JOURNAL OF VIROLOGY, Aug. 1995, p. 4593–4599 Vol. 69, No. 8
0022-538X/95/$04.00+0

Long-Term Expression in Sensory Neurons in Tissue Culture from Herpes Simplex Virus Type 1 (HSV-1) Promoters in an HSV-1-Derived Vector

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Received 11 January 1995/Accepted 19 April 1995

Amplicons, defective herpes simplex virus type 1 (HSV-1) vectors, were constructed to use four HSV-1 promoters, from the immediate-early (IE) 1 IE 3, IE 4/5, and late glycoprotein C (gC) genes, to regulate expression of the *Escherichia coli lacZ* gene, encoding β-galactosidase, and packaged into infectious particles. Infection of sensory neurons in vitro with amplicons containing the IE 1, IE 3, or IE 4/5 promoter resulted in stable long-term expression of β-galactosidase from 2 to 10 weeks after gene transfer. The number of neurons expressing β-galactosidase was not changed by treatments previously shown to produce reactivation of latent HSV-1. In addition, the latency-associated transcript was detected in many of the same neurons that expressed β-galactosidase, indicating that the viral IE promoters in the amplicons can function in the same neurons that harbor latent virus. Delivery of β-galactosidase protein directly into neurons by microinjection indicated that the half-life for histochemical detection of β-galactosidase was between 24 and 48 h, suggesting that the persistence of β-galactosidase histochemical staining cannot be explained by the stability of the reporter protein alone. In contrast to the IE promoters, the gC promoter of the late gene class did not support long-term expression of β-galactosidase; instead, β-galactosidase was detected in only a few neurons per culture at 2 weeks after infection, and superinfection with wild-type HSV-1 did not increase the level of expression from the gC promoter. These results suggest that the HSV-1 IE promoters in the amplicons are not subject to the promoter inactivation that occurs with many types of virus vectors and that the IE promoters in the context of the amplicon avoid the promoter inactivation observed from the same promoters in the HSV-1 genome during latency.

The difficulties involved in transferring genes into neurons by conventional methods have led to increasing interest in the use of virus vectors to introduce foreign genes into neurons. Because of the capability of herpes simplex virus type 1 (HSV-1) to infect and become latent in neurons (34), HSV-1 has recently gained attention as a potential vector for use in the nervous system. To circumvent the complex gene regulation that occurs with HSV-1 infection, amplicons derived from HSV-1 are being developed to investigate neuronal physiology and for gene therapy.

HSV-1 amplicons are based on the original observation and further characterizations that subgenomic fragments of HSV DNA that contain only an HSV-1 origin of replication (ori_S) and the terminal repeat **a** sequence can be replicated and packaged as concatemers into infectious particles by a helper HSV-1 (21, 32). An amplicon that places the *lacZ* gene under the control of the HSV-1 immediate-early (IE) 4/5 gene promoter results in detectable levels of β-galactosidase in dorsal root ganglia (DRG) neurons in culture for at least 2 weeks (12). HSV-1 amplicons have also been used to infect peripheral and central nervous system neurons in culture (2, 3, 12–16) and in vivo (7, 8, 18, 19).

A major obstacle in the development of virus vectors has been in obtaining efficient long-term gene expression. In general, foreign genes inserted into the HSV-1 genome, as recombinant HSV-1 vectors, are inactivated with time (4, 6). Relatively little is known about the quantitative aspects of expression from amplicon vectors, although amplicons have been shown to express over relatively long periods in neurons in vitro (12–15, 19) and in vivo (7, 8, 19).

In this study, we used DRG neurons in vitro to examine the properties of gene expression from several HSV-1 amplicons. In DRG neurons in vitro, a marked shutdown of β-galactosidase expression is observed following infection with the recombinant virus, C-gal$^+$, that contains the human cytomegalovirus IE promoter regulating *lacZ* in a nonessential region of the virus (30). Similar results of promoter inactivation in recombinant HSV-1 have been reported for DRG neurons in vivo (9). Consequently, DRG neurons in vitro provide a model well suited for the examination of the regulation of gene expression from virus vectors. This system allows characterization of the properties of the HSV-1 vectors and analyses of the ability of these amplicons to maintain long-term expression in neurons. In addition, this system allows the comparison of expression from the latent helper HSV-1 genome with expression from the amplicons following coinfection within individual neurons.

MATERIALS AND METHODS

Construction and packaging of the amplicons. The amplicons used in the studies described were constructed by using standard methods of cloning (27), and the HSV-1 regions used for the construction of the amplicons are shown in Fig. 1. The pHSVlac amplicon, containing the HSV-1 IE 4/5 promoter regulating *lacZ*, has been described previously (12). The other HSV-1 amplicons used in these studies contain the same *lacZ* coding region, simian virus 40 polyadenyl-

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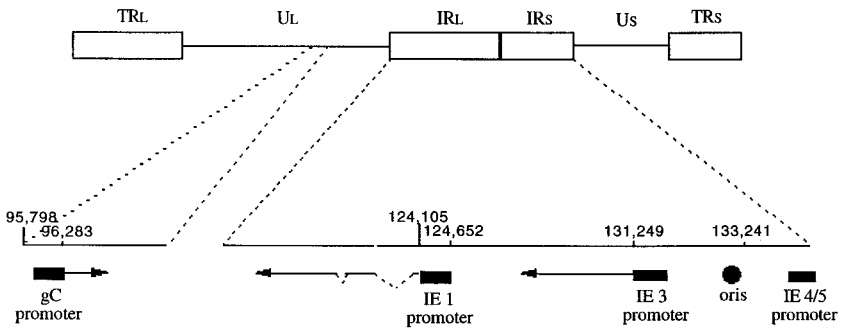

FIG. 1. Schematic representation of the HSV-1 genome indicating the promoter regions used to regulate *lacZ* expression in the amplicons. The solid bars represent the promoter regions used to control the *lacZ* gene in the plasmids for preparation of the amplicons. The closed circle represents the region that included the ori_S used for cloning of the amplicons. The amplicon plasmids containing IE 1, IE 3, and gC promoters were constructed as described in Materials and Methods.

ation signal, **a** packaging site, and bacterial plasmid sequences present in pHSVlac (12). The locations of restriction sites used to construct these amplicons are based on the published HSV-1 strain 17 *syn*$^+$ sequence (24). For the construction of the amplicons described here, HSV-1 DNA encoding the viral promoter regions, the cap site, and 5′ nontranslated regions of the viral RNA were fused to the reporter gene in the noncoding region of the β-galactosidase gene as originally described (33). The IE 3 promoter-containing amplicon (pIE3-lacZ) was constructed by insertion of the ori_S and IE 3 promoter as a single fragment from a *Pvu*II site at nucleotide (nt) 133241 to a *Sal*I site at nt 131249. To construct the IE 1 promoter-containing amplicon (pIE1-lacZ), a *Taq*I fragment containing the ori_S (nt 132555 to 131601) was combined with the IE 1 promoter obtained from a *Bst*EII-to-*Nco*I fragment (nt 124105 to 124652). The glycoprotein C (gC) promoter-containing amplicon (pgC-lacZ) was constructed from the same ori_S (*Taq*I fragment, nt 132555 to 131601) combined with a gC promoter fragment extending from nt 95798 to 96283 subcloned from the *Hin*dIII L fragment (provided by E. K. Wagner, University of California, Irvine).

The amplicons were packaged into infectious particles by transfection of the amplicon DNA followed by infection with the packaging helper virus as described previously (11). The helper virus used for packaging in these studies was the IE 3 deletion mutant, HSV-1 strain 17$^+$ D30EBA, which was propagated on a complementing cell line (RR1) that expresses the IE 3 gene (26). For the colocalization experiments, the wild-type virus, HSV-1 strain F (American Type Culture Collection), was used as the helper, packaging virus.

The ratios of amplicon to helper virus were determined from the titers of the amplicon as blue-forming units (number of β-galactosidase-positive Vero cells), and the titers of the helper virus in the standard plaque formation assay were determined by using the IE 3-complementing cell line, RR1. The pgC-lacZ amplicon required superinfection of Vero cells with wild-type virus for efficient expression of β-galactosidase. Amplicon preparations were tested for the presence of recombinant wild-type virus in the D30EBA helper virus population by plaque formation assay on Vero cells and were negative. The multiplicity of infection used to inoculate the neuronal cultures was 10 relative to the titer of the helper virus. Histochemical detection of β-galactosidase, termed 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) histochemistry, was performed as described by Sanes et al. (28).

Neuronal cultures. Neuronal cultures were prepared from DRG of embryonic day 15 rats as previously described (37). The neuronal cultures described in this study contained approximately 10^3 neurons in each well of a 24-well cluster dish. Latent viral infection in neuronal cultures was established as previously described (37) and maintained in the standard neuronal culture medium (50 ng of 2.5 S mouse nerve growth factor [NGF; provided by E. M. Johnson, Washington University, St. Louis, Mo.] per ml in Eagle's minimal essential medium [Life Technologies] with 10% calf serum [Life Technologies]).

Reactivation stimuli for induction of latent virus. NGF deprivation was achieved by adding 1% guinea pig anti-mouse NGF serum (titer, >20,000) as previously described (36, 37). Superinfection was performed with HSV-1 strain 17$^+$ at a multiplicity of 10 PFU per cell. Drugs were prepared by dilution in standard neuronal culture medium as previously described (31). Chlorophenylthio-cyclic AMP (CPT-cAMP; Sigma) was used at a final concentration of 500 μM. 4-β-Phorbol-12-myristate-13-acetate (PMA; Sigma) was used at a final concentration of 10^{-9} M.

Microinjection of neurons and β-galactosidase detection by using X-Gal histochemistry. Neurons plated on collagen-coated plastic coverslips (Lux) were pressure injected by using micropipettes pulled from 1-mm-microdot glass stock (Glass Company of America) with a single-stage vertical puller (Kopf). The pipettes were prepared as described by Graessmann and Graessmann (17). During microinjection, neurons were maintained in L-15 medium with 5% newborn bovine serum, 50 ng of NGF per ml, and 2× penicillin-streptomycin at room temperature. The neurons were pressure injected with a solution containing 10 mg of rhodamine-labeled dextran (Molecular Probes) per ml and 2.5 mg of β-galactosidase (Sigma) per ml in 48 mM K_2HPO_4–45 mM KH_2PO_4–14 mM NaH_2PO_4 (pH 7.3). X-Gal histochemistry for detection of β-galactosidase was performed. Photomicrographs were taken with a Nikon Optiphot-2 equipped with Hoffman optics and epifluorescence attachment, using Kodak T-64 film.

Colocalization of LAT and β-galactosidase by in situ hybridizations and immunohistochemistry. In situ hybridization for detection of the latency-associated transcript (LAT) was performed as previously described (30). The hybridization probe was pLAT, which contains a 1.2-kb insert from the *Pst*I site to the *Kpn*I site of the LAT region and has no region of overlap with other known genes in the LAT region (25). Immunohistochemistry for detection of β-galactosidase was performed on neuronal cultures fixed with 4% paraformaldehyde in phosphate-buffered saline either directly or following in situ hybridization as described above. For detection of β-galactosidase by immunohistochemistry, cultures were incubated for 4 h at room temperature with a 1/2,000 dilution of a monoclonal anti-β-galactosidase antibody (Promega). Indirect immunohistochemistry was performed by the avidin-biotin-peroxidase complex (Vectastain) method as instructed by the manufacturer (Vector Laboratories, Burlingame, Calif.). The chromogen aminoethyl carbazole was used as the substrate to produce an intense red color in positively stained cells. Photographs of representative fields were obtained by using a Nikon Optiphot-2 equipped with Hoffman optics. The composite images were generated by scanning the 35-mm slides with a Nikon Cool Scan Slide Scanner and produced digitized images on a Macintosh 2X. Colors were recalibrated but not altered, and the final results were printed with a Kodak 7700 printer.

Amplicon DNA quantification. DNA was isolated form neuronal cultures at times indicated following infection with the amplicons by lysis of the cells in 10 mM Tris-HCl (pH 8.5)–0.01 M EDTA–0.5% sodium dodecyl sulfate (SDS). Samples were treated with 0.5 mg of proteinase K (Boehringer Mannheim) per ml for 12 h at 37°C and then subjected to chloroform-phenol extraction and ethanol precipitation. The DNA samples were applied to a nylon membrane (Hybond-N; Amersham) by using a slot blot apparatus (Hoefer Scientific Instruments). As the standard, a plasmid containing the *lacZ* gene, pON1 (1), was serially diluted and applied to the membrane. The probe used to detect the amplicon DNA was the *lacZ* gene labeled by random primer incorporation of [α-^{32}P]dCTP. Hybridizations were done for 18 h at 42°C. Filters were washed at 50°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. The filters were exposed to Kodak X-Omat-AR film with an intensifying screen. The autoradiograms were scanned with a Microteck ScanMaker IIHR and analyzed by using NIH Image software.

RESULTS

Long-term expression from the IE promoters in HSV-1 amplicon vectors. The *lacZ* gene product is detected for up to 2 weeks in neurons infected with pHSVlac (13). pHSV-lac uses the HSV-1 IE 4/5 gene promoter to regulate the expression of β-galactosidase. The observation of expression of β-galactosidase from the amplicon at 2 weeks after infection is unexpected since the HSV-1 IE promoters are thought to be inactive during the latent infection, both in vivo and in vitro. Potentially, this finding could reflect a unique property of the IE 4/5 promoter, a result of a very stable protein product, or an intrinsic ability of viral promoters to avoid inactivation in the amplicon. To address these possibilities, additional amplicons were constructed by using other viral promoters.

Neuronal cultures were infected with the HSV-1 amplicon/

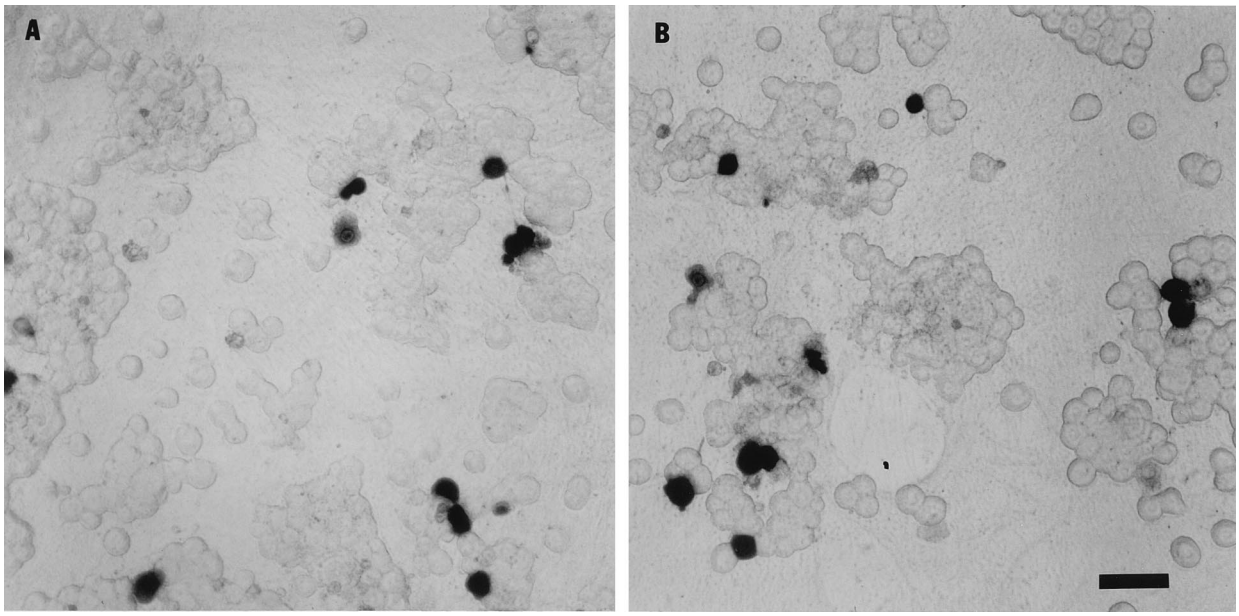

FIG. 2. β-Galactosidase staining of sensory neurons in culture at times after infection with the IE 1 amplicon. Shown are representative cultures at 2 (A) or 10 (B) weeks after infection with the amplicon pIE1lacZ, stained by X-Gal histochemistry. Bar, 50 μm.

helper virus preparations and examined for the expression of β-galactosidase at several times after inoculation. Under carefully controlled culture conditions using plating densities of approximately 1,000 neurons per culture, DRG neurons can be maintained in culture for several months. Using these methods, we examined neuronal cultures infected with the IE 1, IE 3, or IE 4/5 promoter-*lacZ*-containing amplicons packaged with the IE 3 deletion mutant for expression of β-galactosidase activity at 2 or 10 weeks after inoculation. Fig. 2 shows representative fields of view of neuronal cultures at 2 and 10 weeks after infection of the cultures with the pIE1-lacZ amplicon. At the multiplicities of infection used in these experiments, no cytopathic effects were observed following infection. Similar intensities of staining were obtained with the IE 1, IE 3, and IE 4/5 promoter-containing vectors. Under these conditions, the number of neurons expressing β-galactosidase activity remained essentially the same for up to 10 weeks, the longest time examined (Table 1). The IE 1, IE 3, and IE 4/5 promoters produced similar results, indicating that β-galactosidase was expressed from the IE promoters in neurons under conditions analogous to those for the latent infection. Under these conditions during the latent HSV-1 infection, the IE gene products are generally not detected (5, 10, 30, 35). These results suggest that the IE promoters in the amplicons are not subject to inactivation that occurs in the virus during latency and that these amplicons are capable of long-term expression in sensory neurons in vitro.

TABLE 1. Expression of β-galactosidase from HSV-1 promoters in neuronal cultures at 2 weeks and 10 weeks after infection with amplicons

Promoter	No. of β-galactosidase-positive neurons per culture[a] at:	
	2 wk postinfection	10 wk postinfection
IE 1	93 ± 10	75 ± 15
IE 3	87 ± 7	84 ± 12
IE 4/5	85 ± 13	79 ± 9
gC	3 ± 1	ND

[a] DRG neuronal cultures were infected with HSV-1 amplicons containing the promoter indicated. The ratios of helper virus to amplicon were between 50:1 and 100:1 for the amplicon preparations. At the times indicated, the cultures were stained by X-Gal histochemistry and the number of β-galactosidase-positive neurons was counted. The values are the mean numbers of β-galactosidase-positive neurons from five cultures ± standard errors of the means. ND, not determined.

Limited expression from a late gene class promoter. In contrast to the IE promoters, when an amplicon containing the late HSV-1 gene promoter from gC driving *lacZ* was used to infect the neurons, β-galactosidase expression was observed in approximately 10% of the neurons at 48 h postinoculation and in <1% at 2 weeks postinoculation (Table 1). In addition, the expression from the gC amplicon was not induced following superinfection with wild-type virus, suggesting that the promoter activity was uninducible or that the gC amplicon was eliminated from the neurons.

Comparison of the amounts of amplicon DNA. To determine if the loss of expression from the gC amplicon was the result of promoter inactivation or elimination of the DNA, the amounts of amplicon DNA present in the neuronal cultures following infection with the gC amplicon were compared with the amounts of the IE 4/5 amplicon. Comparison of the relative amounts of gC amplicon DNA and IE 4/5 amplicon DNA indicated that the levels of DNA were similar and remained essentially unchanged from 2 days (0.5 pg for IE 4/5 amplicon and 1 pg for gC amplicon) to 2 weeks (0.5 pg for both the IE 4/5 and gC amplicons) postinoculation. These results suggest that the lack of expression of β-galactosidase from the gC promoter was not the result of inefficient infection or degradation of the vector DNA (Fig. 3).

Stimuli that reactivate latent virus do not increase the percentage of neurons expressing β-galactosidase. The observation of long-term expression of β-galactosidase activity in 10% of the neurons did not exclude the possibility that the amplicons were inactivated or repressed in some of the neurons. In neurons in culture, latent HSV-1 has previously been shown to

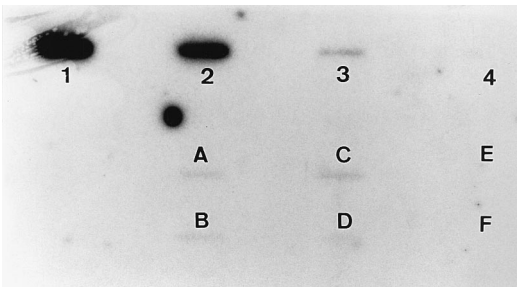

FIG. 3. Comparison of the amounts of gC amplicon DNA with IE 4/5 amplicon DNA following infection of neuronal cultures. The results of DNA slot blot analysis shows the relative amounts of amplicon DNA harvested from neurons at 2 days (A) and 2 weeks (B) after infection with pHSVlac, 2 days (C) and 2 weeks (D) after infection pgClacZ, or 2 days (E) and 2 weeks (F) after mock infection. Slots 1 to 4 are DNA standards (0.2 ng, 0.02 ng, 2 pg, and 0.2 pg, respectively).

TABLE 2. Effects of reactivation stimuli on the number of neurons expressing β-galactosidase following infection with HSV-1 amplicons[a]

Promoter	No. of β-galactosidase-positive neurons				
	Untreated	Superinfection	Anti-NGF	CTP-cAMP	PMA
IE 1	25 ± 5	19 ± 5	29 ± 4	17 ± 3	19 ± 10
IE 3	40 ± 6	38 ± 5	40 ± 9	44 ± 8	45 ± 11
IE 4/5	55 ± 5	70 ± 5	70 ± 8	59 ± 14	ND
gC	2 ± 1	2 ± 1	2 ± 1	ND	ND

[a] DRG neuronal cultures 2 weeks after infection with the HSV-1 amplicon containing the promoter indicated were treated with the reactivation stimulus indicated. The ratios of helper virus to amplicon were between 50:1 and 100:1 for the amplicon preparations. Cultures were infected with the equivalent of a multiplicity of infection of 10 for the helper virus. Two days after the addition of the reactivation stimulus, the cultures were stained by X-Gal histochemistry and the number of β-galactosidase-positive neurons was counted. Values are the mean numbers of β-galactosidase-positive neurons from three cultures ± standard errors of the means. ND, not determined.

be reactivated by a variety of stimuli, including superinfection with HSV-1, NGF deprivation, or addition of activators of protein kinase C or A (31). To determine if β-galactosidase activity could be induced in additional neurons potentially harboring quiescent viral amplicons, cultures were treated by superinfection with HSV-1, NGF deprivation, activation of protein kinase C by treatment with PMA, and treatment with the cell-permeable cAMP analog CPT-cAMP, performed as described in Table 2. The percentage of neurons with positive staining for β-galactosidase was not significantly altered by any of these treatments (Table 2). These results show that β-galactosidase activity was not induced in additional neurons by treatments demonstrated to cause reactivation. That the frequency of expression from the IE promoters was not induced by the reactivation stimuli most likely indicates that the promoter were active and therefore could not be further induced.

Persistence of β-galactosidase activity is not the result of a prolonged half-life of the β-galactosidase protein. In other studies, we have demonstrated that expression of β-galactosidase from a viral mutant, containing the human cytomegalovirus promoter driving *lacZ*, is dramatically repressed within 1 week of establishment of a latent infection in neurons (30). These results suggest that the upper limits of the half-life of the β-galactosidase protein in neurons is less than 1 week; otherwise, a much longer time would be required to observe the disappearance of β-galactosidase. To further delineate the half-life for the detection of β-galactosidase, neurons were directly pressure injected with β-galactosidase protein and rhodamine-labeled dextran. Rhodamine-dextran was selected as a second marker for comparison since it has a relatively long half-life in most systems that have been examined (29). At the times specified, neurons were examined by fluorescence for the presence of rhodamine-dextran, and β-galactosidase activity was detected by using X-Gal histochemistry (Fig. 4). As shown in Fig. 5, the number of X-Gal-positive neurons at 6 h after injection was significantly greater than that observed at 24 h or 3 days. Taken together, these results strongly indicate that the half-life of β-galactosidase cannot explain the long-term signal observed in neurons following infection with the amplicons containing the IE promoters regulating the *lacZ* gene.

An IE promoter in the amplicon functions in neurons that harbor latent HSV-1. In the infections described above, the ratio of helper virus to amplicon was 100:1; consequently, many of the same neurons that harbor an amplicon should also contain helper virus. In these experiments, the helper virus was not competent to reactivate, and it remained possible that a wild-type virus and an amplicon could interact in different manners. To address this possibility, pIE3-lacZ was packaged with wild-type virus (HSV-1 strain F), and after establishment of latency, neurons were examined for coexpression of β-galactosidase protein and the latency-associated transcript (LAT).

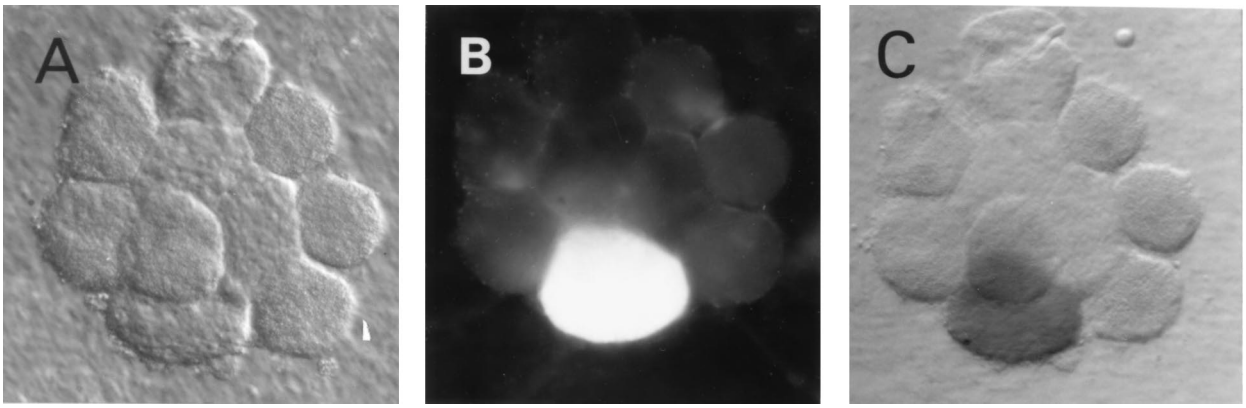

FIG. 4. Detection of rhodamine-dextran- and β-galactosidase-labeled neurons 24 h after microinjection. Rat sensory neurons were pressure coinjected with rhodamine-dextran and β-galactosidase. Twenty-four hours after injection, the neurons were fixed and examined for epifluorescence and X-Gal histochemical staining. The same field is shown under Hoffman optics (A), under epifluorescence (B), or following development to detect β-galactosidase (C).

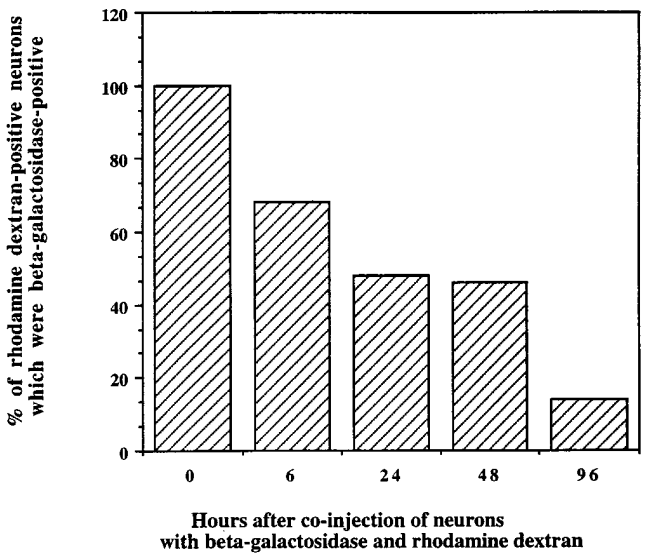

FIG. 5. Half-life of detectable β-galactosidase activity after microinjection into neurons. Neurons coinjected with rhodamine-dextran and β-galactosidase were fixed and analyzed at the times indicated. The number of neurons injected was counted and confirmed by the appearance of intracellular staining with rhodamine-dextran, visualized by epifluorescence. At various times after microinjection, cultures were fixed as described in the text, photographed under epifluorescence, and then stained by X-Gal histochemistry to detect β-galactosidase activity. Results are presented as the number of neurons marked with rhodamine fluorescence that had X-Gal staining.

When neuronal cultures were infected with this amplicon preparation and maintained for 2 weeks, neurons expressing both β-galactosidase and LAT, as well as either LAT or β-galactosidase alone, were detected (Fig. 6). We have detected an increase in the numbers of LAT-positive neurons with time after the establishment of latency; therefore, in these experiments the LAT-negative neurons may also have harbored latent virus (30). However, a population of neurons containing latent virus also contain the amplicon, indicating that stable latency with no evidence for IE gene expression from the helper wild-type virus can occur in the same neurons that express β-galactosidase from an IE promoter in the amplicon. Previously we have reported that during the latent infection in neurons in vitro, RNA from the IE 1 gene is not detected by in situ hybridization and antigens from the IE genes are not detected by immunohistochemistry (30). These results indicate that β-galactosidase can be expressed from the IE promoters in the amplicons in the same neurons that harbor latent HSV-1.

DISCUSSION

Promoter regulation in viral vectors. A major problem in the development of viral vectors for gene transduction into neurons has been promoter inactivation. Promoter inactivation by using recombinant HSV-1 vectors has been reported for DRG neurons in vitro (30) and in vivo (9), although recently, long-term expression was also reported (23). The results presented here indicate that the IE promoters in the amplicons were capable of long-term expression in DRG neurons in culture and were not subjected to inactivation as occurs in the virus genome during the latent infection.

The use of the neuronal culture system allowed demonstration that the maintenance of expression was essentially quantitative between 2 and 10 weeks postinfection. These observations are supported by the demonstration of expression from amplicons in vivo for long periods (7, 19).

Stability of β-galactosidase. β-Galactosidase has been widely used as a reporter gene in studies designed to develop virus vectors. The concern that the continued presence of β-galactosidase is the result of a very long half-life and not continued expression has been raised. Direct pressure injection of β-galactosidase into neurons in culture indicate that the half-life of β-galactosidase detection was between 1 and 2 days. Since the levels of β-galactosidase required for detection are likely to be much less than 50% of the starting amount, the actual half-life of the protein is predicted to be shorter than that estimated by these histochemical methods. Therefore,

FIG. 6. Colocalization of β-galactosidase and LAT during latency. Two weeks after infection with the IE 3 amplicon, neurons were examined for the expression of β-galactosidase and LAT. Shown are mock-infected neurons (A) and neuronal cultures infected with the pIE3-lacZ amplicon/HSV-1 strain F preparation (B and C). In panel B, a neuron positive for β-galactosidase alone (arrow) and a neuron positive for LAT alone (asterisk) are shown. In panel C, a neuron positive for β-galactosidase alone (arrow) and two neurons positive for both β-galactosidase and LAT (arrowhead) are shown. Bar in panel A represents 50 μm and applies to all panels.

while β-galactosidase is clearly a stable protein in neurons, detection for weeks is likely to require continued expression.

It is possible that the damage caused by microinjection of the neurons altered the degradation rate of the protein. However, these results are supported by studies using a recombinant HSV-1 that expresses β-galactosidase. Neurons infected with the mutant HSV-1 containing the human cytomegalovirus IE promoter regulating the *lacZ* gene inserted in a nonessential region of the virus readily establish latent infections with essentially no detectable loss of neurons (30). However, between 3 and 7 days postinfection, an 80% decrease in the number of β-galactosidase-positive neurons was observed, while evidence of latent infections increases, as indicated by the LAT in situ signal (30). These results indicate that the upper limit of the half-life for detection of β-galactosidase activity is days, not weeks, and are in agreement with the results reported here for direct microinjection of β-galactosidase. Together, these results indicate that the β-galactosidase detected at 2 or 10 weeks postinfection with the HSV-1 IE promoter-*lacZ* amplicons required continued expression from the *lacZ* gene.

Long-term IE promoter function and viral latency. By use of double labeling to detect LAT and β-galactosidase, it was demonstrated that the same neurons can harbor both latent HSV-1 and transcriptionally active amplicons. This result is not surprising given the much higher multiplicity of infection of the packaging virus compared with the amplicon for the preparations used in these studies. A common feature of HSV-1 latency in humans and animal models and the in vitro neuronal model of latency is that during latency, viral gene expression, with the exception of LAT expression, is repressed (10). Several mechanisms could potentially explain the long-term activity from the IE promoters in the amplicons and may be relevant to understanding processes that regulate the latent HSV-1 infection in neurons. The sequences in the amplicons may contain elements that exert a dominant effect that prevents inactivation of these promoters from occurring. The observation that the gC promoter in the amplicon transiently expressed β-galactosidase and was then irreversibly inactivated argues against this possibility. The transient expression from the gC promoter was unexpected and may be the result of placement in the amplicon system, similar to results from transient transfection studies.

The amplicons deliver a high gene dose to each infected cell as the result of the concatemeric packaging of multimers of the unit amplicon plasmid (12). This high copy number could act as an amplification system that permits the detection of a low basal level of expression from the IE promoters. This would suggest that the IE promoters may function at low levels during the latent infection with the virus, below the level of detection and below the level that activates viral gene expression leading to reactivation. Consistent with this possibility, a recent study using quantitative reverse transcriptase-PCR detected very low level transcription of the IE 3 gene during the latent infection in mice (20). Alternatively, the high copy number of the amplicon might act to bind a repressor(s), thus resulting in the disinhibition of the IE promoter in the amplicon, similar to findings of the studies on Oct-2 reported by Lillycrop et al. (22).

An intriguing possibility is that *cis*-acting elements of the HSV-1 genome are required to repress the IE promoters during latency. By removing the IE promoter from the context of the viral genome, these promoters may consequently remain active. The process of promoter inactivation has been widely observed with other types of virus vectors. Finding mechanisms to avoid promoter inactivation remains a major challenge in developing effective virus vectors. The results presented here may facilitate the development of viral vectors for long-term expression in neurons.

ACKNOWLEDGMENTS

We thank L. I. Pizer for helpful discussion.

This work was supported by Public Health Service grants NS 01741 to R.L.S. AG10446 and NS29046 to C.L.W., and AG10827 to A.I.G. A.I.G. was also supported by grants from the American Health Assistance Foundation and the Burroughs Wellcome Fund.

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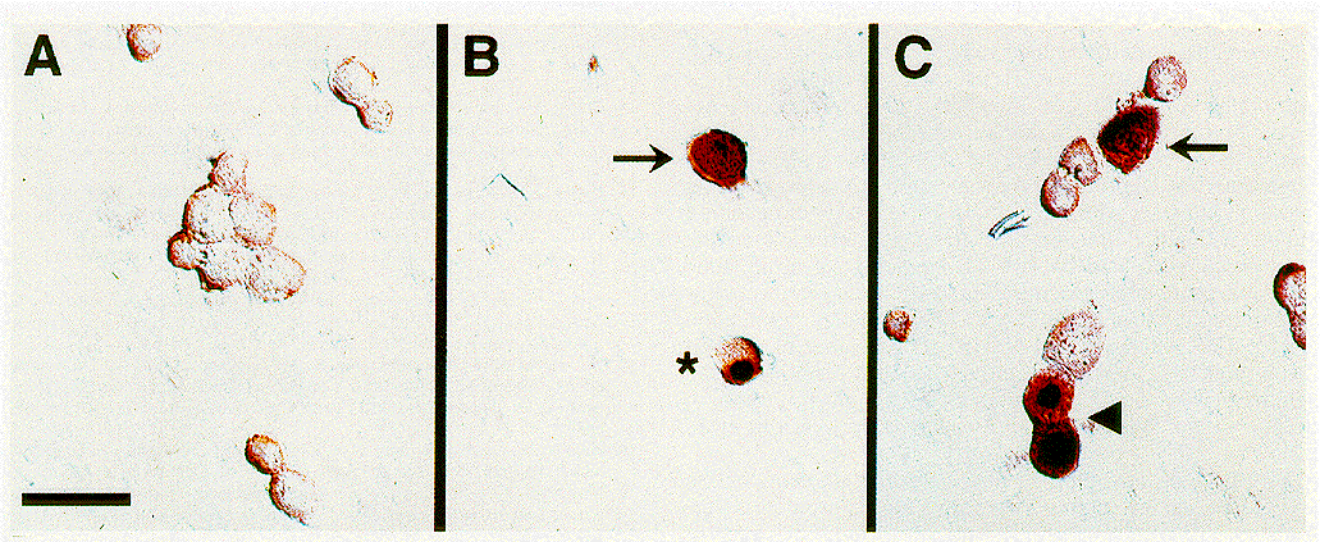
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