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An HSV-1 Vector Expressing Tyrosine Hydroxylase Causes Production and Release of L-DOPA from Cultured Rat Striatal Cells

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Abstract: In this report we demonstrate that a defective herpes simplex virus type one (HSV-1) vector can express enzymatically active tyrosine hydroxylase in cultured striatal cells that are thereby converted into L-DOPA–producing cells. A human tyrosine hydroxylase cDNA (form II) was inserted into an HSV-1 vector (pHSVth) and packaged into virus particles using an HSV-1 strain 17 mutant in the immediate early 3 gene (either ts K or D30EBA) as helper virus. Cultured fibroblasts were infected with pHSVth and 1 day later tyrosine hydroxylase immunoreactivity and tyrosine hydroxylase enzyme activity were observed. The tyrosine hydroxylase enzyme activity directed the production of L-DOPA. pHSVth infection of striatal cells in dissociated cell culture resulted in expression of tyrosine hydroxylase RNA and tyrosine hydroxylase immunoreactivity. Release of L-DOPA and low levels of dopamine were observed from cells in pHSVth-infected striatal cultures. Expression of tyrosine hydroxylase and release of catecholamines were maintained for at least 1 week after infection. **Key Words:** Tyrosine hydroxylase—Defective herpes simplex virus vector—Striatal neuron—Parkinson's disease—Gene therapy.
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The development of vector systems capable of transferring recombinant genes into mammalian neurons has scientific as well as clinical importance. One such approach has used defective herpes simplex virus type one (HSV-1) vectors to express reporter genes in neurons in culture and in vivo (Geller and Breakefield, 1988; Freese et al., 1990*b*; Geller and Freese, 1990; Geller et al., 1990). This methodology appears to support long-term (>2 weeks) expression of *E. coli* β-galactosidase in both PNS and CNS neurons. Recently, several studies have demonstrated that this HSV-1 vector system can be used to introduce functional gene products into a variety of cell types, including PNS and CNS neurons. For example, nerve growth factor was expressed in the superior cervical ganglia of axotomized rats (Federoff et al., 1992), the human nerve growth factor receptor (p75) was expressed in cultured cortical neurons (Battleman et al., 1993), the glucose transporter gene was expressed in hippocampal neurons and glia (Ho et al., 1993), the GluR6 receptor subtype was introduced into these same cell types (Bergold et al., 1993), and an adenylate cyclase was expressed in cultured sympathetic neurons (Geller et al., 1993). In each case, the recombinant gene product produced a functional response. These studies lend impetus to experiments using defective HSV-1 vectors in the development of gene therapy approaches to certain neurological disorders such as Parkinson's disease (PD).

PD is a neurodegenerative disorder resulting from the death of the dopaminergic neurons in the substantia nigra pars compacta, which project to the corpus striatum (for review, see Hornykiewicz, 1988). Current therapy for PD is to elevate striatal dopamine levels by oral administration of L-DOPA, the precursor for dopamine. It is considered that the exogenous L-DOPA is converted to dopamine primarily by the aromatic amino acid decarboxylase (EC 4.1.1.28; AADC) pres-

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Abbreviations used: AADC, aromatic amino acid decarboxylase; ABC, avidin-biotin complex; HSV-1, herpes simplex virus type one; HTH-2, human TH type II; IE, immediate early; IR, immunoreactivity; moi, multiplicity of infection; PBS, phosphate-buffered saline; PD, Parkinson's disease; pfu, plaque forming units; RT, reverse transcription; TH, tyrosine hydroxylase; ts, temperature sensitive.

ent in the surviving dopaminergic neurons (Melamed, 1988), although additional sites for conversion of L-DOPA to dopamine may also exist (Tashiro et al., 1989; Li et al., 1992). Due to the loss of efficacy of L-DOPA over time, various cell transplantation procedures have been developed to achieve in situ production of L-DOPA. In general, although transplantation of L-DOPA– or dopamine-producing cells into the striatum has been successful in animal models of PD and even in some human studies (Yahr and Bergmann, 1987; Lindvall et al., 1990), there are several potential obstacles to the widespread application of this technique, particularly as it applies to the acquisition of human fetal neuronal tissue. In contrast, genetically engineered cells producing the L-DOPA biosynthetic enzyme, tyrosine hydroxylase (EC 1.14.16.2; TH) have proven to be effective in the rat model of PD (for review, see Suhr and Gage, 1993). Fibroblasts, myoblasts, pituicytes, and glia can support expression of recombinant TH and consequent production of L-DOPA (Horellou et al., 1989; Wolff et al., 1989; Owens et al., 1991; Jiao et al., 1993). However, because many of these cell types are not normally found in the CNS, long-term problems associated with proliferation, extracellular matrix deposition, and/or loss of TH gene expression have been observed (Gage et al., 1991).

An alternative approach to implanting heterologous cells expressing TH is to use the defective HSV-1 vector system to introduce the TH gene directly into striatal cells, thereby converting a fraction of the striatal cells into L-DOPA–producing cells (Freese et al., 1990*b*; O'Malley and Geller, 1992). The potential advantages of this approach include first, L-DOPA is produced proximal to its required site of action, and second, there is no potential problem of either graft rejection or tumor formation from graft cells. A potential disadvantage is that striatal neurons normally use other neurotransmitters; therefore, regulation of catecholamine release may be altered or suppressed. Although nonneuronal cell types, including fibroblasts, myoblasts, pituicytes, and glia, can support expression of recombinant TH and consequent production of L-DOPA (Horellou et al., 1989; Wolff et al., 1989; Owens et al., 1991; Jiao et al., 1993), it has not been established that expression of TH in striatal cells can direct the production of L-DOPA in these cells. To begin to explore the feasibility of this approach, TH was expressed in cultured cells from an HSV-1 vector, and production and release of L-DOPA was examined. Expression of recombinant TH in cultured fibroblasts resulted in the production of TH RNA, TH immunoreactivity (TH-IR), and TH enzyme activity, which directed the production of L-DOPA. Expression of TH in cultured striatal cells resulted in production of TH RNA and TH-IR and release of L-DOPA into the media.

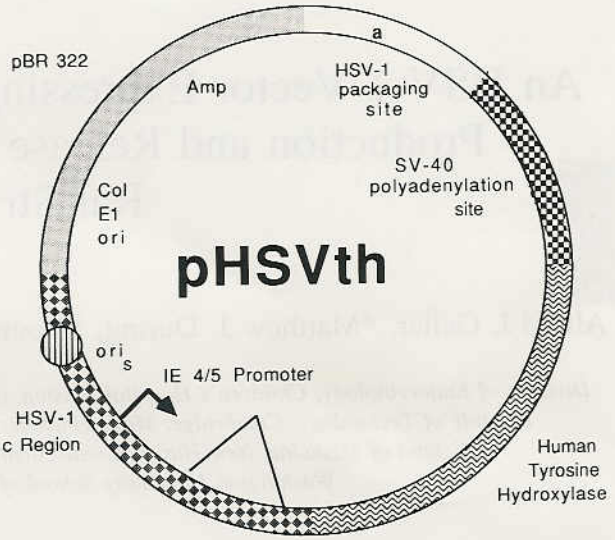

FIG. 1. The structure of pHSVth. The transcription unit in pHSVth contains the HSV-1 IE4/5 promoter (arrow), the intervening sequence following that promoter (triangle), the HTH-2 cDNA (O'Malley et al., 1987; wavy line segment), and the SV-40 early region polyadenylation site (checkerboard). An HSV-1 origin of DNA replication (ori_s, circle with vertical lines) and the HSV-1 packaging site [**a** sequence, clear segment] support packaging into HSV-1 particles; pBR322 sequences (dotted).

MATERIALS AND METHODS

Construction of pHSVth and packaging into HSV-1 particles

Constructions were performed by standard recombinant DNA procedures (Maniatis et al., 1982). The human TH type II (O'Malley et al., 1987; HTH-2), one of the two predominant forms of TH in the human CNS (Coker et al., 1990; Lewis et al., 1993), was inserted into an HSV-1 vector, pHSVlac (Geller and Breakefield, 1988). pHSVlac was digested to completion with *Hin*dIII and partially with *Eco*RI to remove the 3.3-kb *E. coli LacZ* gene fragment, which was replaced with the 1.7-kb TH cDNA form II fragment (O'Malley et al., 1987; Ginns et al., 1988) using the same restriction endonuclease sites. The resulting vector was designated pHSVth (Fig. 1). The control vectors used in this study were pHSVpUC and pHSVlac, which express the pUC19 polylinker and *E. coli* β-galactosidase, respectively. pHSVth and control vectors were packaged into HSV-1 particles using our established procedures (Geller, 1988; Geller et al., 1990). Initially we used HSV-1 strain 17 ts K (Davison et al., 1984), which harbors a temperature-sensitive (ts) mutation in the immediate early (IE) 3 gene, as helper virus. Subsequent packaging procedures used a deletion mutant in the IE3 gene, D30EBA (Paterson and Everett, 1990). The titers of the pHSVth virus stocks were as follows: 5×10^6 plaque-forming units (pfu)/ml ts K and 4×10^6 infectious particles/ml pHSVth or 1×10^7 pfu/ml D30EBA and 9×10^6 infectious particles/ml pHSVth.

PCR analysis of pHSVth DNA in HSV-1 particles

CV1 monkey fibroblasts (1×10^7) were infected with pHSVth [multiplicity of infection (moi) 5.0] and the cells

were incubated at 31°C for 24 h. Cells were lysed (60°C, 1 h) in a PCR-compatible buffer containing nonionic detergents (50 m*M* KCl, 10 m*M* Tris, pH 8.3, 2.5 m*M* $MgCl_2$, 0.1 mg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween 20) and proteinase K (Higuchi, 1989). After incubation, the proteinase K was inactivated by heating (95°C, 10 min) and an aliquot of the cell lysate subjected to PCR (30 cycles; 1 min 94°C, 1 min 60°C, 1 min 72°C) using ^{32}P-end-labeled primers from exon 1 (OHTH-116, nucleotides 36–65) and exon 2 (OHTH-193, nucleotides 156–185) of the human TH gene; the predicted PCR product is 150 bp. Reaction products were electrophoresed on a 5% polyacrylamide gel and the gel was subjected to autoradiography.

TH immunofluorescence in CV1 cells

Cultures (1×10^5 cells/0.5 ml) were infected with pHSVth (moi 0.1). One day later, the cells were fixed with 4% paraformaldehyde in 0.1 *M* $NaPO_4$, pH 7.4, the cells were washed with phosphate-buffered saline (PBS; three times, 5 min each), and immunocytochemistry was performed (Huettner and Baughman, 1986). The cells were incubated first in PBS containing 1% goat serum and 0.1% Triton X-100 for 30 min at room temperature and then in the same solution containing the primary antibody (mouse monoclonal anti-rat TH antibody, 1:100 dilution) overnight at 4°C. The cells were washed (three times, 5 min each) in PBS containing 1% goat serum and 0.1% Triton X-100, and then incubated (2 h, room temperature) in the same buffer containing fluorescein isothiocyanate–conjugated goat $F(ab')_2$ anti-mouse $F(ab')_2$ antibody (1:200 dilution). The cells were washed as described above, and the coverslips were mounted in PBS/glycerol (1:1) containing 0.4% *n*-propyl gallate. Photomicrographs were taken with Kodak P800-1600 film. To determine the percentage of cells containing TH-IR, at least 500 cells in a culture were scored; the experiment was repeated four times with similar results; and the results from one experiment are presented.

TH enzyme and catecholamine assays in fibroblasts

CV1 cells (3×10^6) were infected (moi 0.2) with pHSVth or ts K, or mock infected, and incubated for 1 day. TH enzyme activity was determined in cell lysates using a nonenzymatic coupled decarboxylation assay (Waymire et al., 1971). To analyze catecholamine metabolites, from 16 to 24 h after infection, the cultures were incubated in defined medium consisting of 135 m*M* NaCl, 3 m*M* KCl, 1 m*M* $MgCl_2$, 1.2 m*M* $CaCl_2$, 2 m*M* $NaPO_4$, pH 7.4, 10 m*M* glucose (Geller et al., 1993), and 0.5 m*M* methyltetrahydropterine was added as indicated. Cells were lysed in 1 ml of 0.1 *M* $HClO_4$, 1% $Na_2S_2O_5$ by sonication for 2 min. Cell lysates were clarified by centrifugation (2,500 *g*, 5 min) and assayed for catecholamines by HPLC (Matson et al., 1987). Catecholamine analysis was performed by HPLC followed by a serial array of 16 electrode sensors. A gradient method (Dr. I. N. Acworth of ESA Inc.) was used to separate catecholamines by HPLC; a 15 × 0.45-cm, 5 μm Nikko Bioscience column was eluted with a gradient from 0.1 *M* NaH_2PO_4 (pH 3.45), 10 mg/L dodecyl sulfonic acid, 0.1 μ*M* nitrilotriacetic acid (mobile phase A) to 0.1 *M* NaH_2PO_4 (pH 3.35), 50 mg/L dodecyl sulfonic acid, 0.1 μ*M* nitrilotriacetic acid, 50% methanol (vol/vol) (mobile phase B). The gradient increased phase B from 6% at 0 min to 40% at 15 min, followed by a second gradient to 90% phase B at 21 min with an 11-min reequilibration period. The electrochemical detector (CEAS no. 55-0650, ESA) contained a serial array of 16 electrode sensors set at 50 mV increments with sensor 1 set at 0 mV. The identity of peaks was determined by comparison with standards (match criteria: retention time ±2%; peak width ±3 s; peak ratio between sensors >80%). The amount of L-DOPA or dopamine in each sample was quantitated by comparing the peak height in the dominant sensor relative to the standards. The data were expressed as picograms of catecholamine × 10^{-3}/cell.

Striatal cultures

Dissociated striatal cell cultures were prepared from the striatum of 2-day-old Sprague–Dawley rats by the method of Freese et al. (1990*a*); i.e., 24-well plates were treated with 1 ml of 20 mg/ml poly-L-lysine and washed with H_2O just before use. The anterior striatum was removed and placed into a Petri dish containing Tyrode's buffer (140 m*M* NaCl, 4 m*M* KCl, 360 μ*M* NaH_2PO_4, 180 μ*M* KH_2PO_4, 11 m*M* glucose, pH 7.4) on ice. After all the striata were dissected, the buffer was aspirated and ~1 ml of media [45% Dulbecco's modified Eagle medium, 45% Ham's F12, 10% heat-inactivated horse serum, 4 g/L glucose, and 100 U/ml penicillin/streptomycin] was added, the tissue was dissociated by trituration with a flame-polished Pasteur pipette, the cells were diluted with medium to a concentration of 3×10^5/ml, and 0.5 ml was added to each well of a 24-well plate. Cultures were incubated at 37°C in the presence of 5% CO_2. The cultures were treated on days 5–6 with 40 μ*M* cytosine arabinoside. Subsequently, the cultures were fed every third day by replacing two-thirds of the medium with fresh medium. Cultures were infected either 7 or 21 days after plating.

RNA analysis in striatal cultures

Cultures (0.5 ml) of striatal cells were infected with pHSVth (moi 0.2) and the cells were incubated at 37°C for 5 days. RNA was extracted as described (Chomczynski and Sacchi, 1987), for each condition the RNA obtained from six cultures was combined, and then treated with RNase-free DNase (Krug and Berger, 1987) to remove any contaminating DNA. Reverse transcription (RT) was performed after normalizing RNA samples with an 18S ribosomal RNA probe (Coker et al., 1990), and PCR was performed as described above. Reactions were also performed in the absence of reverse transcriptase to quantitate any PCR products arising from pHSVth vector DNA. PCR products were analyzed on a 5% polyacrylamide gel and the expected product of 150 bp was observed. Autoradiograms were subjected to densitometry scanning to quantitate the levels of the PCR products.

TH immunoreactivity in striatal cultures

Cultures (1×10^5 cells/0.5 ml, day 14) were infected on day 14 with pHSVth (moi 0.05). One week later the cells were fixed with 4% paraformaldehyde in PIPES, pH 7.5, and immunocytochemistry was performed using the avidin-biotin complex (ABC) procedure (Hsu et al., 1981). The primary antibody was mouse monoclonal anti-rat TH antibody (1:2,000 dilution in PBS containing 1% bovine serum albumin and 0.1% Triton X-100). The secondary antibody was biotinylated goat $F(ab')_2$ anti-mouse IgG (1:100 dilution). After washing (three times, 5 min each) the cells were incubated with ExtrAvidin (1:30 dilution) and the horserad-

ish peroxidase reaction was performed. At least 500 cells containing TH-IR were counted and the neuronal or glial morphology of each cell was scored. To determine the total number of cells examined, the number of cells in each of 10 microscopic fields was counted, and the average number of cells in a field and the number of fields examined was used to calculate the total number of cells examined. The number of cells containing TH-IR and the total number of cells examined were used to calculate the percentage of cells containing TH-IR. The experiment was repeated three times with similar results and the results from one experiment are shown.

Catecholamine release assays from striatal cells

Striatal cultures (2×10^5 cells/0.5 ml, day 14) were infected (day 14 or 19) with pHSVth (moi 0.2) and catecholamine release assayed on day 21 (2 and 7 days after infection). Cells were incubated in defined medium for 8 h (Bottenstein and Sato, 1979); the TH cofactor methyltetrahydropterine (0.5 m*M*) and tyrosine (1 m*M*) were added where indicated to the defined medium. Release assays were performed by incubating cells (30 min, 37°C) in 200 μl of release buffer containing 135 m*M* NaCl, 3 m*M* KCl, 1 m*M* $MgCl_2$, 1.2 m*M* $CaCl_2$, 2 m*M* $NaPO_4$, pH 7.4, 10 m*M* glucose (Geller et al., 1993). The buffer was centrifuged (2,500 *g*, 5 min), and 20 μl of 2 *M* $HClO_4$ and 20 μl of 1% $Na_2S_2O_5$ were added. High K release buffer contained 56 m*M* KCl, 80 m*M* NaCl, and the other components of release buffer. Catecholamines were assayed by HPLC as described above (Matson et al., 1987). Because neurotransmitter release was measured over a 30-min period, this assay measures steady-state changes in release rather than changes in the initial rate of release.

Materials

Cell culture reagents, including horse serum, were obtained from GIBCO (Long Island, NY, U.S.A.). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA, U.S.A.). Taq DNA polymerase was obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Mouse monoclonal anti-rat TH antibody was obtained from Boehringer Mannheim, fluorescein isothiocyanate–conjugated goat $F(ab')_2$ anti-mouse $F(ab')_2$ antibody was from Cappel (Durham, NC, U.S.A.). HPLC colums were obtained from Nikko Bioscience (Tokyo, Japan) and catecholamines were detected with an electrochemical detector (CEAS no. 55-0650; ESA, Bedford, MA, U.S.A.). Radioisotopes were obtained from New England Nuclear (Boston, MA, U.S.A.). Other reagents, antibodies, and drugs were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

RESULTS

Packaging of pHSVth into HSV-1 particles

pHSVth and control vectors were packaged into HSV-1 particles by our established procedure, using HSV-1 ts K as helper virus (Geller, 1988). To determine if pHSVth was properly packaged into HSV-1 particles, DNA was prepared from pHSVth- or ts K–infected cells, or mock infected cells (Geller and Breakefield, 1988), and analyzed using the PCR and primers specific to the human TH gene. PCR analysis of the DNA isolated from pHSVth-infected cells, but not the negative controls, yielded a band of the expected size that comigrated with the PCR products obtained using the HTH-2 cDNA (Fig. 2, lanes pHSVth and human th, respectively).

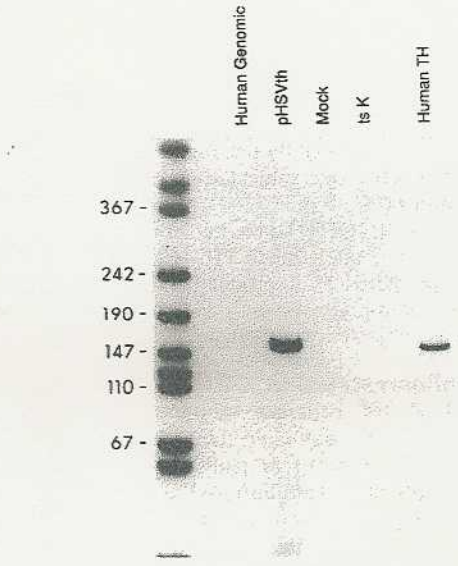

FIG. 2. pHSVth is properly packaged into HSV-1 particles. CV1 cells were infected with pHSVth and incubated at 31°C for 1 day. DNA was isolated and subjected to PCR using primers from exons 1 and 2 of the human TH gene; the predicted PCR product is 150 bp. Reaction products were resolved on a polyacrylamide gel with size standards (bp) as shown. Lanes: Human Genomic, human genomic DNA; pHSVth, DNA isolated from pHSVth-infected cells; ts K, DNA isolated from ts K–infected cells; Mock, DNA isolated from uninfected CV1 cells; Human TH, *E. coli* plasmid DNA of HTH-2 cDNA.

Expression of TH in fibroblast cells

The ability of pHSVth to direct production of TH was investigated. CV1 monkey fibroblasts were infected with pHSVth or pHSVpUC, or were mock infected, 1 day later the cells were fixed, and expression of TH was detected by immunocytochemistry. TH-IR was detected using a monoclonal mouse anti-rat TH antibody, which was visualized with a fluorescein isothiocyanate–conjugated goat anti-mouse IgG antibody. Cultures infected with pHSVth (moi 0.1) contained ~2% TH-IR cells (16 cells contained TH-IR, 737 cells were scored; Fig. 3); in contrast, pHSVpUC or mock infected cultures contained <0.2% (0 cells contained TH-IR, 500 cells scored) TH-IR cells. pHSVth-infected cultures assayed with preimmune primary serum followed by the secondary antibody lacked cells (<0.2%) containing TH-IR (not shown). The CV1 cells maintained normal morphology and no cytopathic effects were apparent during the 1-day incubation after infection with either pHSVth or pHSVlac. RNA was isolated from infected cells and analyzed by RT-PCR using human TH-specific primers; pHSVth-infected CV1 cells contained TH RNA, whereas

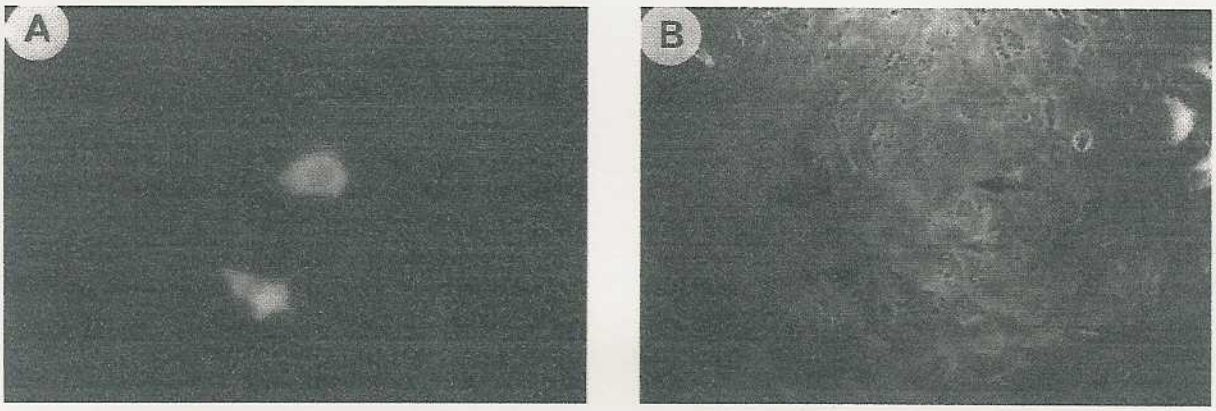

FIG. 3. pHSVth expresses TH in cultured fibroblasts. CV1 cells were infected with pHSVth (moi 0.1) and 1 day later immunocytochemistry was performed (Geller and Breakefield, 1988; Geller and Freese, 1990) using a mouse anti-rat TH antibody and TH-IR was visualized with a fluorescein isothiocyanate–conjugated goat anti-mouse IgG. The width of each photomicrograph represents 230 μm. **A:** TH-IR. **B:** Phase-contrast photomicrograph of the same field.

pHSVpUC- or mock infected CV1 cells lacked TH RNA (not shown).

The ability of pHSVth to direct the production of TH enzyme activity was investigated. CV1 cells were infected with pHSVth or ts K, or were mock infected, 1 day later cell extracts were prepared, and TH enzyme activity was measured. Cultures infected with pHSVth, but not ts K, contained a 90-fold increase in TH enzyme activity compared with mock infected cultures, and this increase was statistically significant ($p < 0.01$, Table 1). The amount of TH activity per microgram of protein per hour in the pHSVth-infected fibroblasts was ~40% of the TH activity measured in rat striatal tissue assayed concurrently.

TABLE 1. *TH enzyme activity and catecholamine synthesis in CV1 monkey fibroblasts 1 day after pHSVth infection*

Condition	TH activity (pmol L-DOPA/ μg of protein/h)	pg of catecholamines × 10^{-3}/cell	
		L-DOPA	Dopamine
pHSVth	0.181 ± 0.025[a]	0.19 ± 0.01[b]	0.19 ± 0.03[c]
pHSVth + cofactor		1.4 ± 0.2[c]	0.27 ± 0.04[c]
ts K	0.002 ± 0.003	0.06 ± 0.01	0.02 ± 0.01
Mock	0.002 ± 0.004	0.02 ± 0.007	0.03 ± 0.01
Rat striatum	0.457 ± 0.063		

CV1 cells were infected with pHSVth or ts K, or were mock infected, and incubated for 1 day. TH enzyme activity was determined in cell lysates, using a nonenzymatic coupled decarboxylation assay (Waymire et al., 1971). Catecholamines were assayed by HPLC (Matson et al., 1987) as described in Materials and Methods. Mock and ts K values were not altered by cofactor; values shown are from experiments without cofactor. For both TH enzyme activity and catecholamine synthesis assays, each experimental condition was performed in duplicate, each experiment was performed three times, and the standard deviations are shown.

[a] $p < 0.01$ or [b] $p < 0.05$ or [c] $p < 0.001$, compared with either ts K or mock conditions using repeated-measures ANOVA with Dunnett's post hoc test.

TH directs production of catecholamines

To determine if expression of recombinant human TH directed the production of L-DOPA, CV1 cells were infected, 1 day later cell extracts were prepared, and L-DOPA and dopamine levels were measured by HPLC (Matson et al., 1987). Cultures infected with pHSVth, but not ts K, contained a 10- and 7-fold increase in L-DOPA and dopamine, respectively, compared with mock infected cultures (Table 1). Addition of tyrosine and methyltetrahydropterine, the cofactor for TH, to the culture medium increased L-DOPA and dopamine levels in pHSVth-infected cells 68- and 10-fold, respectively, above levels in mock infected cells. Cultures infected with pHSVth showed a statistically significant increase in the biosynthesis of L-DOPA compared with cultures either infected with ts K or mock infected (without cofactor, $p < 0.05$; with cofactor added, $p < 0.001$, Table 1).

Expression of TH in striatal cells

We examined the ability of pHSVth to express human TH RNA and protein in striatal cells in dissociated cell culture (Freese et al., 1990*a*). Five days after infection total cellular RNA was extracted, cDNA was synthesized using reverse transcriptase, and the RT products were amplified using PCR and human TH-specific primers. The reaction products were subjected to Southern analysis using a human TH-specific probe, and the TH-specific band on the resulting autoradiogram was quantitated by densitometry scanning. To quantitate any background due to PCR amplification of pHSVth vector DNA, reverse transcriptase was omitted from parallel assays performed on aliquots of the same RNA preparations. As shown in Fig. 4, the

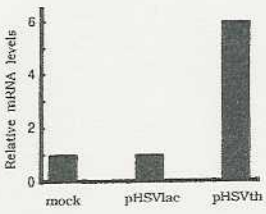

FIG. 4. Relative levels of TH RNA in striatal cultures 5 days after pHSVth infection. RNA was isolated from striatal cells; human-specific TH RNA was detected by RT-PCR and quantitated by densitometry scanning of autoradiograms, as described in Materials and Methods. Duplicate reactions with or without reverse transcriptase were performed using the RNAs prepared from the pHSVth- or pHSVlac-infected, or mock infected cultures.

pHSVth-infected cells contained fivefold more human TH RNA than the pHSVlac- or mock infected striatal cells. The presence of any detectable human TH RNA in these controls is surprising but may be due to the comigration of a nonspecific background band or to a low level of contamination from TH plasmids present in the laboratory.

To determine if pHSVth directed expression of TH-IR in striatal cells, 1 week after infection cultures were fixed and assayed for TH-IR using a mouse monoclonal anti-TH antibody, which was visualized by the ABC method. This time period was chosen because Watson et al. (1980) have shown that by 1 week after injection of HSV-1 ts mutants into the brain, a latent infection has been established. This experiment was performed at a low ratio of virus to cells (moi 0.05), to clearly distinguish positive from negative cells. As shown in Fig. 5A–C, TH-IR was observed in ~1% of the cells (1,000 positive cells of ~149,000). Approximately 90% of the cells with TH-IR displayed neuronal morphology and 10% displayed glial morphology; whereas ~80% of the cells in a culture displayed neuronal morphology and 20% displayed glial morphology. This 10% difference between the percentage of cells containing TH-IR with neuronal morphology and the percentage of cells in the culture with neuronal morphology might be due to either errors in scoring the cell type or a slight preference for HSV-1 particles to infect neurons in this culture system. The types of cells containing TH-IR were not confirmed by costaining these cells with antibodies specific for neurons or glia; however, we have previously shown that pHSVlac supports expression of β-galactosidase–IR in cultured striatal cells that contain (or lack) neurofilament-IR (Freese and Geller, 1991). pHSVth-infected cultures assayed with preimmune primary serum

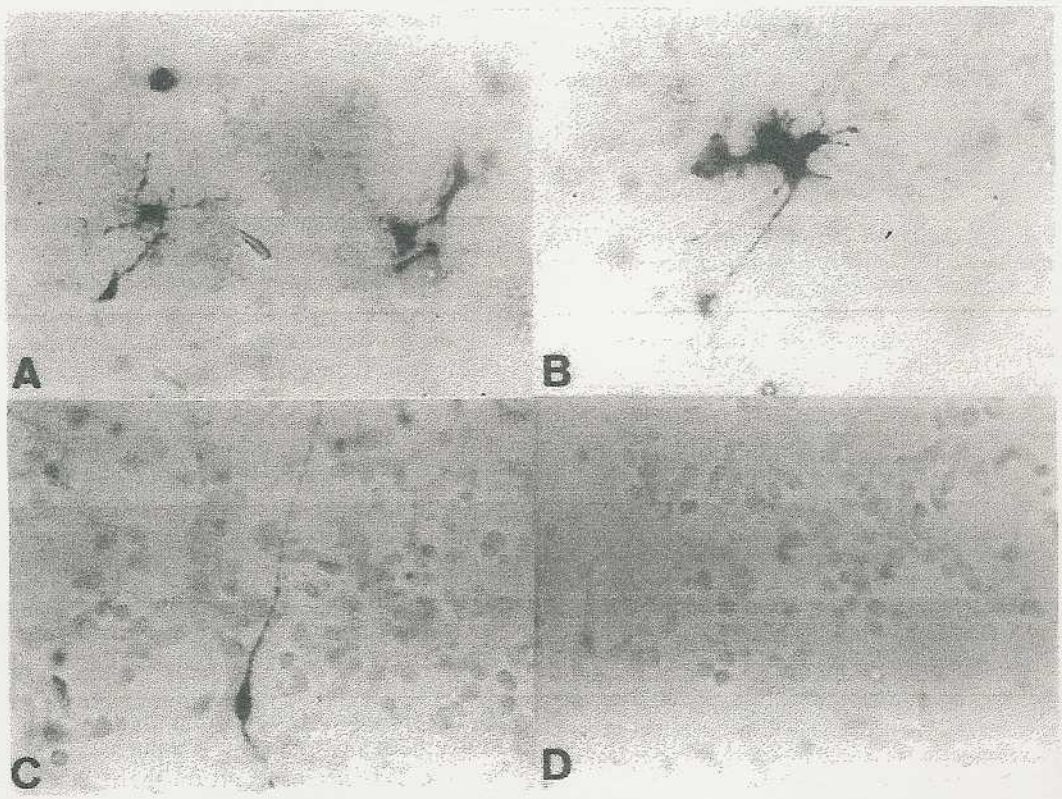

FIG. 5. TH-IR in cultured striatal cells 1 week after pHSVth infection. Cultured striatal cells (Freese et al., 1990a) were infected with pHSVth. One week later immunocytochemistry was performed using a mouse anti-rat TH antibody and the ABC procedure. The width of A represents 420 μm and the widths of B–D represent 210 μm. **A:** Low-power view of TH-IR in cells with either neuronal or glial morphology. **B:** High-power view of TH-IR in a cell with glial morphology. **C:** High-power view of TH-IR in a cell with neuronal morphology. **D:** TH-IR is absent from cells in a mock infected culture.

TABLE 2. *Catecholamine release from cultured striatal cells 2 days or 1 week after pHSVth infection*

		pg of catecholamine released/min/10^6 cells			
		L-DOPA		Dopamine	
Condition	Additions	High K	Low K	High K	Low K
pHSVth 1 wk	cof	28 ± 3.1[a]	16 ± 2.9[a]	<1	<1
pHSVth 1 wk	cof + Tyr	144 ± 16[a]	78 ± 10[a]	6 ± 1.4[a]	2 ± 0.8[a]
pHSVth 2 days	cof	26 ± 3.0[a]	12 ± 1.9[a]	<1	<1
pHSVth 2 days	cof + Tyr	142 ± 15[a]	74 ± 9.1[a]	6 ± 0.8[a]	4 ± 1.1[a]
pHSVpUC 1 wk	cof	<1	<1	<1	<1
pHSVpUC 1 wk	cof + Tyr	<1	<1	<1	<1
Mock	cof	<1	<1	<1	<1
Mock	cof + Tyr	<1	<1	<1	<1

Cultured striatal cells (Freese et al., 1990*a*) were infected with pHSVth; 2 and 7 days later catecholamine release was assayed by HPLC (Matson et al., 1987), and data (pg of catecholamine/μl sample) were converted to picograms of catecholamines released per minute per 10^6 cells. Methyltetrahydropterine (cof) and tyrosine were added as indicated. Each condition was repeated twice, the experiment was performed five times, and the standard deviations are shown.

[a] $p < 0.001$, compared with either pHSVpUC or mock with the same additions and the same release buffer (high K or low K) using repeated-measures ANOVA with Dunnett's post hoc test.

followed by the secondary antibody lacked cells (<0.2%) containing TH-IR (not shown). Cultures infected with pHSVlac (not shown) or mock infected (Fig. 5D) lacked cells containing TH-IR (<0.2%, 0 cells contained TH-IR, ~150,000 cells scored). The experiment in Fig. 5 was performed 1 week after infection; cells containing TH-IR were also observed 1 day after pHSVth infection (not shown). The cells in the infected cultures maintained normal morphology and no cytopathic effects were apparent throughout the 1-week period after infection with either pHSVth or pHSVlac. Infection with a higher moi did result in an increased percentage of cells containing TH-IR (not shown); however, some cells appeared less healthy and some neurite fragmentation was apparent.

Release of catecholamines from striatal cells

To determine if the pHSVth-infected striatal cells synthesized and released L-DOPA, 2 days or 1 week after infection, the cells were incubated for 30 min in a defined release buffer containing physiological ion concentrations (Geller et al., 1993), and the release of both L-DOPA and dopamine was measured by HPLC (Matson et al., 1987). pHSVth, but not pHSVpUC, caused the release of L-DOPA (Table 2). L-DOPA was released under normal physiological conditions and release was stimulated by depolarization (56 m*M* K^+). Cultures infected with pHSVth supported a statistically significant increase in L-DOPA release compared with cultures either infected with pHSVpUC or mock infected ($p < 0.001$, Table 2). This observation is consistent with expression of TH in both neurons and glia. Furthermore, a low level of dopamine was released from pHSVth-infected cultures (Table 2) to which both cofactor and substrate (tyrosine) were added for 8 h before catecholamine release was measured. In addition, both L-DOPA and dopamine release were dependent on calcium (not shown). This neurotransmitter release assay does not correct for reuptake of catecholamines and therefore may underestimate the release rate.

DISCUSSION

In this report we demonstrate that an HSV-1 vector containing the human TH gene (type II) can express TH in cultured fibroblast cells as well as in striatal cells, resulting in the production of L-DOPA. Specifically, we show that pHSVth was efficiently packaged into HSV-1 particles. One day after infection of fibroblast cells with pHSVth, we observed TH-IR, TH enzyme activity, and production of L-DOPA. In cultured striatal cells, pHSVth directed production of TH RNA and TH-IR, which resulted in the release of L-DOPA. TH expression and release of L-DOPA were maintained for at least 1 week. The TH gene has previously been delivered into several cell types, including retrovirus vector–mediated gene transfer into fibroblasts, pituicytes, and glia (Horellou et al., 1989; Wolff et al., 1989; Owens et al., 1991) as well as lipofection-mediated transfection of muscle cells (Jiao et al., 1993). In each case, TH was expressed and L-DOPA was produced. However, this report is the first demonstration that TH can be efficiently expressed in cultured striatal cells that normally do not contain TH or produce L-DOPA.

Expression of recombinant TH was monitored for a period of 1 week because by 1 week after injection of

HSV-1 ts mutants into the brain, infectious HSV-1 particles are no longer detected and a latent infection has been established (Watson et al., 1980). Because expression of TH RNA and TH-IR was maintained in striatal cells for up to 1 week after gene transfer, these results suggest that pHSVth can persist in striatal cells and can continue to express TH RNA and TH enzyme. Similarly, we have reported that pHSVlac can support expression of β-galactosidase, for at least 2 weeks, in cultured PNS (Geller and Breakefield, 1988) and CNS (Geller and Freese, 1990) neurons, including striatal neurons (Freese and Geller, 1991). Furthermore, we have found that β-galactosidase can be expressed in cultured sensory neurons for up to 3 months after infection with pHSVlac (C. L. Wilcox, A. I. Geller, and R. L. Smith, unpublished data). Currently, experiments are in progress to determine the stability of pHSVth-directed TH expression in striatal cells in the adult rat brain.

The percentage of TH-positive CV1 or striatal cells was about fivefold lower than predicted from the moi. Because pHSVth was titered on CV1 cells, the fivefold range in the number of TH-positive CV1 cells could be due to variations in culture and/or infection conditions, or a small loss of pHSVth virus due to multiple freeze–thaw cycles. The fivefold lower than predicted number of TH-positive striatal cells may reflect the inherent variations in the system that are also observed with CV1 cells, and may also be due to differences in infection and/or expression levels obtained with pHSVth in the CV1 cells compared with the striatal cells.

Many of the expression experiments in this study were performed at a low ratio of vector to cells (moi 0.05–0.2). This was done for two reasons. First, using either CV1 or PC12 cells, we have established that the amount of β-galactosidase activity expressed from pHSVlac increases linearly with the moi (range 0.02–0.32) of pHSVlac (Geller, 1991). Therefore, to ensure accurate quantitation in the immunocytochemical experiments and the enzyme assay experiments it was necessary to perform these experiments in the linear response range for defective HSV-1 vectors. Using the infection conditions reported in this study, at the relatively low moi of pHSVth, no cell damage was observed in the striatal cultures. Second, after infection of striatal cells at higher moi (0.2–0.8), correspondingly more cells containing TH-IR were observed (not shown); however, in at least one preparation of pHSVth virus, the higher moi resulted in some cytopathology in some striatal cultures. This cell damage could be due to the virus preparation procedure used in this study, a crude cell lysate that contains fetal bovine serum, cell fragments, and other material that could potentially damage CNS cells.

Alternatively, limitations of the current packaging system, such as the $\sim 1 \times 10^{-5}$ reversion frequency of the helper virus to wild-type HSV-1, could explain the striatal cell damage observed with higher moi of some preparations of pHSVth. It is interesting that no cytotoxic effects were observed when this same virus (moi 0.1) was used to infect cultures of superior cervical ganglia (not shown). In addition, we have reported that pHSVlac can be used at higher moi (e.g., moi 1) in cultures of PNS and CNS cells (Geller and Breakefield, 1988; Geller and Freese, 1990) including striatal cells (Freese and Geller, 1991). Striatal cells in the adult brain may be more resistant to these effects because they maintain their normal connections and environmental influences.

The ability of pHSVth, even at low moi, to direct production of reasonable levels of both TH enzyme activity (Table 1) and L-DOPA (Tables 1 and 2) might be explained by the high levels of expression obtained from the IE4/5 promoter, and by the concatenation of the vector during the packaging procedure, which results in packaging ~15 copies of the vector into each HSV-1 particle. In this regard, it is interesting that as few as 100 HSV-1 particles of a vector that expresses the GluR6 kainate receptor subtype were sufficient to induce a significant loss of CA3 hippocampal neurons (Bergold et al., 1993).

Low levels of dopamine were detected in both fibroblast cells infected with pHSVth (Table 1) and in media collected from striatal cells infected with pHSVth (Table 2). It is unclear what mechanism generates dopamine in the fibroblasts other than nonspecific decarboxylation. In striatal cells, two possible pathways could explain the production of dopamine; i.e., L-DOPA could be converted to dopamine in the TH-expressing cell in which the L-DOPA is synthesized; alternatively, the L-DOPA could be released, taken up by other cells in the culture, and converted to dopamine in these cells; or both of these pathways may contribute to the synthesis of dopamine. The enzymatic mechanism by which L-DOPA is converted to dopamine in these striatal cells is not clear. However, lesioning studies have suggested that a significant amount of AADC activity is produced in the striatum. Moreover, several recent studies have more directly shown that AADC is expressed in both a subpopulation of medium spiny neurons (Tashiro et al., 1989) as well as in primary striatal astrocytes (Li et al., 1992). Conceivably, such cells contribute to the production of the dopamine detected in the pHSVth-infected cultures (Table 2). The possibility that L-DOPA itself can function as a neurotransmitter has been reviewed recently (Misu and Goshima, 1993). Although not conclusive, available evidence does suggest that L-DOPA can be released in a K^+-evoked, Ca^{2+}-dependent manner in vitro (Goshima et al., 1988) and in vivo (Nakamura et al., 1992). These studies support our finding that L-DOPA can be converted to dopamine and released from cells in pHSVth-infected striatal cultures. Because HSV-1 vectors have the potential to express more than one gene, the possibility exists to coexpress

both TH and AADC in the same cell to increase the efficiency of dopamine production.

The results in cultured cells reported here suggest that pHSVth might be evaluated in a rodent model of PD. In fibroblasts, the amount of TH enzyme produced by pHSVth was comparable with that found in catecholaminergic neurons (Table 1). The rate of L-DOPA production in striatal cells reported here (Table 2) is comparable with that obtained with genetically engineered cells that express TH, and transplantation of such cells into the 6-hydroxydopamine–lesioned rat restores function (Wolff et al., 1989; Horellou et al., 1990; Uchida et al., 1990; Jiao et al., 1993).

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