoters.

Two promoters that support long-term expression in HSV-1 vectors lack putative E2F-1/DP-1 transcription factor binding sites. First, a 2.4 kb fragment of the phosphate-activated glutaminase (PAG) promoter lacks putative E2F-1/DP-1 transcription factor binding sites; in a HSV-1 vector, this promoter supports long-term (2 months) glutamatergic-specific expression (Rasmussen et al., 2007). Second, the deletion analysis of the 6.3 kb TH upstream fragment yielded two proximal ~100 bp fragments that each support long-term expression after fusion to the NFH promoter (Gao et al., 2007). The other ~100 bp fragment from the TH promoter lacks putative E2F-1/DP-1 transcription factor binding sites. However, this ~100 bp fragment contains eight putative transcription factor binding sites, and the cognate transcription factors for three of these sites are expressed in the brain. Interestingly, putative cognate sites for two of these three transcription factors are also present in the PAG promoter fragment (Rasmussen et al., 2007). Thus, it appears that more than one transcription factor can support long-term expression from HSV-1 vectors.

3. Discussion

In this study, we showed that a 42 bp sequence, located ~3 kb upstream (5') from the TH promoter transcription start site, supports long-term, neuron-specific expression, upon fusion to the NFH promoter, in a helper virus-free HSV-1 vector system. Further, analysis of five clustered sets of point mutations defined a 16 bp maximum critical sequence that supports long-term expression. Computer searches of a transcription factor database revealed that this 16 bp sequence contains putative binding sites for two known transcription factors, only one of which is expressed in the brain, E2F-1/DP-1. Putative E2F-1/DP-1 sites are present in other specific promoters that support long-term expression from HSV-1 vectors, and are absent from the NFH promoter, which supports only short-term expression.

The TH-NFH promoter, and a derivative, the INS-TH-NFH promoter, support long-term expression in multiple types of neurons in multiple brain areas; thus, the relevant transcription factor(s) are likely widely expressed in the brain. The TH-NFH promoter supports expression in the striatum for 6 months, in the hippocampus for 2 months, and in perirhinal and postrhinal cortices for 1 month; these were the longest times tested (Zhang et al., 2000). The INS-TH-NFH promoter supports expression in the striatum for 14 months (Sun et al., 2004), in the hippocampus for 1 month (Zhang et al., 2009), in postrhinal cortex for 1.75 months (Zhang et al., 2010), and in the substantia nigra for 1 month (Cao et al., 2008); these were the longest times tested. The INS-TH-NFH promoter supports expression in GABAergic neurons in the striatum (Sun et al., 2004), both GABAergic and glutamatergic neurons in the hippocampus (Zhang et al., 2009); predominately GABAergic and glutamatergic, but also cholinergic, neurons in postrhinal cortex (Zhang et al., 2005); and in catecholaminergic neurons in the substantia nigra (Cao et al., 2008). Because the TH-NFH and INS-TH-NFH promoters support long-term expression in multiple types of neurons in multiple brain areas, the transcription factor(s) that support long-term expression from the upstream TH promoter fragment are likely expressed in multiple types of neurons, in multiple brain areas. It is possible that an unknown transcription factor is responsible for the long-term expression supported by the 16 bp maximum critical sequence, but because expression is required in multiple types of neurons, in multiple brain areas, it seems more plausible that such a widely expressed transcription factor is already known. Of note, both subunits of the E2F-1/DP-1 transcription factor are expressed widely in the brain, E2F-1 at high levels, and DP-1 at mid-range levels. Thus, this transcription factor, and its cognate

binding site, is a strong candidate for supporting long-term expression from HSV-1 vectors. However, the direct tests of fusing a canonical E2F-1/DP-1 binding site, and canonical binding sites for other members of the E2F/DP family, to the NFH promoter remain to be performed.

We previously identified two ~100 bp fragments that each support long-term, neuron-specific expression, upon fusion to the NFH promoter (both fragments are located ~3 kb upstream from the TH promoter transcription start site). Each ~100 bp fragment supports levels of long-term expression similar to that supported by the entire 6.3 kb TH fragment (Gao et al., 2007); long-term expression was assayed as the percentage of expressing striatal cells at 1 or 2 months compared to 4 days. Further, the 42 bp sequence studied here, and the larger fragments previously examined (Gao et al., 2007; Zhang et al., 2000), support similar levels of long-term expression; as determined by comparing the amounts of vector particles injected (IVP) and the numbers of expressing cells at 2 months. Thus, it appears that the 42 bp sequence, and the 16 bp maximum critical sequence contained therein, support long-term expression in many types of striatal neurons; similarly, the other ~100 bp sequence (which has yet to be analyzed in detail) also supports long-term expression in many types of striatal neurons. The vast majority of striatal neurons are GABAergic medium spiny neurons, but within this classification there are multiple subtypes of neurons that differ in peptide neurotransmitters, other molecular markers, and projection sites (Graybiel, 1990). Consistent with an element that supports long-term expression in multiple types of neurons, putative E2F-1/DP-1 binding sites are found in the 16 bp maximum critical sequence identified here, and in the GAD and VGLUT1 promoters that support long-term expression in neocortical neurons (Rasmussen et al., 2007). Analogously, the other ~100 bp sequence contains putative binding sites for three transcription factors that are expressed in the brain, and putative binding sites for two of these transcription factors are also found in the PAG promoter, that supports long-term expression in neocortical neurons (Rasmussen et al., 2007). Additional studies are indicated to determine if the 16 bp maximum critical sequence supports long-term expression in multiple brain areas, and to isolate the elements that support long-term expression from these other promoters.

The TH promoter has been extensively analyzed for elements that support either catecholaminergic-specific expression or short-term changes in expression levels in response to physiological events. As previously discussed (Gao et al., 2007), all of these known elements are outside of the sequence studied here.

The 16 bp sequence identified here can potentially be used to improve long-term expression above the levels currently supported by the INS-TH-NFH promoter. The INS-TH-NFH promoter has supported studies on gene therapy for Parkinson's disease, including coexpression of up to four genes from two transcription units (Sun et al., 2004), and expression for 7, 8, or 14 months (Sun et al., 2003; Sun et al., 2004; Sun et al., 2005). Further, the INS-TH-NFH promoter has supported studies that identified a small ~500 neuron circuit in rat postnatal cortex that can encode some of the essential information for performing specific visual object discriminations (Zhang et al., 2005; Zhang et al., 2010). Moreover, this promoter has supported studies that corrected deficits in spatial learning, by gene transfer into neurons in the hippocampal dentate area (Zhang et al., 2009). Identification of specific transcription factor binding sites, and the cognate transcription factors, that support long-term expression will illuminate the mechanisms that can support long-term expression from HSV-1 vectors, and thereby benefit both gene therapy and basic neuroscience studies.

4. Materials and Methods

4.1. Materials

Oligonucleotides were synthesized by Invitrogen. Restriction endonucleases and calf intestinal phosphatase (CIP) were obtained from New England BioLabs. Dulbecco's modified minimal essential medium (DMEM), OPTI-MEM I, glutamine, penicillin/streptomycin, and fetal bovine serum (FBS) were from Invitrogen. G418 was obtained from RPI. X-Gal was obtained from Sigma. Rabbit or mouse anti-*E. coli* β -gal antibodies was obtained from Sigma, and mouse monoclonal anti-NeuN was obtained from Millipore. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Ig) G, biotinylated goat anti-mouse IgG, the ABC reagent, Texas red-conjugated horse anti-mouse IgG, and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were from Vector Labs.

4.2. Cells

BHK21 cells (Yang et al., 2001) or 2-2 cells (Smith et al., 1992) 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. Cells were grown in humidified incubators containing 5 % CO₂ at 37°C.

4.3. Vectors

pNFHlac has been described (Wang et al., 1999), and pTHoligo-NFHlac is diagrammed in Fig. 2. The transcription unit contains an oligonucleotide with 42 bp of TH enhancer sequence (detailed next), a 0.6 kb fragment of the mouse NFH promoter (from plasmid pH-615 (Schwartz et al., 1994)), the *Lac Z* gene, and an intron and polyadenylation site. To reduce interactions between genetic elements in the vector backbone and the transcription unit, the HSV-1 ori_s fragment, which contains the HSV-1 IE 4/5 promoter, is followed by a cassette of three SV40 early region polyadenylation sites (Wang et al., 1999). Replication and packaging of the vector into HSV-1 particles is supported by an HSV-1 origin of DNA replication (ori_s) and the HSV-1 *a* sequence, which contains the packaging site, respectively.

To construct pTHoligo-wt-NFHlac, pNFHlac was digested with *Bam*H I, which cuts once at the 5' end of the NFH promoter fragment, the DNA was treated with CIP, and a phosphorylated oligonucleotide was inserted that destroys the *Bam*H I site at the 5' end of the oligonucleotide, contains an *Asc* I site to determine orientation of the oligonucleotide, and contains 42 bp of rat TH enhancer sequence from the f4-2-C fragment, located ~3 kb upstream from the TH promoter transcription start site (Gao et al., 2007) (sense strand 5' GATCGGGCGCGCCTAGGCTCTGCGGCTTGCTTCCAGCCTGTACGCTGTCCAGAG 3').

To construct pTHoligo-Mut-1-NFHlac to pTHoligo-Mut-5-NFHlac, we inserted oligonucleotides between the *Asc* I and *Bam*H I sites in pTHoligo-wt-NFHlac. Each oligonucleotide contains an *Fse* I site near the 5' end to verify both insertion of the oligonucleotide and orientation. The oligonucleotides, for the sense strand, were Mut-1 5' CGCGCCGGCCGGCCCTGTAGCAGCGGCTTGCTTCCAGCCTGTACGCTGTCCAGAG 3', Mut-2 5' CGCGCCGGCCGGCCCTAGGCTCTCTGTAGCACTTCCAGCCTGTACGCTGTCCAGAG 3', Mut-3 5' CGCGCCGGCCGGCCCTAGGCTCTGCGGCTTGCTGTAGCACTGTACGCTGTCCAGAG 3', Mut-4 5' CGCGCCGGCCGGCCCTAGGCTCTGCGGCTTGCTTCCAGCCTGTAGCATGTCCAGAG 3', and Mut-5 5'

CGCGCCGGCCGGCCTAGGCTCTGCGGCTTGCTTCCAGCCTGTACGCCTGTAGCA G 3'. The TH enhancer sequences in the resulting vectors were verified by DNA sequencing. The TH enhancer sequences in these vectors are shown in Fig. 1.

The control vector for the β -gal-IR assay was pINS-TH-NFHth/ires/aadc; this vector expresses TH and AADC, but does not express β -gal (Sun et al., 2003).

4.4. Packaging vectors into HSV-1 particles

Vectors were packaged into HSV-1 particles using our helper virus-free packaging system (Fraefel et al., 1996), including an optimized 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 transducing BHK fibroblast cells and 1 day later quantifying the numbers X-gal positive cells (IVP/ml). The titering used BHK cells as the best available assay because these fibroblast cells form a monolayer; in contrast, most neuronal cell lines do not form a monolayer, and BHK cells support higher titers than PC12 cells (Yang et al., 2001; Zhang et al., 2000). The activity of the TH-NFH promoter in fibroblast cells represents ectopic activity that declines rapidly at longer times after gene transfer. Before injection into the brain, each vector stock was diluted as indicated to achieve a titer of 1×10^6 IVP/ml. 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 Long Evans rats (175–200 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) +0.7, medial-lateral (ML) ± 3.0 , 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 (Paxinos and Watson, 1986). 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

At 2 months after gene transfer, rats were perfused, and immunohistochemistry assays were performed, as described (Zhang et al., 2000). 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 mouse anti- β -gal antibody (1:500 dilution) that was visualized using a biotin-conjugated goat anti-mouse IgG (1:200 dilution), 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 Texas red-conjugated secondary antibodies, as described (Zhang et al., 2000).

4.7. Cell counts

Twenty-five μ m coronal sections that contained the area proximal to each injection site were prepared from the striatum; the β -gal-IR cells were found in ~ 35 sections per hemisphere, with the majority of the cells in ~ 20 sections per hemisphere. Every 4th section was analyzed for β -gal-IR. Cells were counted under 60x magnification; each section was counted at least twice, on different days, and the values differed by $<10\%$ for each section.

To quantify neuron-specific expression, photomicrographs were captured using a video camera, under 60x magnification. The β -gal-IR cells in each section were scored for

costaining with NeuN-IR. All the cells containing β -gal-IR in a section were scored; each section was counted at least twice, on different days, and the values differed by <10 % for each section.

Statistical comparisons were performed using ANOVAs (SigmaStat).

4.8. Computer analyses

Putative transcription factor binding sites were analyzed using Genomatix software (<http://www.genomatix.de/cgi-bin/.eldorado/main.pl>). Expression levels in the mouse brain were analyzed using the Allen Brain Atlas (<http://mouse.brain-map.org/welcome.do?sessionId=B4CD215888034060325A29D70FA0EED1>).

Acknowledgments

We gratefully thank Dr. A. Davison for HSV-1 cosmid set C, Dr. K. O'Malley for the TH promoter, and Dr. W. Schlaepfer for the NFH promoter. This work was supported by AG025894 (G.Z.) and NS045855, and NS057558 (A.I.G.).

References

- Brown ER, Coker GTD, O'Malley KL. Organization and evolution of the rat tyrosine hydroxylase gene. *Biochemistry*. 1987; 26:5208–12. [PubMed: 2889468]
- Cao H, Zhang GR, Wang X, Kong L, Geller AI. Enhanced nigrostriatal neuron-specific, long-term expression by using neural-specific promoters in combination with targeted gene transfer by modified helper virus-free HSV-1 vector particles. *BMC Neurosci*. 2008; 9:37. [PubMed: 18402684]
- Dobson AT, Margolis TP, Gomes WA, Feldman LT. In vivo deletion analysis of the herpes simplex virus type 1 latency-associated transcript promoter. *J Virol*. 1995; 69:2264–70. [PubMed: 7884873]
- During MJ, Naegel JR, O'Malley KL, Geller AI. Long-term behavioral recovery in parkinsonian rats by an HSV vector expressing tyrosine hydroxylase. *Science*. 1994; 266:1399–403. [PubMed: 7669103]
- 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; 70:7190–7. [PubMed: 8794366]
- Fritschy JM, Brandner S, Aguzzi A, Koedood M, Luscher B, Mitchell PJ. Brain cell type specificity and gliosis-induced activation of the human cytomegalovirus immediate-early promoter in transgenic mice. *J Neurosci*. 1996; 16:2275–82. [PubMed: 8601807]
- Gao Q, Sun M, Wang X, Geller AI. 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. *Brain Res*. 2007; 1130:1–16. [PubMed: 17169349]
- Geller AI, Breakefield XO. A defective HSV-1 vector expresses Escherichia coli beta-galactosidase in cultured peripheral neurons. *Science*. 1988; 241:1667–9. [PubMed: 2843986]
- Graybiel AM. Neurotransmitters and neuromodulators in the basal ganglia. *Trends Neurosci*. 1990; 13:244–54. [PubMed: 1695398]
- 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; 7:2015–24. [PubMed: 8930662]
- 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 USA*. 1994; 91:8979–83. [PubMed: 8090756]

- Lachmann RH, Efsthathiou S. Utilization of the herpes simplex virus type 1 latency-associated regulatory region to drive stable reporter gene expression in the nervous system. *J Virol.* 1997; 71:3197–207. [PubMed: 9060683]
- 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; 20:460–9. [PubMed: 8679207]
- Loiacono CM, Myers R, Mitchell WJ. Neurons differentially activate the herpes simplex virus type 1 immediate-early gene ICP0 and ICP27 promoters in transgenic mice. *J Virol.* 2002; 76:2449–59. [PubMed: 11836423]
- Loiacono CM, Taus NS, Mitchell WJ. The herpes simplex virus type 1 ICP0 promoter is activated by viral reactivation stimuli in trigeminal ganglia neurons of transgenic mice. *J Neurovirol.* 2003; 9:336–45. [PubMed: 12775417]
- Mitchell WJ. Neurons differentially control expression of a herpes simplex virus type 1 immediate-early promoter in transgenic mice. *J Virol.* 1995; 69:7942–50. [PubMed: 7494307]
- Paxinos, G.; Watson, C. The rat brain in stereotaxic coordinates. Academic Press; Sidney: 1986.
- Ptashne M. How eukaryotic transcriptional activators work. *Nature.* 1988; 335:683–9. [PubMed: 3050531]
- Rasmussen M, Kong L, Zhang G, Liu M, Wang X, Szabo G, Curthoys NP, Geller AI. The phosphate-activated glutaminase, vesicular glutamate transporter-1, or glutamic acid decarboxylase promoters support long-term, glutamatergic- or GABAergic-specific expression from helper virus-free HSV-1 vectors. 2006 In preparation.
- Rasmussen M, Kong L, Zhang G, Liu M, Wang X, Szabo G, Curthoys NP, Geller AI. Glutamatergic or GABAergic neuron-specific, long-term expression in neocortical neurons from helper virus-free HSV-1 vectors containing the phosphate-activated glutaminase, vesicular glutamate transporter-1, or glutamic acid decarboxylase promoter. *Brain Res.* 2007; 1144:19–32. [PubMed: 17331479]
- Roizman, B.; Sears, AE. Herpes simplex viruses and their replication. In: Roizman, B.; Whitley, RJ.; Lopez, C., editors. The human herpesviruses. Raven Press; New York: 1993. p. 11–68.
- Schwartz ML, Katagi C, Bruce J, Schlaepfer WW. Brain-specific enhancement of the mouse neurofilament heavy gene promoter in vitro. *J Biol Chem.* 1994; 269:13444–50. [PubMed: 8175776]
- 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; 68:1792–803. [PubMed: 9109503]
- 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; 10:2005–11. [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; 14:415–24. [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; 1052:119–29. [PubMed: 16018990]
- Tao Y, Kassatly RF, Cress WD, Horowitz JM. Subunit composition determines E2F DNA-binding site specificity. *Mol Cell Biol.* 1997; 17:6994–7007. [PubMed: 9372931]

- Taus NS, Mitchell WJ. The transgenic ICP4 promoter is activated in Schwann cells in trigeminal ganglia of mice latently infected with herpes simplex virus type 1. *J Virol.* 2001; 75:10401–8. [PubMed: 11581408]
- 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; 10:1763–71. [PubMed: 10446916]
- 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. *Molec Brain Res.* 2001; 90:1–16. [PubMed: 11376851]
- 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. *Molec Brain Res.* 2000; 84:17–31. [PubMed: 11113528]
- Zhang G, Wang X, Kong L, Lu X, Lee B, Liu M, Sun M, Franklin C, Cook RG, Geller AI. Genetic enhancement of visual learning by activation of protein kinase C pathways in small groups of rat cortical neurons. *J Neurosci.* 2005; 25:8468–81. [PubMed: 16162929]
- Zhang G, Liu M, Cao H, Kong L, Wang X, Cook RG, Geller AI. Improved spatial learning in aged rats by genetic activation of protein kinase C in small groups of rat hippocampal neurons. *Hippocampus.* 2009; 19:413–23. [PubMed: 18942114]
- Zhang G, Cao H, Kong L, O'Brien J, Baughns A, Jan M, Zhao H, Wang X, Lu X, Cook RG, Geller AI. Identified circuit in rat postrhinal cortex encodes essential information for performing specific visual shape discriminations. *Proc Natl Acad Sci USA.* 2010; 107:14478–14483. [PubMed: 20660720]
- Zhang G, Geller AI. An HSV-1 vector containing the VGLUT1 promoter is expressed only in VGLUT1-, and not VGLUT2-, containing glutamatergic neurons. *Brain Res.* 2010; 1331:12–19. [PubMed: 20307509]
- Zhang G, Li X, Cao H, Zhao H, Geller AI. The vesicular glutamate transporter-1 upstream promoter and first intron each support glutamatergic-specific expression in rat postrhinal cortex. *Brain Research.* 2011; 1377:1–12. [PubMed: 21172319]

Highlights

Promoters that support long-term expression are desirable

A 16 bp sequence that supports long-term expression was identified

This 16 bp enhancer sequence is from the TH promoter

This 16 bp sequence contains a putative E2F-1/DP-1 binding site

WT CTAGGCTCTGCGGCTTGCTTCCAGCCTGTACGCTGTCCAGAG
Mut-1 .CT.TAG.A.....
Mut-2 CT.TAGCA.....
Mut-3 GTAGCA.....
Mut-4 GCA.....
Mut-5 CTGTAGC..
Maximum Critical Sequence CTTCCAGCCTGTACGC

Fig. 1.

The 42 bp 5' upstream enhancer sequence from TH promoter, the mutations studied here, and the maximum critical sequence, as determined from the analysis. The 42 bp enhancer sequence was derived from a previous study in which a specific fragment (f4-2-C) supported long-term expression, and the two adjacent, partially overlapping fragments did not support long-term expression (Gao et al., 2007). The 42 bp sequence studied here contains 23 bp of sequence unique to f4-2-C, and the underlined flanking sequences on each end are also present in either adjacent fragment (9 bp at the 5' end and 10 bp at the 3' end). The sequences of the five mutations studied here are shown underneath the wt sequence. The 16 bp maximum critical sequence, determined from this analysis, is presented at the bottom.

pTHoligo-NFHIac

Fig. 2.

Schematic diagram of pTHoligo-NFHIac. The transcription unit contains an oligonucleotide with 42 bp of 5' upstream enhancer sequence from TH promoter that is flanked by restriction sites for insertion into the vector (brick segment, detailed in methods and Fig. 1), the NFH promoter (wavy line segment), the *Lac Z* gene (black segment), the second intron from the mouse α -globin gene (triangle), and the SV40 polyadenylation signal (cross-stippled segment). A cassette of 3 polyadenylation sites (tri A, clear segment) was placed 5' to the TH enhancer oligonucleotide to reduce any effects on expression from the HSV-1 IE 4/5 promoter (diagonal line segment). A HSV-1 origin of DNA replication (*ori_s*, black circle in diagonal line segment) and the HSV-1 α sequence, which contains the packaging site (diagonal line segment), support replication and packaging of the vector into HSV-1 particles, respectively. Genetic elements from pBR322 (short line segment) support growth of the vector in *E. coli*.

Fig. 3.

Low and high power views of β -gal-IR striatal cells from rats sacrificed at 2 months after gene transfer with vectors containing the wt TH enhancer sequence or each of the five mutations. 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 photomicrographs show areas proximal to the needle tracks. (A and B) pTHoligo-wt-NFHLac, (A) low power or (B) high power. (C and D) pTHoligo-Mut-1-NFHLac, (C) low power or (D) high power. (E and F) pTHoligo-Mut-2-NFHLac, (E) low power or (F) high power. (G and H) pTHoligo-Mut-3-NFHLac, (G) low power or (H) high power. (I and J) pTHoligo-Mut-4-NFHLac, (I) low power or (J) high power. (K and L) pTHoligo-Mut-5-

NFHLac, (K) low power or (L) high power. pTHoligo-wt-NFHLac and pTHoligo-Mut-1, 2, or 5-NFHLac support numerous β -gal-IR cells; in contrast, pTHoligo-Mut-3 or 4-NFHLac support few β -gal-IR cells. Controls: (M and N) pTHoligo-wt-NFHLac, no primary antibody, (M) low power or (N) high power. (O and P) A control vector, pINS-TH-NFHth/ires/aadc, (O) low power or (P) high power. Scale bars: (A,C,E,G,I,K,M, and O) 100 μ m; (B,D,F,H,J,L,N, and P) 50 μ m.

Fig. 4. β -gal-IR striatal cells that contain NeuN-IR from a rat sacrificed at 2 months after gene transfer with pTHoligo-wt-NFHlac. 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 Texas red-conjugated secondary antibody. (A–C) pTHoligo-wt-NFHlac; (A) β -gal-IR, (B) NeuN-IR, and (C) merged. Numerous β -gal-IR cells were observed, and most of these cells contained NeuN-IR (arrows). (D–F) Omitting the primary antibodies from the assay resulted in background levels of fluorescence; (D) fluorescein-conjugated secondary antibody-IR, (E) Texas red-conjugated secondary antibody-IR, and (F) merge. Scale bar: 50 μ m.

TABLE 1

The numbers of β -gal-IR cells, at 2 months after gene transfer, supported by HSV-1 vectors containing the wt sequence or specific mutations in the upstream TH enhancer.

TH sequence	Average β -gal-IR cells per hemisphere
WT	298 $\pm$ 36
Mut-1	307 $\pm$ 15
Mut-2	282 $\pm$ 48
Mut-3	83 $\pm$ 14
Mut-4	46 $\pm$ 5
Mut-5	262 $\pm$ 66

Four striata were analyzed for each vector. The sequences of the wt TH fragment and the mutations are shown in Fig. 1. The mean $\pm$ s.e.m. are shown.

TABLE 2

For specific vectors that contain different fragments of the upstream TH enhancer, the amount of vector injected, the numbers of expressing cells at specific times after gene transfer, and the ratio of expressing cells to IVP injected

Vector	TH fragment ^a	Brain area	Survival time	IVP injected	Cells per hemisphere	Cells/10 ³ IVP	Reference
pTHoligo-wt-NFHlac	3,910–3,951	striatum	2 months	3 × 10 ³	298±36	99	this study
pTH-NFHlac exp. 1	1–6342	striatum	2 months	5 × 10 ³	125±23	25	(Gao et al., 2007)
pTHf4-NFHlac exp. 1	3,344–4,563	striatum	2 months	5 × 10 ³	107±31	25	(Gao et al., 2007)
pTH-NFHlac exp. 2	1–6342	striatum	2 months	6 × 10 ³	189±64	21	(Gao et al., 2007)
pTHf4-NFHlac exp. 2	3,344–4,563	striatum	2 months	6 × 10 ³	198±119	32	(Gao et al., 2007)
pTHf4-2-NFHlac exp. 2	3,771–4,090	striatum	2 months	5 × 10 ³	188±48	33	(Gao et al., 2007)
pTH-NFHlac exp. 3	1–6342	striatum	2 months	6 × 10 ³	230±84	38	(Gao et al., 2007)
pTHf4-2-NFHlac exp. 3	3,771–4,090	striatum	2 months	5 × 10 ³	195±42	38	(Gao et al., 2007)
pTHf4-2-A-NFHlac exp. 3	3,771–3,854	striatum	2 months	7 × 10 ³	152±50	39	(Gao et al., 2007)
pTHf4-2-C-NFHlac exp. 3	3,879–3,982	striatum	2 months	6 × 10 ³	184±57	22	(Gao et al., 2007)
pTH-NFHlac	1–6342	striatum	2 months	9 × 10 ⁴	230±35	31	(Zhang et al., 2000)
pTH-NFHlac	1–6342	hippocampus	2 months	9 × 10 ⁴	789±215	8.8	(Zhang et al., 2000)
pTH-NFHlac	1–6342	perirhinal cortex	1 month	9 × 10 ⁴	123±26	1.4	(Zhang et al., 2000)
pTH-NFHlac	1–6342	posthinal cortex	1 month	9 × 10 ⁴	165±25	1.8	(Zhang et al., 2000)
PkcΔ vector ^b	1–6342	posthinal cortex	7 weeks	2 × 10 ⁴	428±25	21	(Zhang et al., 2010)
PkcΔ vector ^b	1–6342	hippocampus	1 month	9 × 10 ⁴	4,879±429	54	(Zhang et al., 2009)
4-gene-vector ^c	1–6342	striatum	6 months	2 × 10 ⁵	11,400±2,850	57	(Sun et al., 2004)

In this study and (Gao et al., 2007), β-gal-IR cells were assayed in the striatum; in (Zhang et al., 2000), (Zhang et al., 2010), and (Zhang et al., 2009), X-gal cells were assayed in specific brain areas; in (Sun et al., 2004), flag-IR cells (flag-TH) were assayed in the striatum. The β-gal-IR assay is more sensitive than the X-gal assay. In (Gao et al., 2007) and in (Zhang et al., 2000), the same vector was often assayed in the same brain area at multiple time points; in these experiments, the 2 month time point, or the closest time point to 2 months, is shown. Three to six hemispheres were assayed for each condition; mean±s.e.m. are shown, except mean±s.d. are shown for conditions from (Zhang et al., 2000).

^aNucleotides refer to a 6.3 kb Bam HI fragment, from –0.5 to –6.8 kb in the TH promoter, that supports long-term expression in pTH-NFHlac (Zhang et al., 2000).

^bpINS-TH-NFHpkcΔ/INS-TH-NFHlac

^cpINS-TH-NFHflag-th/ires/aadc/INS-TH-NFHha-vmat/ires/gtpch