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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

(gene therapy/opiates)

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ABSTRACT **We have previously used a defective herpes simplex virus vector to express a foreign gene in the adult rat brain. One application of this technology would be the *in vivo* analysis of promoter function in brain after *de novo* transfer, which would allow the rapid generation of vectors with localized application in a broad range of mammalian species while avoiding influences of other nearby promoters. A 2.7-kb fragment of the rat preproenkephalin promoter was placed upstream of the bacterial *lacZ* gene in our herpes simplex virus amplicon. A restricted pattern of *lacZ* expression was observed *in vivo*, which follows previously observed patterns of endogenous preproenkephalin expression. These results, from the direct gene transfer into an adult animal brain for *in vivo* promoter analysis, demonstrate that sequence information that influences restricted expression of preproenkephalin is located within 2.7 kb upstream of transcriptional initiation. *lacZ* expression was also observed in rat brain for 2 months after direct transfer, and PCR analysis confirmed the continued presence of amplicon DNA in *lacZ*-positive sections. Restricted and long-term expression observed with an endogenous promoter has important implications for gene therapy using viral vectors.**

The herpes simplex virus (HSV) amplicon was developed as a eukaryotic cloning–expression vector (1–4). The amplicon is a plasmid that contains an HSV origin of DNA replication and cleavage/packaging signal, but by itself expresses no HSV gene products. Any promoter and reporter gene combination can be placed in the amplicon according to the desired application, resulting in the transfer of only a single selected functional transcription unit. In the presence of a helper virus, the amplicon is amplified to genome size (≈150 kb) and packaged into an HSV particle, creating a defective viral vector (1–6). The defective HSV vector is thus incapable of autonomous replication in the absence of helper virus. Defective HSV vectors express foreign genes in both nonneuronal (2) and neuronal (7) cells in tissue culture and *in vivo* in the rat central nervous system (5) and peripheral nervous system (8).

One use of the defective HSV vector would be as a vehicle for the analysis of promoter function *in vivo*. This technique should combine the advantages of plasmid-based systems, which have been widely utilized for promoter analysis in tissue culture (9, 10), with the ability to assay promoter function *in vivo*. This would be of particular utility in the central nervous system and would provide an alternative to the transgenic mouse (11), which is currently the major vehicle for *in vivo* promoter analysis. Potential benefits of the defective HSV system for promoter analysis include rapid and simple generation of vectors, and compatibility with many mammalian species, due to the broad host range of HSV (12), whereas transgenic technology is usually limited to mice. Finally, as an episomal vector injected into the fully developed adult animal, the defective HSV vector should not be subject to variables such as effects of insertion within the host chromosome or developmental modification.

The rat preproenkephalin (PPE) promoter was chosen for analysis due to the complex patterns of endogenous PPE expression (13–16) and regulation in neural and neuroectoderm-derived tissues (13, 15, 16). Within the brain, PPE is produced in a highly specific pattern, with expression limited to discrete brain regions (13–19), including caudate nucleus, amygdala, piriform cortex, olfactory tubercle, and ventromedial hypothalamus (15, 16).

The current study reports the use of direct gene transfer for analysis of the activity of an endogenous cellular promoter *in vivo*. The results demonstrate that a 2.7-kb fragment of the rat PPE promoter yields substantial expression of the *lacZ* reporter gene in several brain regions that express endogenous PPE transcripts, whereas no *lacZ* expression was noted in a brain region that lacks endogenous PPE transcripts.

MATERIALS AND METHODS

Construction of Plasmid pHENK and Derivatives. A 2.7-kb *Avr* II–*Sac* I fragment of the rat PPE promoter was isolated from plasmid pRESS1 (14). This fragment was inserted into the polylinker of plasmid pSL301 (Invitrogen) and then removed by cutting with *Avr* II and *Msc* I. To create pHENK.2700, this fragment was ligated to a 4.3-kb *Stu* I–*Pst* I fragment from plasmid pCH110 (Pharmacia), containing the *lacZ* gene and simian virus 40 polyadenylylation signal, in the amplicon pSRa-ori (5). Deletions were then introduced into the PPE promoter. pSRa-ori.s1, which was used as the basic amplicon for pHENK derivatives, is pSRa-ori containing a large polylinker ("superlinker") from pSL301. pHENK.1431 was created by digestion of pHENK.2700 with *Bam*HI, which cuts at bp −1431 within the PPE promoter, and *Csp* I, which cuts once within the vector. This was ligated to pSRa-ori.s1 cut with *Bgl* II and *Csp* I. pHENK.841 was created by ligation of an *Sph* I–*Csp* I fragment from pHENK.2700 to pSRa-ori.s1 cut with *Sph* I and *Csp* I. Similarly, a *Bgl* II–*Csp* I fragment from pHENK.2700 was used to create pHENK.500. The promoter orientation was the same for all amplicons.

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Abbreviations: HSV, herpes simplex virus; PPE, preproenkephalin; HCMV, human cytomegalovirus; IE, immediate early.

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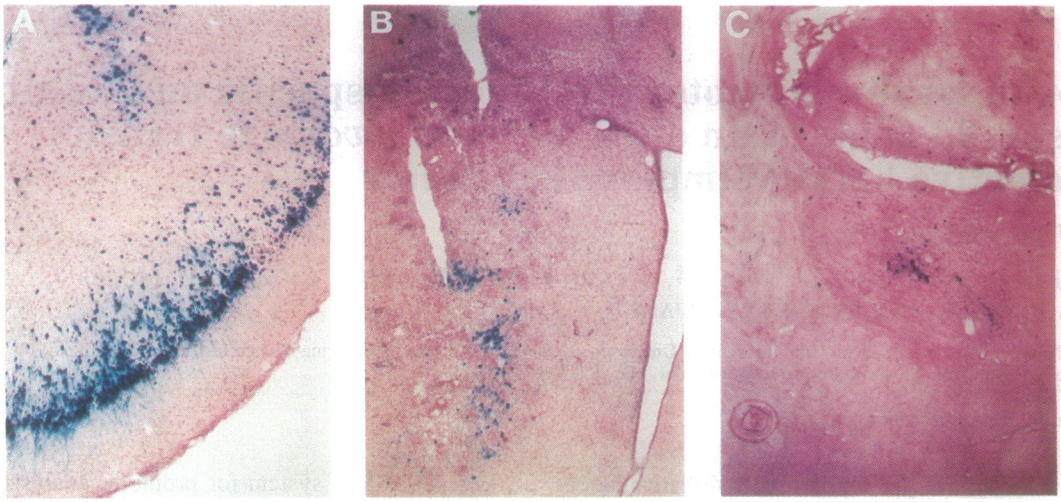

FIG. 1. Region-specific *lacZ* expression in rat brain under control of the 2.7-kb fragment of rat PPE promoter. dvHENK was stereotaxically microinjected into the piriform cortex (*A*) and the caudate nucleus (*B*). Both are regions with endogenous PPE expression, and significant *lacZ* expression was observed in these regions. In the piriform cortex (*A*), the majority of endogenous PPE expression is observed in the pyramidal cell layer, and the local pattern of *lacZ* expression was also predominantly seen as a band in this layer. (*C*) *lacZ* expression in dorsolateral thalamic region demonstrates that some cells in this region are capable of supporting PPE promoter function. (×60.)

Cell Culture and Generation of Defective Viral Vector dvHENK. Defective vector dvHENK was created using pHENK.2700, as described (5). Plaque assay of the resulting stock at a nonpermissive temperature (37°C) demonstrated that the reversion of temperature-sensitive helper virus tsK to wild-type virus was at a rate of $<10^{-7}$ ml^{-1}, consistent with previous observations (5). Defective virus (dvHENK) titer was estimated from the ratio of amplicon/helper virus DNA, as described (1–3).

Infusion of dvHENK into Rat Brain and Tissue Preparation. Female Sprague–Dawley rats (250–300 g) were used in all experiments. dvHENK was stereotaxically microinjected into brain regions according to coordinates of Paxinos and Watson (20). Three animals were tested per region, and the pattern of expression for each region was reproduced in all animals. For regional distribution, animals were sacrificed 3 days after surgery, while long-term expression was examined after 2 months. The titer of dvHENK.2700 was 4×10^7 defective particles per ml, and the titers of the deleted forms were 1×10^8 defective particles per ml. All brains were processed and stained for 5-bromo-4-chloro-3-indolyl β-D-galactoside histochemistry (5).

PCR Analysis of Defective Vector DNA from Fixed and Stained Sections. After photography, coverslips were floated off and sections were rehydrated. Regions consisting of ≈10 mm^2 around the injection site were scraped off, placed into 0.6-ml tubes, and processed for "nested" PCR as described (21). Primers were directed against the *lacZ* gene. Primers for reaction A were lacZ182 (5′-CCGACTGATGCCTTCTGAACAA-3′) and lacZ560 (5′-GACGACAGTATCGGCCTCAGGA-3′). A 1:1000 dilution (1 μl) of products from reaction A was utilized for reaction B, with primers lacZ211 (5′-GCATTATTGCCGTAAGCCGTGG-3′) and lacZ470 (5′-ATTCAGGCTGCGCAACTGTTGG-3′). Other components were from a standard PCR kit (Cetus). The cycling parameters were 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C for 35 cycles. Reaction products were analyzed by electrophoresis on a 1.5% agarose gel.

***In Situ* Hybridization.** *In situ* hybridization to detect endogenous PPE transcripts was performed as described (16, 22). Probes were generated from a PPE cDNA generated by reverse transcriptase/PCR and obtained from P. J. Brooks (Rockefeller University).

RESULTS

The 2.7-kb Rat PPE Promoter Drives *lacZ* Expression in Regions That Contain Endogenous PPE Transcripts. dvHENK was stereotaxically microinjected into regions of the rat brain that express endogenous PPE mRNA (15, 16), including the caudate nucleus, piriform cortex, and amygdala. Numerous cells containing β-galactosidase activity were observed in each of these regions (Figs. 1 *A* and *B*, 2*A*, and 3*C*). The efficiency of expression in these regions was 1–5%, which is not significantly different from the

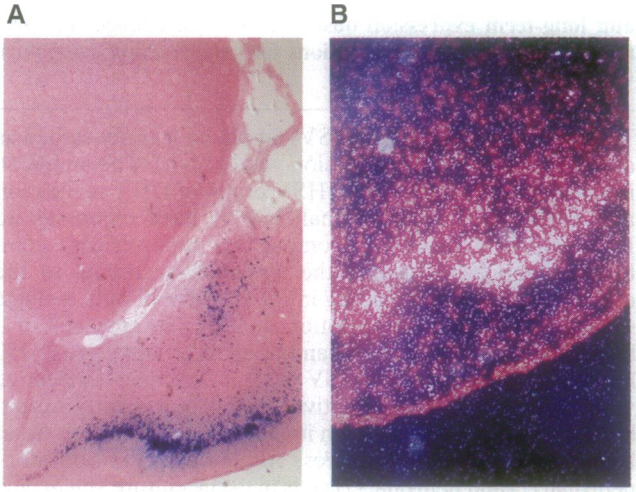

FIG. 2. dvHENK expression in piriform cortex compared with *in situ* hybridization for PPE mRNA. (*A*) β-Galactosidase expression in the piriform cortex after microinjection of dvHENK into rat brain. The majority of cells are limited to pyramidal neurons of the piriform cortex. In particular, note the curvature in the layer of positive cells near the left border of the picture. Above the piriform cortex, a region of positive cells can be seen in the anterior amygdaloid area, which also contains neurons expressing endogenous PPE transcripts (16). (*B*) Dark-field photomicrograph of an *in situ* hybridization for PPE mRNA in piriform cortex from a separate animal. A single-stranded PPE antisense probe labeled with ^{125}I was generated via amplification in a thermal cycler (22). The band of grains (white dots in the dark field) at the bottom of the photograph covers neurons of the piriform cortex containing PPE mRNA. Again, note the curvature of the region of positive cells in the piriform cortex, near the left border of the picture. (*A*, ×20; *B*, ×30.)

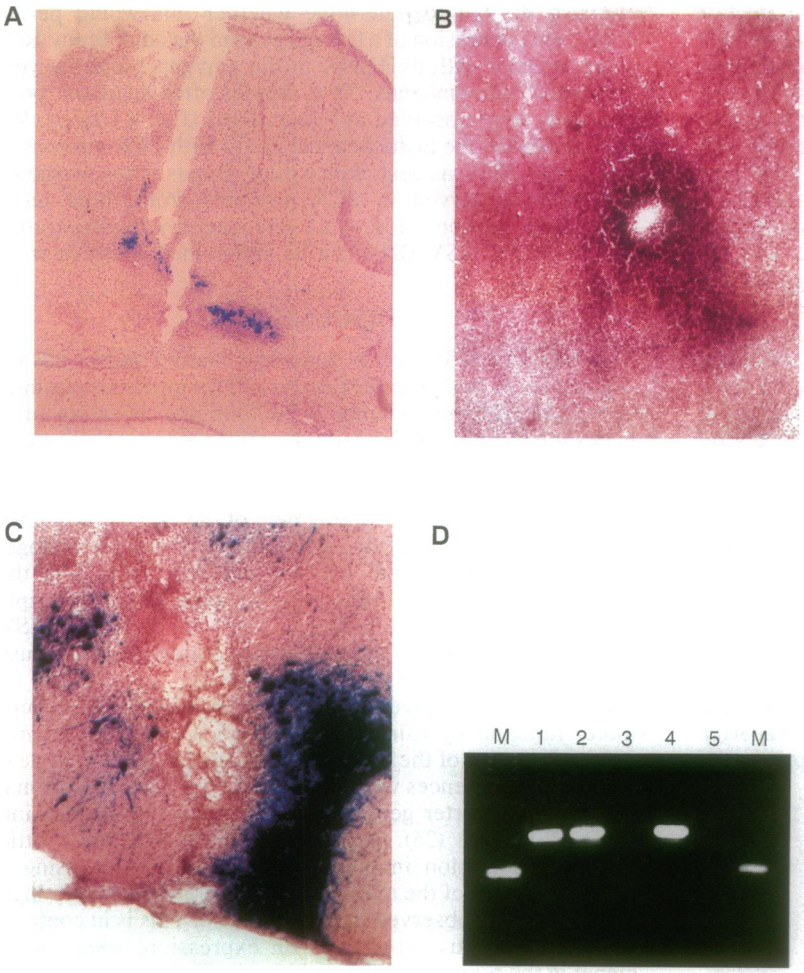

FIG. 3. Demonstration of restricted expression from rat PPE promoter *in vivo*. (*A*) Positive cells in the dorsolateral neocortex after injection with dvHCL. This vector contains the *lacZ* gene under the control of the HCMV IE promoter (6), which is a strong nonspecific promoter. This demonstrates that the defective HSV vector is capable of transferring and expressing a foreign gene in this region. (*B*) Absence of β-galactosidase expression in dorsolateral neocortex after injection with dvHENK. This region does not contain endogenous PPE transcripts. The injection track can be seen in the center. The 2.7-kb PPE promoter fragment, which supports expression in other regions, therefore, was not functional in dorsolateral neocortex, consistent with endogenous PPE expression. (*C*) Numerous positive cells demonstrated near the needle track in the amygdala, including cells in the cortical and medial nuclei, consistent with endogenous patterns of PPE expression. dvHENK was infused into the amygdala on the opposite side of same brain as in *B*. (*A*, ×20; *B*, ×30; *C*, ×30). (*D*) Presence of dvHENK DNA in injected brain regions demonstrated by "nested" PCR analysis of stained sections. Lanes: 1, *lacZ*-expressing amygdala seen in *C*; 2, dorsolateral neocortex seen in *B* that contained no *lacZ*-expressing cells; 3, uninjected negative control region from same brain as in *B* and *C*; 4, positive control using plasmid pHENK; 5, no-tissue/DNA blank control; M, 123-bp ladder. All positive bands were at the expected 269-bp position.

efficiencies that we have observed with studies using the cytomegalovirus immediate-early (IE) promoter (5). This pattern is not only comparable to the known pattern of PPE expression, but local β-galactosidase production within injected sites appears to be restricted to a nonrandom pattern dictated by cell-group boundaries. For example, positive cells were found dispersed in the caudate nucleus after vector application there (Fig. 1*B*), which is expected since cells that express endogenous PPE are distributed throughout this region (15, 16). In the piriform cortex, the majority of positive cells were observed within the pyramidal cell layer (Figs. 1*A* and 2*A*). Localized expression within this region was further analyzed through direct comparison with production of endogenous PPE transcripts (Fig. 2). *In situ* hybridization revealed that endogenous PPE mRNA was localized to the pyramidal cell layer of the piriform cortex (Fig. 2*B*), which directly paralleled the pattern of *lacZ* expression under the control of the 2.7-kb PPE promoter in a separate animal (Fig. 2*A*). These data are consistent with a specific pattern of endogenous PPE expression characteristic of cells within this region (15, 16).

The PPE Promoter Yields Some Expression in the Thalamus but No Expression in the Dorsolateral Neocortex. To further analyze PPE promoter function, dvHENK was injected into the dorsolateral thalamic region and dorsolateral neocortex, two regions that contain few cells that express normal PPE as determined by immunocytochemistry and *in situ* hybridization (15, 16). Some positive cells were observed in the dorsolateral thalamus (Fig. 1*C*). In contrast, no positive cells were observed in the dorsolateral neocortex of any animal injected in this region (Fig. 3*B*), an absence that would be predicted from the lack of endogenous PPE transcripts in this region. Despite the presence of a needle track within the substance of the neocortex, it was still possible that a negative result was due to physical loss of defective viral particles during or after surgery. To address this question, coverslips were floated off stained sections and "nested" PCR analysis was performed. "Nested" PCR involves two successive reactions, with the second reaction using a sample of DNA from the first reaction. The primers from the second reaction are internal to the first, which increases the specificity of the final amplified product, since it is extremely unlikely that a nonspecific product from reaction A would be reamplified with the internal primers from reaction B. The results show that dvHENK DNA was present in both the amygdala and the dorsolateral cortex (Fig. 3*D*), even though *lacZ* expression was only observed in the amygdala. No DNA was present in an uninjected region of the same brain, as expected.

Several controls were performed to eliminate the possibility that nonspecific factors from the vector influenced the observed expression pattern. As a positive control for expression in the dorsolateral neocortex, defective vector dvHCL was injected. This vector contains the human cytomegalovirus (HCMV) IE promoter driving expression of *lacZ* and has been described (5). Numerous positive cells were repeatedly observed (Fig. 3*A*), demonstrating that there is no inability for defective HSV vectors to transfer and express the *lacZ* gene in this region. As a negative control for expression in the caudate nucleus and amygdala/piriform region, deletions were generated in the enkephalin promoter. Three vectors were generated that contain 1431 bp, 841 bp, or 500 bp upstream from the PPE transcriptional start site. Vectors were titered in the manner used to titer the vector

with a 2700-bp fragment. Approximately 200,000 defective vector particles were infused into each region of each subject in a total volume of 2 μl, and all animals were sacrificed 3 days after vector injection. In the amygdala/piriform region, $>10^4$ positive cells were observed using dvHENK.2700, while an average of 132, 58, and 107 positive cells were observed with vectors containing 1431-bp, 841-bp, and 500-bp PPE promoter fragments, respectively ($n = 3$). Similar results were obtained in the caudate nucleus. Again $>10^4$ positive cells were observed using dvHENK.2700, while 66, 32, and 128 positive cells were observed with vectors containing 1431-bp, 841-bp, and 500-bp PPE promoter fragments, respectively ($n = 2$). Finally, we have earlier generated vectors that utilized a 1.1-kb fragment of the rat vasopressin promoter (obtained from D. Richter, University of Hamburg, Germany) to drive expression of the *lacZ* gene. The amplicon used was identical to that used in the current study, and although packaged viral vectors were obtained, no *lacZ* expression was ever observed after injection into any rat brain region, including those containing vasopressin-expressing cells. In total, the above data suggest that the absence of positive cells after injection of dvHENK into dorsolateral neocortex was due to a lack of adequate PPE promoter activity, and *lacZ* expression observed in the caudate nucleus and amygdala/piriform cortex was driven primarily by elements contained within the 2700-bp rat PPE promoter.

Defective HSV Vector DNA Is Stable *in Vivo* and the PPE Promoter Permits Long-Term Expression of the Reporter Gene. Since the PPE promoter is an endogenous promoter, we also wanted to determine whether this would permit long-term expression of a foreign gene in the adult brain. Rats were injected with dvHENK in the amygdala and the ventromedial hypothalamus. Two months after injection, positive cells were observed in both regions (Fig. 4 *Upper*). We then extended the histochemical assay with PCR analysis of dvHENK DNA, as described earlier. Bands of the predicted size were observed in PCR products from three *lacZ*-expressing sections (Fig. 4 *Lower*), thereby demonstrating that defective HSV DNA can be maintained stably *in vivo*.

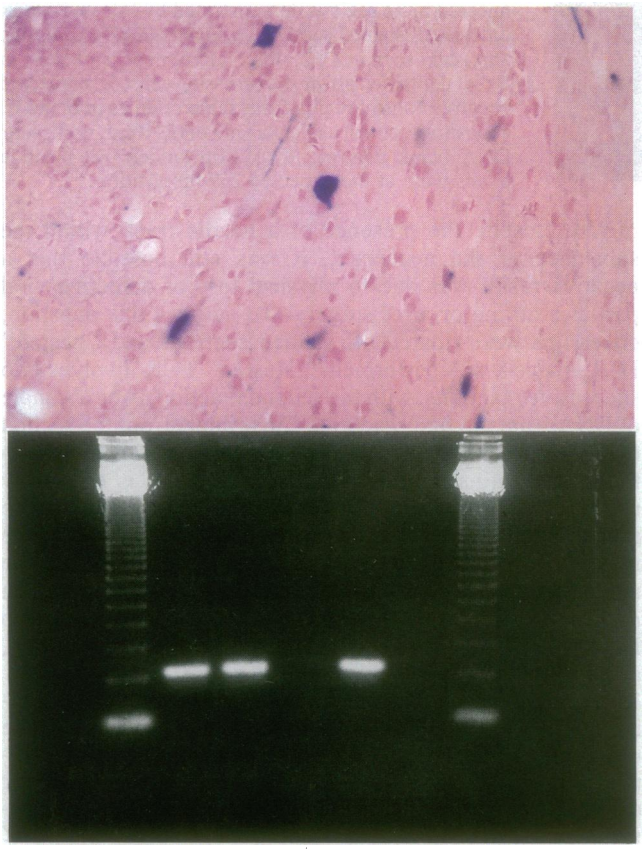

FIG. 4. Long-term expression of *lacZ* in rat brain. (*Upper*) Positive cells in central nucleus of the amygdala. Positive cells were also observed in the ventromedial nucleus of the hypothalamus after injection there. Animals were sacrificed 2 months after infusion of dvHENK. (×250.) (*Lower*) After PCR analysis of sections from animals with long-term expression. Lanes: 1, from positive amygdala; 2, from positive hypothalamus; 3, from positive amygdala from a different subject; 4, negative control uninjected brain region; 5, plasmid pHENK as positive control; 6, no-tissue/DNA control; M, 123-bp ladder.

DISCUSSION

The defective HSV vector possesses many features advantageous for *in vivo* promoter analysis. This system is plasmid-based and episomal; potentially confounding cis-acting influences are thereby minimized (23, 24). Moreover, promoters from any mammalian species can be studied within the same species. Using the defective HSV vector system, we have demonstrated that a 2.7-kb fragment of the rat PPE promoter is functional in several discrete regions where the endogenous gene is expressed, while it did not function in the dorsolateral neocortex, where endogenous PPE transcripts are also not normally observed. Thus, the defective HSV vector system can provide an alternative to transgenic mouse technology for *in vivo* promoter analysis.

Recently, there have been attempts to examine PPE promoter function by using transgenic mice (25, 26). When a 200-bp fragment of the human PPE promoter was employed, significant differences were noted between the gross regional patterns of reporter gene and endogenous PPE expression within the brain (25). Another study reported very little reporter expression in brains of transgenic mice using a 1.6-kb fragment of the rat PPE promoter, although very high expression was observed in the testis (26). This is in contrast to the endogenous pattern of PPE expression, which is far higher in the brain than in testis (27, 28). In the current study, a larger PPE promoter fragment was employed, and the rat promoter was studied within the rat brain. Both factors may have contributed to our observations of restricted expression consistent with endogenous PPE distribution. We have also observed that deletion of the PPE promoter in dvHENK to 1.4 kb upstream of transcription initiation results in an ≈100-fold decrease in *lacZ* expression in the caudate and amygdala/piriform cortex. This is consistent with the low level of expression in brains of transgenic mice with the 1.6-kb fragment of the rat PPE promoter. Furthermore, limited expression from deleted forms of the PPE promoter and a complete absence of *lacZ* expression when a small fragment of the rat vasopressin promoter was used indicate that the majority of expression from our vector is due to elements within the 2.7-kb rat PPE promoter fragments with little influence from other sequences within the vector. Finally, while the defective HSV vector carries within the virion a transcriptional activating protein, VP16, this protein requires a specific HSV recognition sequence (29), which is not present in either the HSV sequences in our plasmid or within the known PPE promoter sequence. It is therefore unlikely that this factor significantly influenced our results.

Positive cells were unexpectedly observed in the dorsolateral thalamic region, suggesting that this region contains factors that permit transcription from the PPE promoter. It is possible that an element that silences transcription in thalamus is present beyond the 2.7-kb sequence used in this study, and complex combinations of enhancers and silencers have been found in regulatory regions of other genes expressed in brain (30, 31). In addition, observations of PPE RNA in brain cell types previously thought to lack such transcripts, including the reticular nucleus of the thalamus, indicate that previous hybridization studies may not have completely char-

acterized endogenous PPE expression (refs. 32 and 33; P. J. Brooks, personal communication). In fact, slot blot analysis has revealed normal PPE mRNA in extracts from rat thalamus (17). In contrast, the lack of transcription in the dorsolateral neocortex indicates that this region is incapable of supporting PPE promoter activity, which is consistent with the absence of endogenous PPE transcripts. We have more recently obtained additionally supportive findings in the hypothalamus. There is intense endogenous PPE expression in the ventromedial hypothalamus, whereas few transcripts have been noted in the lateral hypothalamic area (16). Injection of dvHENK into the ventromedial hypothalamus can result in many positive cells, but injection into the lateral hypothalamic area yields far fewer positive cells (J. Yin, M.G.K., A.D.K., and D.W.P., unpublished data).

Using the PPE promoter, we also report the demonstration of long-term expression of a foreign gene in the adult rat brain after direct *in vivo* transfer via a defective HSV vector. Earlier, we observed expression in rat brain of *lacZ* under the control of the HCMV IE promoter (5), which was limited to 2 weeks after viral injection. This was consistent with other *in vivo* studies using the HCMV IE promoter (34, 35). Although down-regulation of promoter function would have explained this result, it was also possible that either the vector DNA was degraded with time or that cells containing the transferred gene may have died. Stable maintenance of defective HSV DNA has not previously been reported, but PCR analysis of stained sections confirms that defective HSV vector DNA is stable within at least some cells of the rat brain. The replacement of a viral IE promoter with a promoter for an endogenous cellular gene resulted in long-term (2 month) expression. It should be noted that long-term expression in the rat brain has recently been reported using a recombinant adenoviral vector (36). The current system is a defective virus with a plasmid-based genome, however, and it is unclear as to whether factors influencing the stability of such genomes would be analogous to those influencing recombinant adenovirus or herpesvirus genomes. Although there appears to be a decrease in the number of positive cells with time, the data reported here indicate that amplicon-based genomes are stable for at least 2 months and that long-term expression can be achieved through the use of appropriate promoters.

Our results also have implications for the emerging field of direct gene transfer as a therapeutic modality. Although regulating and maintaining expression of a foreign gene *in vivo* is difficult, it is central to the continued development of approaches to genetic therapy (37). The observation that defective HSV vector DNA can be stably maintained *in vivo* suggests that such vectors are applicable for long-term expression studies. Locally restricted long-term PPE promoter-driven expression in brain further indicates that certain promoters may allow specifically targeted and stable production of a foreign gene product *in vivo*. The combination of defective HSV vectors and endogenous cellular promoters may provide both additional approaches to understanding gene regulation *in vivo* and opportunities for genetic therapy through stable modification of the physiology of nondividing terminally differentiated cells.

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