Induction of Herpes Simplex Virus Type 1 Immediate-Early Gene Expression by a Cellular Activity Expressed in Vero and NB41A3 Cells after Growth Arrest-Release

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Infected cell protein 0 (ICP0), a major immediate-early regulatory protein of herpes simplex virus type 1 (HSV-1), activates expression of all classes of HSV genes as well as a variety of heterologous viral and cellular genes. Previous studies have shown that a cellular activity expressed maximally in Vero cells 8 h after release from growth arrest in the G₀/G₁ phase of the cell cycle can enhance plaque formation and gene expression of a mutant virus (7134) lacking both copies of the gene encoding ICP0 (W. Cai and P. Schaffer, *J. Virol.* 65:4078–4090, 1991). This observation suggests that the cellular activity can substitute for ICP0 to activate viral gene expression. To further characterize this cellular activity, Vero and NB41A3 (mouse neuroblastoma) cells were transfected at various times after release from growth arrest with promoter-chloramphenicol acetyltransferase (CAT) constructs containing promoters representing the major kinetic classes of HSV genes, and CAT activity was measured from 2 to 24 h postrelease. The results of these tests demonstrate that CAT expression from immediate-early promoter-CAT plasmids was enhanced 10- and 3-fold when Vero and NB41A3 cells were transfected at 6 and 2 h postrelease, respectively. In contrast, only low levels of immediate-early promoter-driven CAT activity were apparent when cells were transfected at later times postrelease. No significant stimulation of CAT activity was observed from promoter-CAT plasmids containing representative early or late HSV promoters or a heterologous viral (simian virus 40 early) promoter. Differences in the efficiency of uptake of plasmid DNA by cells at various times postrelease did not account for the observed differences in CAT expression. Unlike Vero cells, in which cell division resumed after release from growth arrest, division of NB41A3 cells did not resume. Rather, these cells displayed morphological features suggestive of a differentiated phenotype. Collectively, these findings demonstrate that a cellular activity expressed in Vero and NB41A3 cells after release from growth arrest can activate HSV gene expression by enhancing immediate-early gene expression.

The hallmark of herpesvirus infection is the establishment of latency associated with periodic reactivation (reviewed in references 36 and 72). In acute infection with herpes simplex virus type 1 (HSV-1), this phenomenon is clinically apparent as acute gingivostomatitis or pharyngitis followed by periodic recurrences at the initial site of inoculation, resulting in recurrent disease (72). During acute infection, the virus enters dendritic termini and travels via retrograde axonal transport to neuronal cell bodies in ganglia. Viral DNA exists in a quiescent, probably episomal state in neuronal nuclei, where gene expression is restricted to the latency-associated transcripts (LATs) (25, 59–61). At some later time (weeks to years), viral gene expression is derepressed in response to a variety of stress-associated stimuli, resulting in the resumption of viral gene expression, synthesis of new virus, anterograde axonal transport of virus to the initial site of infection, and recurrent infection of epithelial cells at this site (15).

The molecular mechanism by which expression of the HSV-1 genome is repressed and subsequently reactivated in neurons is unknown. Although cellular factors are likely responsible for the suppression of productive infection leading to latency and the resumption of productive infection leading to reactivation, viral gene expression is thought to be mediated

through the activities of viral immediate-early regulatory proteins in response to these cellular factors (41, 62). Although not expressed during latent infection, immediate-early genes are the first to be expressed during productive infection, and immediate-early proteins are required for the timely and coordinate expression of early and late viral genes (37, 38). Consequently, the activities of immediate-early proteins during productive infection must be repressed during the establishment of latency and reinitiated during reactivation.

The roles of immediate-early proteins in regulating viral gene expression during productive infection have been fairly well characterized; however, the molecular mechanisms underlying these roles have not. Of the five immediate-early proteins (infected cell protein 0 [ICP0], ICP4, ICP22, ICP27, and ICP47) only two, ICP4 and ICP27, are essential for virus replication (22, 47). ICP4 activates transcription of early and late genes and represses its own transcription as well as that of other immediate-early genes (19, 20, 30, 53). ICP27 is also involved in regulating expression of early and late genes, possibly by altering the stabilities of mRNAs whose synthesis is activated by ICP4 and ICP0, by modifying the rates of transcription of late messages, or by a combination of both activities (39, 49, 58, 73). Very little is known about the functions of immediate-early proteins ICP22 and ICP47. Available information suggests, however, that ICP22 is also a viral regulatory protein (3, 69). Although not essential for viral growth in cell culture, ICP0 is the only immediate-early protein able to activate expression from immediate-early promoters,

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and thus ICP0 is the only immediate-early protein able to activate expression of all classes of HSV-1 genes (11, 26, 27, 30). Despite its broad and potent transactivating activity, the mechanism by which ICP0 activates viral gene expression is not known. Because of its strong activating activity, especially for immediate-early genes, ICP0 is an excellent candidate protein to initiate the cascade of viral gene expression at the onset of reactivation. If this is the case, one could postulate that the transactivating activity of ICP0 is repressed by cellular factors during the establishment and maintenance of latency and derepressed by cellular factors at the onset of reactivation.

As noted above, a role for cellular factors in mediating the switch from productive infection to latency and vice versa has been postulated. Indeed, cellular proteins involved in the activation and repression of immediate-early gene expression have been described. First, the virion-associated protein, VP16, and the cellular octamer-binding factor, Oct-1, activate immediate-early genes through sequence-specific interactions with viral promoters (48, 52, 57). VP16 alone exhibits no transactivating activity. Second, repression of transcription from the ICP4 promoter was shown to be mediated by a factor in neuroblastoma cells that interacts with the same sequence that binds the VP16/Oct-1 complex in immediate-early promoters (40). A central question that remains to be answered is, What cellular factors are involved in the initial event that signals reactivation of HSV gene expression? Recently, Cai and Schaffer have shown that a cellular activity expressed maximally 6 h after release of Vero cells from growth arrest in the G₀/G₁ stage of the cell cycle can substitute operationally for ICP0 to enhance viral gene expression (12). This cellular activity is of special interest with regard to the mechanism of reactivation, since it is ultimately able to enhance expression of all classes of viral genes in ICP0-deletion mutant-infected cells. The activity is expressed only after release of cells from growth arrest in G₀/G₁ of the cell cycle. Neurons latently infected with HSV in vivo are growth arrested in G₀ and are induced to enter G₁ following stress-associated stimulation (14, 32). Events at the molecular level associated with the transition of neurons from G₀/G₁ to later stages of the cell cycle may well play a role in reactivation of HSV from latency.

As a first step in characterizing the cellular activity initially described by Cai and Schaffer (12), we examined whether the activity expressed by growth-arrested-released Vero cells is able to stimulate expression from individual classes (immediate-early, early, and late) of HSV-1 promoters. We also examined whether a similar activity is expressed in cells of neural origin following growth arrest-release. Our results indicate that an activity able to stimulate immediate-early, but not early or late, promoters is expressed in both Vero and NB41A3 cells after release from growth arrest. Notably, the stimulatory activity was expressed in NB41A3 cells despite the fact that these cells ceased to divide but remained fully viable after release from growth arrest.

MATERIALS AND METHODS

Cells. African green monkey kidney cells (Vero; ATCC CCL 81) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. NB41A3 cells (ATCC CCL 147) were propagated in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum. All cells were grown and maintained as monolayer cultures at 37°C in 5% CO₂.

Construction of plasmids. Plasmid pWRICP0 expresses chloramphenicol acetyltransferase (CAT) under control of the wild-type promoter/regulatory sequences of the ICP0 gene

(45) (nucleotides 125062 to 124105 by the numbering system of Perry et al. [55, 56]) (Fig. 1B). pWRICP0 was constructed by removing the 1,597-bp *SacI*-*Bam*HI fragment of pW3 (which contains the entire ICP0 promoter, leader, first exon, and part of the first intron) and cloning it into the *SacI*-*Bam*HI site of pUC19 (New England Biolabs, Beverly, Mass.) to create pW3SB (63). The unique *NcoI* site in pW3SB, which includes the initiation codon of the ICP0 open reading frame, was cleaved with *NcoI*, blunt ended following incubation with the Klenow fragment of DNA polymerase I, ligated to *Hind*III linkers, and recircularized to create plasmid pW3SH (55). A 955-bp *SacI*-*Hind*III fragment was isolated from pW3SH and cloned into the *SacI*-*Hind*III site of the CAT expression vector pGemCAT (5), placing the ICP0 promoter/regulatory sequences (−803 to +152 relative to the start of transcription [45, 56]) immediately 5' to the open reading frame of the CAT gene (Fig. 1B). Three tandem copies of the simian virus 40 (SV40) polyadenylation signal included in an 810-bp fragment were inserted immediately 5' of the ICP0 promoter to minimize the influence of readthrough transcripts originating from cryptic promoters within vector sequences (46). The sequence content and orientation of ICP0 promoter/regulatory sequences in this plasmid were confirmed by dideoxynucleotide sequencing (Promega, Madison, Wis.).

The ICP0-luciferase reporter gene plasmid, pMCICP0, was constructed by isolating a 955-bp *SacI*-*Hind*III fragment from pWRICP0, which contains the ICP0 promoter/regulatory sequences, and cloning it into the *SacI*-*Hind*III site of the luciferase expression vector pGL2-Basic (Promega). pGL2-Control, an SV40 early promoter-luciferase reporter plasmid, was obtained from Promega.

Deletion mutations were introduced into the 5' region of ICP0 sequences as follows. pWRICP0 was doubly digested with *Stu*I and *Sma*I or with *Sac*I and *Sma*I, ligated to *Xba*I linkers or *Sac*I linkers, and recircularized to create plasmids pStu/*Sma* and p*Sma*, respectively (Fig. 1B). The deletion in pStu/*Sma* is 431 bp in length (−559 to −128), and that in p*Sma* is 675 bp (−803 to −128). pWR574 was constructed by synthesizing a 574-bp *Stu*I-*Hind*III fragment by PCR (Perkin-Elmer Cetus, Norwalk, Conn.), using pWRICP0 as a template and 20-nucleotide primers (5'-TGCAAGAGGCCTTGTTTCGC-3' and 5'-TTAAGCTTGCGACCAGACTGTCTGGGC CA-3') designed to specify the boundaries of the fragment as −559 and +7 of the wild-type sequence (55). The amplified fragment was cloned into the *Stu*I-*Hind*III site of pWRICP0, replacing the wild-type sequence and resulting in a plasmid lacking 145 bp of the 152-bp 5' untranslated leader sequence of ICP0. The identity of all mutations was confirmed by dideoxynucleotide sequencing (Promega).

The construction and characterization of HSV-1 promoter-CAT plasmids containing the promoter of the early gene UL9 (pL9CAT2.2), the delayed-early gene encoding glycoprotein B (pKXXBI-CAT), the gene specifying the LATs (pLHp5CRE), the immediate-early gene encoding ICP4 (pIE3-CAT), the early gene encoding thymidine kinase (pTK-CAT), and the true late gene, L42 (pL42-CAT), of HSV-1 have been described previously (5, 11, 21, 42). CAT plasmids pLSHp5 (17), pICP22/47-CAT, and pHCMV-CAT (79) contain the SV40 early promoter, the promoter/regulatory sequences of the immediate-early genes encoding ICP22/47, and the human cytomegalovirus (HCMV) transcriptional regulatory region of IE1, respectively.

Effect of isoleucine deprivation on cell growth. The growth of Vero and NB41A3 cells in medium containing and lacking isoleucine was assessed by seeding 3 × 10⁵ cells in 35-mm-diameter petri dishes in appropriate growth medium for 24 h,

FIG. 1. Wild-type and mutant promoter-CAT plasmids used in this study. (A) Diagram of the HSV-1 genome showing the locations of both copies of the ICP0 transcriptional unit within the long repeats of HSV DNA. Beneath the diagram of the HSV-1 genome is an expanded diagram of the ICP0 transcriptional unit showing the promoter region (dotted line), exons (thick horizontal lines), and two introns (thin diagonal lines). (B) Expanded diagram of the wild-type ICP0 promoter/regulatory region from -803 to +152 in pWRICP0. Relevant restriction sites are shown above the line. The black box on the right represents the CAT gene, including its 5' untranslated leader and coding sequences. The locations of deletions in ICP0 regulatory sequences in plasmids pSma, pStu/Sma, and pWR574 are shown beneath the diagram of pWRICP0. Below the plasmids are symbols representing previously identified and potential regulatory elements within the ICP0 promoter region. 18bp repeat; an 18-bp repeat sequence not previously described; EGR, *cis*-element target for cellular immediate-early proteins Egr-1 (mouse) and EGR2 (human) (54); ICP4, binding site for HSV-1 regulatory protein ICP4; GCGGAAC, GA-rich element (4, 10, 75, 80). (C) Diagram of promoter/regulatory sequences in immediate-early (ICP4, pIE3CAT; ICP22/47, pICP22/47CAT), early (TK, pTKCAT; UL9, pCAT22), delayed-early (gB, pKXXB1-CAT), and late (L42, pL42CAT) reporter genes. Also shown

transferring the cells to medium lacking isoleucine for 42 h, and then returning the cells to medium containing isoleucine. Cells were counted in triplicate before, during, and after cultivation in medium lacking isoleucine.

CAT assays in growth-arrested-released Vero and NB41A3 cells. (i) **Growth arrest-release.** Vero and NB41A3 cells were growth arrested by isoleucine deprivation essentially as described by Cai and Schaffer (12), Hamlin and Pardee (34), and Ley and Tobey (43). Briefly, cells were seeded in 100-mm-diameter petri dishes at densities of 2.5×10^6 Vero cells or 4.0×10^6 NB41A3 cells in their respective growth media and incubated at 37°C for 24 h. Growth medium was then removed and replaced with medium lacking isoleucine and supplemented with 10% dialyzed fetal bovine serum (for Vero cells) or 15% dialyzed horse serum and 2.5% dialyzed fetal bovine serum (for NB41A3 cells). Isoleucine-free Dulbecco's modified Eagle's medium (product no. 57-861-501) and isoleucine-free Ham's F-10 (product no. 57-965-501) were prepared as instructed by the manufacturer (JRH Biosciences, Lenexa, Kans.). Following incubation for 42 h in medium lacking isoleucine, cells were returned to isoleucine-containing medium to reverse the block and were transfected at the designated times. Vero cells incubated in medium lacking isoleucine have been shown to be growth arrested at a point within the G₀/G₁ stage of the cell cycle (12).

(ii) **Transfection.** In an effort to maximize the efficiency of transfection while minimizing the time needed to achieve detectable levels of CAT activity, we used Lipofectin (Bethesda Research Laboratories, Gaithersburg, Md.) to introduce plasmid DNA into cells (28). Twenty micrograms of plasmid DNA in 2.5 ml of the appropriate growth medium was mixed with 2.5 ml of growth medium containing 75 mg of Lipofectin and incubated at 25°C for 15 min. At the designated times after return of cultures to isoleucine-containing medium, growth medium was removed from monolayers and replaced with 5 ml of the DNA-Lipofectin mixture. DNA and cells were incubated at 37°C in 5% CO₂, and transfected cells were harvested and processed for CAT activity or luciferase activity 6 h after transfection.

(iii) **CAT assays.** Extracts of transfected cells were prepared by scraping monolayers in 100-mm-diameter petri dishes into 6 ml of CAT buffer (0.25 M Tris-HCl [pH 7.4]), concentrating the cell pellet by centrifugation at $4,000 \times g$, and resuspending the pellet in 100 μ l of CAT buffer. After cell lysis in a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for 1 min at 60% efficiency, the soluble protein fraction was separated from cellular debris by centrifugation at $16,000 \times g$ for 15 min, and the protein concentration in the soluble extract was determined by the method of Bradford (8). CAT assays were carried out by the addition of 200 mg of protein extract to 30 ml of 20 mM acetyl coenzyme A (Boehringer Mannheim, Indianapolis, Ind.) and 100 nCi of [¹⁴C]chloramphenicol (Dupont, NEN Research Products, Boston, Mass.). Samples were incubated at 37°C for 90 min and analyzed by thin-layer chromatography (Eastman Kodak, Rochester, N.Y.) after ethyl acetate extraction. The results of CAT assays are presented as the percent conversion of substrate to acetylated forms of chloramphenicol. All assays were repeated at least three times. Because absolute values varied among tests, meaningful averages could not be calculated. Relative values were highly reproducible, however.

is a diagram of the promoter regulatory region of the gene specifying the LATs in reporter plasmid pLHp5CRE.

(iv) **Luciferase assays.** Extracts of transfected cells were prepared by scraping monolayers in 100-mm-diameter petri dishes into 1 ml of extraction buffer (100 mM KPO_4 [pH 7.8], 1 mM dithiothreitol), concentrating the cell pellet by centrifugation at $4,000 \times g$, and resuspending the pellet in 100 μl of extraction buffer. After cell lysis in a sonicator (Heat Systems-Ultrasonics) for 1 min at 60% efficiency, the soluble protein fraction was separated from cellular debris by centrifugation at $16,000 \times g$ for 15 min, and the protein concentration in the soluble extract was determined by the method of Bradford (8). Luciferase assays were performed by adding 50 μl of cell extract to 100 μl of luciferase assay reagent (Promega) and measuring the reaction in an LKB-Wallac 1251 luminometer (BioOrbit, Turku, Finland).

Slot blot hybridization. To measure transfection efficiency, cells in either 100- or 60-mm-diameter petri dishes were washed in ice-cold phosphate-buffered saline (PBS; 0.13 M NaCl, 2.6 mM KCl , 10 mM Na_2HPO_4 , 1.3 mM KH_2PO_4 [pH 7.4]), scraped into ice-cold PBS, transferred to a 15-ml conical tube (Corning Glass Works, Corning, N.Y.), and centrifuged at $2,000 \times g$. After the supernatant fluid was removed, the cell pellet was washed three times in PBS, and the final pellet was resuspended in three times the packed cell volume of ice-cold hypotonic buffer (10 mM NaCl, 10 mM Tris [pH 7.4], 3 mM MgCl_2). After incubation on ice for 10 min, the swollen cells were transferred to an ice-cold Dounce homogenizer (Wheaton) and homogenized with 10 strokes of a type B pestle, releasing nuclei into the medium. The mixture was then transferred to an Eppendorf microfuge tube and centrifuged at $16,000 \times g$ for 10 min at 4°C , pelleting nuclei and cellular debris. Following removal of the supernatant fluid, the pellet was resuspended in 200 μl of TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 8.0]) and 400 μl of Nonidet P-40 lysis buffer (10 mM NaCl, 10 mM Tris [pH 7.4], 3 mM MgCl_2 , 0.5% Nonidet P-40) and allowed to incubate at 25°C for 1 min to disrupt nuclear membranes. Ten microliters of RNase A (Sigma, St. Louis, Mo.) was then added, and the reaction mixture was incubated for an additional 5 min and then subjected to extraction with an equal volume of a 25:24:1 mixture of phenol, chloroform, and isoamyl alcohol (Sigma). Four hundred fifty microliters of the aqueous phase containing both cellular and plasmid DNA was transferred to a fresh Eppendorf Microfuge tube and treated with 0.1 volume of 3 M NaOH for 5 min at 68°C to denature DNA. After neutralization by the addition of 1.0 volume of sodium acetate (pH 5.0), DNA was transferred to a nylon membrane in a slot blot apparatus (Minifold II; Schleicher & Schuell, Inc., Keene, N.H.) as instructed by the manufacturer. Known amounts of purified plasmid DNA were also transferred to serve as standards. The blot was probed with purified plasmid DNA radiolabeled by nick translation (65), and the amount of hybridizing radiolabeled DNA in each slot was measured by PhosphorImager (model 400E; Molecular Dynamics, Sunnyvale, Calif.) scanning. The amount of plasmid DNA in the nuclei of transfected cells was calculated by comparison with a standard curve generated from the signals of known concentrations of plasmid DNA used as standards.

RESULTS

Activation of an immediate-early promoter in growth-arrested-released Vero cells. Results of previous studies in this laboratory have demonstrated that the plating efficiency of a mutant virus (7134) lacking both copies of the ICP0 gene was enhanced approximately fourfold when plated on Vero cell monolayers 8 h after release from growth arrest relative to 24

FIG. 2. CAT expression in Vero cells transfected with pWRICP0 at various times after release from growth arrest induced by isoleucine deprivation. Vero cells (2.5×10^6 cells per 100-mm-diameter petri dish) were growth arrested by incubation in isoleucine-free medium for 42 h (12). At 42 h, isoleucine-free cell culture medium was replaced by isoleucine-containing medium, and cells were transfected with 20 μg of pWRICP0 at various times thereafter. Six hours after transfection, cells were harvested and processed for CAT activity. Percent conversion of unacetylated to acetylated substrate was used as the measure of promoter activity over the 6-h interval between the time of transfection and the time of harvest. This experiment was repeated four times.

h after release (12). This difference in plating efficiency was not observed with the wild-type virus or with the mutant virus plated on ICP0-expressing cells. The time of maximum expression of this plaque-enhancing activity (8 h postrelease) correlated with progression from G_0/G_1 to the S phase of the cell cycle, as determined by flow cytometry of cells synchronized by isoleucine deprivation. These observations suggested the possibility that a factor (or factors), expressed maximally in Vero cells 8 h after release from growth arrest in G_0/G_1 , can substitute for the defect in the mutant by enhancing viral gene expression, and hence plaque formation, in the absence of functional ICP0. In an effort to better understand the basis of this enhancing cellular activity, we attempted to determine whether a factor(s) in Vero cells could enhance gene expression from each of the major classes of viral promoters (immediate-early, early, and late) 8 h after growth arrest-release.

To determine whether the cellular activity is able to enhance HSV immediate-early gene expression, we constructed a chimeric ICP0 promoter-CAT plasmid, pWRICP0 (Fig. 1B), and tested its inducibility following transfection of Vero cells at 2-h intervals after release from growth arrest (Fig. 2). Under the conditions of growth arrest-release used in these tests, greater than 80% of cells would be expected to be in synchrony throughout the 24-h test period (12). Each time point shown in Fig. 2 represents cumulative CAT activity over the 6-h interval between the time of transfection with pWRICP0 and the time of harvest of cells for CAT assays. This relatively short interval allowed us to monitor CAT expression roughly as a function of the time after release of transfected cells from growth arrest. In this assay, maximum expression of CAT from pWRICP0 occurred when Vero cell monolayers were transfected 6 h after release from growth arrest. These findings are consistent with results obtained in previous studies in which the maximum enhancing effect on the plating efficiency of 7134 in Vero cells

occurred when monolayers were infected 8 h after release from growth arrest (12).

Expression of the cellular enhancing activity was not observed in exponentially growing cells that had not been growth arrested, nor was the activity detected again when growth-arrested-released Vero cells were tested after completion of one synchronous growth cycle (24 h) (data not shown). Thus, as in the case of the plaque-enhancing activity described by Cai and Schaffer (12), the cellular activity appears to be expressed specifically following release from growth arrest near the G₀/G₁ border and not in actively cycling cells.

Transfection efficiency of growth-arrested-released Vero cells. To test the possibility that the differences in CAT expression observed were due to differential uptake of plasmid DNA by Vero cells transfected at different times after release from growth arrest, and not to differences in the ability of cells to enhance gene expression at these times, we measured the level of plasmid DNA in nuclei of cells transfected at 6 and 24 h postrelease by slot blot hybridization (Table 1). Although the 6-h samples contained an average of 1.7 μ g of DNA (8.6% of input DNA), the 24-h samples contained an average of 4.9 μ g of DNA (24% of input DNA). This greater than twofold increase in the amount of DNA was anticipated, given that at 24 h after release from growth arrest the cell population had completed one round of cell division and twice as many cells were available to take up DNA (12) (Table 1). This observation indicates that the efficiency of uptake of plasmid DNA was slightly greater in cells transfected at 24 h than in cells transfected at 6 h after release from growth arrest. However, Vero cells transfected at 6 h postrelease exhibited approximately 16-fold-greater CAT activity per microgram of nuclear plasmid DNA than cells transfected at 24 h postrelease (Table 1). Thus, the increase in CAT activity at 6 h in Fig. 2 was due not to greater efficiency in the uptake of transfected pWRICP0 DNA but to the enhanced ability of this DNA to be expressed at 6 h.

Response of other HSV-1 and heterologous viral promoters to the cellular activity. To assess the responsiveness of other HSV-1 promoter-CAT constructs to the cellular activity, CAT plasmids containing promoters representing each of the major kinetic classes of HSV genes were tested in Vero cells after growth arrest-release (Fig. 3). In these studies, cells were transfected only during the 6-h periods postrelease that corresponded to maximum (6 to 12 h) and minimum (24 to 30 h) expression of the cellular activity in assays with the ICP0 promoter (Fig. 2). The results of these tests show that only the reporter plasmids containing the immediate-early ICP0 and ICP4 promoters responded to the cellular activity in Vero cells (Fig. 3A). ICP0 promoter-driven CAT activity was sixfold greater at 6 h than at 24 h after release from growth arrest, and ICP4 promoter-driven activity was fourfold greater. No signif-

icant differences in CAT expression in cells transfected at 6 and 24 h postrelease were observed for plasmids driven by an early (UL9) or late (L42) promoter (Fig. 3A). Studies of plasmids in which CAT expression was driven by non-HSV viral promoters revealed that SV40 early promoter-driven CAT activities were similar at 6 and 24 h postrelease and that HCMV immediate-early promoter-CAT activity was 2.3-fold greater at 6 than at 24 h (Fig. 3B). Collectively, these results indicate that a cellular activity induced in Vero cells transfected 6 h after release from growth arrest is selective in its ability to enhance expression from at least two immediate-early promoters of HSV and (to a lesser extent) HCMV.

Growth properties of a neural cell line following release from growth arrest induced by isoleucine deprivation. In an effort to determine whether cells of neural origin also specify an enhancing activity for HSV-1 immediate-early promoters, we performed studies similar to those described above, using NB41A3 cells. Originally established from a murine neural neoplastic tumor (C1300), this clonal cell line (41A3) shares many properties of sympathetic neuronal cells, including the development of neurofilaments and neurotubules as well as the synthesis and storage of catecholamines (9, 68). Because of difficulties associated with the growth arrest-release of primary neurons, we chose to use NB41A3 cells because they have proven useful for studies of biochemical and metabolic processes associated with neurons (9, 51, 70, 76).

Prior to conducting CAT assays in growth-arrested-released NB41A3 cells, we examined the effect of the growth arrest-release procedure on NB41A3 cell growth and viability. The growth properties of these cells were examined by comparing the number of viable cells at regular intervals before, during, and after incubation in medium lacking isoleucine with the number of viable cells grown continuously in medium containing isoleucine (Fig. 4A). The results of similar tests of Vero cells are provided for comparison (Fig. 4B). Figure 4 shows that neither NB41A3 nor Vero cells increased in number during the 42-h incubation in medium lacking isoleucine. However, unlike Vero cells, which resumed cell division within 36 h after return to isoleucine-containing medium, NB41A3 cells remained growth arrested for the remainder of the test. Flow cytometric analysis of NB41A3 cells following isoleucine deprivation confirmed the aborted progression through the cell cycle after return to isoleucine-containing medium (data not shown). Because these cells failed to progress through the cell cycle, it was difficult to assess the stage of the cycle at which cells were arrested. Microscopically, these cells exhibited altered morphology (Fig. 5B) relative to cells that had not been subjected to growth arrest (Fig. 5A). Thus, the cells appeared enlarged and flattened and exhibited limited neurite outgrowth. Although they failed to resume cell division, 100% of the cells shown in Fig. 5B excluded trypan blue for up to

TABLE 1. Transfection efficiency of growth-arrested-released Vero cells

Time (h) of transfection after release from growth arrest	Cell no. (10 ⁶ ; avg $\pm$ SD) ^a	Total protein (mg; avg $\pm$ SD) ^b	Protein used in CAT assay (mg)	% Conversion ^c (avg $\pm$ SD)	Input plasmid DNA ^d (μ g) at time of:		% of input plasmid DNA in nuclei at time of harvest
					Transfection	Harvest (avg $\pm$ SD)	
6	2.4 $\pm$ 0.1	0.58 $\pm$ 0.03	0.2	14.1 $\pm$ 3.8	20	1.72 $\pm$ 0.32	8.6
12	4.8 $\pm$ 0.2	1.32 $\pm$ 0.03	0.2	2.6 $\pm$ 2.0	20	4.87 $\pm$ 0.26	24.3

^a Values are calculated averages of determinations in three 100-mm-diameter petri dishes each seeded with 2.5×10^6 cells and treated to achieve growth arrest-release as outlined in Materials and Methods.

^b Per culture, as determined by the method of Bradford (7).

^c See Materials and Methods.

^d See Materials and Methods.

FIG. 3. CAT expression in growth-arrested-released Vero cells transfected with reporter plasmids containing promoters representing the major HSV genes or non-HSV viral promoters. Plasmids containing the promoter/regulatory regions of HSV immediate-early (ICP0 and ICP4), early (UL9), and late (L42) genes (A) and SV40 late and HCMV immediate-early genes (B) were transfected at 6 and 24 h after addition of isoleucine-containing medium. Experiments were performed essentially as described in the legend to Fig. 2 except that cells were transfected only at the times of maximum (6 h) and minimum (24 h) ICP0 promoter-CAT activity. Experiments were repeated at least two times.

300 h after return to isoleucine-containing medium, demonstrating that they were viable. We conclude from these observations that isoleucine deprivation irreversibly growth-arrested NB41A3 cells but not Vero cells. Whether the cessation of cell division is a result of terminal differentiation is unclear.

Activation of the ICP0 promoter in NB41A3 cells following incubation in medium lacking isoleucine. Although NB41A3

FIG. 4. Effects of isoleucine deprivation on the growth of NB41A3 and Vero cells. NB41A3 (A) or Vero (B) cells (3×10^5 cells per 60-mm-diameter petri dish) were growth arrested as described in the legend to Fig. 2. After seeding, cells were harvested and counted at the indicated times before, during, and after incubation at 37°C in isoleucine-free medium. Each point represents the average number of cells in each of three dishes. Squares, cells incubated in isoleucine-free medium; diamonds, cells incubated continuously in isoleucine-containing medium.

cells ceased to divide following incubation in medium lacking isoleucine, we were interested in determining what effect growth arrest would have on expression of the CAT gene from pWRICP0 transfected at various times after return to medium containing isoleucine. The time course experiment presented in Fig. 6 was conducted in a manner identical to that described above for Vero cells (Fig. 2). The results of this test demonstrate that as for Vero cells returned to isoleucine-containing medium, a single peak of CAT activity was evident. However, NB41A3 cells reproducibly exhibited peak CAT expression

FIG. 5. Morphological changes in NB41A3 cells following incubation in isoleucine-free medium. Cells were photographed before (A) and after (B) 42 h of incubation in isoleucine-free medium. Magnification, $\times 63$. Arrows indicate neurite outgrowth.

when transfected at 2 h after return to isoleucine-containing medium (i.e., when CAT activity was measured from 2 to 8 h). Thus, peak ICP0 promoter-activating activity was detected at an earlier time after return to isoleucine-containing medium in NB41A3 than in Vero cells.

Responses of other HSV promoters to the cellular activity expressed by NB41A3 cells. Reporter CAT plasmids containing HSV promoters representing the major kinetic classes of HSV genes were screened for the ability to respond to the cellular activity in NB41A3 cells (Fig. 7). The two 6-h intervals chosen for testing represented the periods of maximal (2 to 8 h) and minimal (8 to 14 h) expression of the ICP0 promoter-activating activity (Fig. 6). The results of these tests demonstrated that the promoter-CAT plasmids containing the immediate-early promoters, ICP0, ICP4, and ICP22/47, responded to the cellular activity more strongly when transfected at 2 h than at 8 h after return to isoleucine-containing medium, although the absolute levels of CAT activity varied from promoter to promoter. The results of slot blot analysis of pWRICP0 DNA recovered from the nuclei of NB41A3 cells transfected at 2-h intervals after return to isoleucine-containing medium revealed no significant differences in the accumulation of plasmid DNA (data not shown). Promoter-CAT

plasmids pTK-CAT (TK, early) and pKXXBI-CAT (gB, delayed-early) as well as pLHp5CRE (LATs) were minimally responsive to the cellular activity expressed from 2 to 8 h postrelease in NB41A3 cells. Notably, the basal activity of the LAT promoter in NB41A3 cells was significantly higher than the basal activities of the gB and TK promoters (Fig. 7).

We conclude from these studies that the cellular activity in NB41A3 cells as in Vero cells is specific for immediate-early promoters. Moreover, the cellular activity enhanced CAT expression from the ICP0 and ICP4 promoters twice as efficiently in NB41A3 cells as in Vero cells.

In an effort to confirm the specificity of the cellular activity for immediate-early promoters, we examined the responses of the ICP0 and SV40 early promoters to the cellular activity in cells cotransfected with plasmids containing each promoter. The SV40 early promoter had been shown to be nonresponsive to the cellular activity in previous tests. Thus, NB41A3 cells were cotransfected at either 2 and at 8 h after release from growth arrest with ICP0-CAT (pWRICP0) and SV40-luciferase (pGL2-Control) reporter plasmids, and extracts were tested for both CAT and luciferase activities. To control for differences in the sensitivities of the two assays, cells were also cotransfected with ICP0-luciferase (pMCICP0) and SV40-

FIG. 6. CAT expression in NB41A3 cells transfected with pWRICP0 at various times after addition of isoleucine-containing medium to cells grown in isoleucine-free medium for 42 h. NB41A3 cells (4.0×10^6 cells per 100-mm-diameter petri dish) were growth arrested as described in the legend to Fig. 2. At various times after addition of isoleucine-containing medium, cells were transfected with 20 μ g of pWRICP0. Six hours after transfection, cells were harvested and processed for CAT activity. Percent conversion was used as the measure of promoter activity during the 6-h interval between the time of transfection and the time of harvest. This experiment was repeated three times.

CAT (pCAT-Control) reporter plasmids, and extracts were tested for both CAT and luciferase activities. In these experiments, the ICP0 promoter, whether linked to the luciferase (Fig. 8A) or CAT (Fig. 8B) reporter gene, directed expression

FIG. 7. CAT expression in NB41A3 isoleucine-deprived cells transfected 2 or 8 h after addition of isoleucine-containing medium. Experiments were performed essentially as described in the legend to Fig. 2 except that cells were transfected only at 2 or 8 h after addition of isoleucine-containing medium. CAT expression from HSV promoter-CAT plasmids representing immediate-early (ICP0, ICP4, and ICP22/47), early (TK), and delayed-early (gB) genes and LATs (LAT) is shown. Experiments were repeated twice.

TABLE 2. Induction of CAT expression from wild-type and mutant ICP0 promoter test plasmids in growth-arrested NB41A3 cells after addition of isoleucine-containing medium

Plasmid ^a	% Conversion after transfection at 2 and 8 h after release from growth arrest ^b (avg $\pm$ SD)		Fold induction ^c
	2 h	8 h	
pWRICP0	6.9 $\pm$ 0.1	0.8 $\pm$ 0.01	8.5
pStu/Sma	1.0 $\pm$ 0.1	0.8 $\pm$ 0.3	1.3
pSma	0.8 $\pm$ 0.01	0.60 $\pm$ 0.01	1.3
pWR574	5.3 $\pm$ 0.4	0.7 $\pm$ 0.03	7.5

^a Plasmids are depicted in Fig. 1.

^b See Materials and Methods.

^c Level of CAT activity at 2 h relative to the level of CAT activity at 8 h after release from growth arrest.

more efficiently when cells were transfected at 2 h than when they were transfected at 8 h after release from growth arrest. In contrast, the levels of expression of the SV40 early promoter within the same cells were similar at the two time points. In contrast to experiments mentioned above, in which approximately sixfold induction was routinely observed, induction of the ICP0 promoter was only approximately threefold in these cotransfection experiments. This reduction in expression is likely due to the fact that only 10 μ g of each reporter plasmid was used in cotransfection experiments, compared with 20 μ g in the aforementioned single-plasmid transfection experiments. Taken together, these results support the conclusion that immediate-early promoters are specifically responsive to a cellular activity induced in neural cells (NB41A3) after addition of isoleucine-containing medium to cells irreversibly growth arrested by isoleucine deprivation.

The region of the ICP0 immediate-early promoter responsive to the cellular activity. To identify elements within the promoter/regulatory region of pWRICP0 that respond to the enhancing activity expressed in NB41A3 cells, several mutant ICP0 promoter-CAT plasmids were constructed. Each plasmid contains a unique deletion mutation engineered in either the 5' upstream or 5' untranslated region of the ICP0 gene with respect to the transcriptional start site at +1 (45, 55) (Fig. 1B). The uninduced levels of expression of all of the mutants were comparable to that of the wild-type plasmid (pWRICP0), as determined by standard CAT assays in exponentially growing (i.e., non-growth-arrested) NB41A3 cells (data not shown). To assess the responsiveness of each mutant promoter to the cellular activity, NB41A3 cells were growth arrested in medium lacking isoleucine and returned to isoleucine-containing medium essentially as described above. Growth-arrested-released cells were then transfected at the times of maximum (2 h) and minimum (8 h) activation of the ICP0 promoter by the cellular activity (Fig. 6).

In this series of experiments, NB41A3 cells transfected with pSma and pStu/Sma at 2 h exhibited nearly the same levels of CAT activity as cells transfected at 8 h after release from growth arrest. In contrast, the levels of CAT activity in cells transfected with pWRICP0 and pWR574 at 2 h were 8.5- and 7.5-fold greater than cells transfected at 8 h after release from growth arrest (Table 2). The observation that nearly equal levels of CAT expression were induced from pWRICP0 and pWR574 when cells were transfected at 2 h after return to isoleucine-containing medium indicates that the *cis*-acting elements that respond to the cellular activity are located in the promoter and not the 5' untranslated region of the ICP0 gene. Moreover, the failure to detect CAT activity in cells trans-

fected with pSma or pStu/Sma at 2 h at levels above those obtained 8 h after return to isoleucine-containing medium indicates that *cis*-acting elements distinct from those responsible for basal activity are responsive to the cellular activity and that these responsive elements lie between nucleotides -550 and -129 upstream of the ICP0 transcriptional start site.

DISCUSSION

An understanding of the molecular mechanisms that underlie the establishment and reactivation of HSV-1 latency will require a better understanding of the interplay between cellular and viral factors that mediate the repression and activation of viral genes. Because immediate-early viral genes are required to orchestrate the expression of early and late genes, cellular factors that affect the expression of this class of immediate-early genes are of special significance to studies of latency. In this study, we have demonstrated that a cell cycle-regulated activity expressed in cells of neural and non-neural origin is able to enhance expression from immediate-early, but not early or late, HSV-1 promoters.

The identification of a cell cycle-regulated factor(s) able to complement an ICP0 null mutant (7134) for plaque formation on Vero cell monolayers was first described by Cai and Schaffer (12). These investigators demonstrated that impairment of the plating efficiency and synthesis of viral proteins characteristic of the ICP0 null mutant, 7134, on 24-h-old monolayers can be overcome by plating virus on synchronized Vero cell monolayers 8 h after cells have entered the cell cycle at some point in G₀/G₁. In contrast to 7134, the plating efficiency of the wild-type virus was independent of cell cycle status. A reasonable explanation for this observation is that upon entry into the post-G₀/G₁ stage of the cell cycle, Vero cells express factors that facilitate replication of HSV-1 genomes unable to express ICP0. Because the wild-type virus expresses ICP0, it is able to replicate efficiently in cells independent of the expression of such factors and, hence, independent of the stage of the cell cycle. Because we wish to isolate, clone, and characterize the cellular gene(s) whose protein products are able to substitute for ICP0, we have attempted to identify the class(es) of viral genes, and the subset of sequences within the regulatory regions of these genes, that respond to the cellular activity.

Time of peak expression of the cellular activity in Vero cells and NB41A3 cells. The observation that expression of an ICP0 promoter-CAT test plasmid was maximal when Vero cells were transfected 6 h after release from growth arrest induced by isoleucine deprivation is in excellent agreement with the results of Cai and Schaffer (12), in which the peak in 7134 plating efficiency occurred in Vero cell monolayers at 8 h after release from growth arrest. The 2-h difference in the times of peak promoter activity and peak plating efficiency is most likely due to differences in the times required for the two assays. Plating efficiency was measured during the 1-h window needed for virus adsorption (12), whereas CAT assays measured cumulative promoter activity in a 6-h time period. The 6-h period of maximum CAT activity in transient assays with Vero cells (6 to 12 h) includes the 1-h window of maximum plating efficiency (8 to 9 h).

Because the existence of a cellular activity able to enhance HSV-1 gene expression may be significant with regard to the mechanism of reactivation of HSV from latency, we attempted to determine whether cells of neural origin are capable of expressing a similar activity. For this purpose, we extended our experiments to include tests in the mouse neuroblastoma cell line, NB41A3. This clonal cell line and its parent have been

used to study several metabolic processes associated with cells of neural origin (i.e., nerve growth factor secretion, catecholamine metabolism, choline metabolism, and neurotoxicity) as well as to model certain interactions of HSV with neurons (2, 9, 18, 24, 51, 70, 78). Like Vero cells, NB41A3 cells deprived of isoleucine entered a state of growth arrest. However, upon return to isoleucine-containing medium, NB41A3 cells remained growth arrested for at least 6 days, whereas Vero cells resumed growth within 24 h. This observation demonstrates that isoleucine deprivation can induce physiological alterations in NB41A3 cells leading to irreversible growth arrest. Whether the suspended growth of NB41A3 cells reflects a state of terminal differentiation is not known. The morphological changes observed following return to isoleucine-containing medium (i.e., flattening of cells and limited neurite outgrowth) are, however, suggestive of a differentiated phenotype, in that they are similar to changes observed in neuroblastoma cells after treatment with sodium butyrate, an agent reported to induce differentiation of neuroblastoma cells, inhibit cell division, and increase the activity of enzymes involved in neurotransmitter metabolism (2, 24, 67, 68).

Like growth-arrested Vero cells, growth-arrested NB41A3 cells express an enhancing activity for the immediate-early promoters after addition of isoleucine. The pattern of ICP0-driven CAT expression over a 24-h period was similar to that observed in Vero cells, except that maximum promoter enhancing activity occurred in the period 2 to 8 h after return to isoleucine-containing medium. This period of peak CAT expression occurs 4 h earlier than it does in Vero cells, which may reflect differences in the overall kinetics of expression of the cellular activity in the two cell types. Alternatively, NB41A3 cells may simply be more efficient in the uptake and expression of plasmid DNA than Vero cells. The observed shift of the peak of CAT enhancing activity observed in Vero cells (high, 6 h; low, 10 h) to a time period 4 h earlier in NB41A3 cells (high, 2 h; low, 6 h) suggests that the overall kinetics of expression of the respective cellular activities, and not just DNA uptake, occur at an earlier time in NB41A3 cells than in Vero cells. Although the stimulatory activities in the two cell types exhibit the same immediate-early promoter specificity, it is not clear whether the activity expressed in NB41A3 cells represents the neural counterpart of the same activity observed in Vero cells. That the activity in NB41A3 cells is expressed at significant levels despite the inability of these cells to resume cell division after reintroduction of isoleucine to the growth medium is notable because it demonstrates that resumption of cell division is not a requirement for expression of the activity.

The cellular activity is specific for the promoters of immediate-early genes. Tests of the promoter specificity of the cellular activity in Vero and NB41A3 cells indicate that of the classes of HSV-1 promoters tested, only those of the immediate-early class responded to the cellular enhancing activity in the time window used to study the ICP0 promoter-CAT plasmid, pWRICP0. In addition, we observed that expression from an immediate-early promoter (IE1) of HCMV, a lymphotropic betaherpesvirus, was also enhanced, although only twofold in Vero cells. In contrast, the SV40 early promoter expressed alone or coexpressed with the ICP0 promoter was not enhanced (Fig. 3 and 8).

It should be noted that plasmid pLHp5CRE, used in these studies to assess the responsiveness of the promoter of the LATs to the cellular activity in NB41A3 cells, contains only 203 bp of its 5' regulatory sequence (+66 to -137), with respect to the start site of the primary LAT [23]). Several reports have demonstrated the requirement of *cis*-acting sequences upstream of this basal promoter for both neural cell-specific

FIG. 8. CAT expression and luciferase expression in NB41A3 isoleucine-deprived cells. Experiments were performed essentially as described in the legend to Fig. 2 except that cells were cotransfected only at 2 and 8 h with either 10 μ g of pMCICP0 and 10 μ g of pCAT-Control (A) or 10 μ g of pWRICP0 and 10 μ g of pGL-Control (B). CAT expression (solid bars) and luciferase expression (stippled bars) are shown.

activity and induced activity in neural cells (6, 7, 29, 81). In light of these findings, conclusions drawn concerning the lack of inducibility of the LAT promoter to the cellular activity (Fig. 7) would be premature.

The results presented in this report demonstrate that (i) a cellular activity expressed in cells of neural and nonneural origin is promoter specific, (ii) this activity may function as a specific enhancer of herpesvirus immediate-early gene expression during productive infection and reactivation from latency, and (iii) the activities observed in Vero and NB41A3 cells may represent cell-type-specific homologs of the same activity.

cis-acting response elements in the promoters of HSV-1

immediate-early genes. We have shown that deletion of sequences -550 to -129 of the ICP0 promoter/regulatory region render the ICP0 promoter unresponsive to the cellular activity in NB41A3 cells (Table 2). Contained within this region of the ICP0 promoter are *cis*-acting response elements or binding sites for several previously identified cellular activators as well as potential *cis* elements for other possible regulators of gene expression (Fig. 1). Deletion of this region effectively removes all but one of the overlapping octamer/TAATGARAT motifs and all of the single octamer and TAATGARAT consensus motifs (Fig. 1B), potentially impairing the ability of plasmid pSma or pStu/Sma to respond to activation by the Oct-1/VP16 complex (10, 31, 45, 52). In the absence of VP16, however, Oct-1 does not appear to activate transcription of most polymerase II-responsive promoters that contain TATAA elements (66, 74). Thus, it is unlikely that Oct-1 alone is responsible for the immediate-early promoter-enhancing activity observed in NB41A3 cells, in which no VP16 is present. The possibility remains, however, that (i) a cellular factor expressed in NB41A3 cells can enhance gene expression in combination with Oct-1 or (ii) octamer-binding proteins other than Oct-1 can function as transcriptional activators in the absence of VP16. Indeed, He et al. have described a large family of POU domain regulatory proteins in mammalian brain tissue that are expressed transiently during development of the nervous system, many of which have binding affinity for the Oct-1 binding motif (35).

Potential role of the cellular activity in the life cycle of HSV-1. The demonstration of a cellular activity able to activate immediate-early promoters in growth-arrested-released neural cells suggests the possibility that this activity functions in the reactivation of HSV from latency in neurons following stimulation by stress-related factors. Isoleucine deprivation causes NB41A3 cells to enter a state of growth arrest that may have features similar to the growth-arrested state of differentiated neurons. Addition of isoleucine to growth-arrested NB41A3 cells may trigger an increase in metabolic activity similar to that which occurs in stimulated neurons (14). Changes in the microenvironment associated with neuronal stimulation include increases in second messenger molecules, such as cyclic AMP (cAMP), cGMP, and protein kinase C, among others (1, 13, 44, 76). Treatment of neuronal cells in culture with a variety of these second messenger molecules as well as the stimulation of neuron-specific receptors have been shown to elicit transient expression of certain transcription factors (e.g., c-Fos, c-Jun, and Oct-1) able to regulate gene expression in such diverse processes as cellular differentiation and mitogenesis (16, 32, 33, 50, 51). As in the case of growth-arrested-released NB41A3 cells, latently infected neurons may also express an activity that enhances immediate-early gene expression. Expression of immediate-early genes is a prerequisite for reactivation of HSV from latency since it constitutes a resumption of the program of gene expression characteristic of productive infection. Evidence to support the notion that latent viral gene expression is regulated by cellular factors is available from *in vitro* models of HSV latency in which reactivation has been shown to occur in response to the activation of second messenger pathways (64, 71). Moreover, Wheatley et al. have used immortalized sensory neurons to show that agents that raise intracellular levels of cAMP also increase expression of the ICP0 gene as well as the permissivity of these cells for HSV infection (77), and Leib et al. have demonstrated that the reactivation of HSV-1 from dissociated latently infected murine trigeminal ganglia is accelerated by cAMP analogs or adenylate cyclase activators (42). Although the cellular pathway leading to activation of immediate-early genes in NB41A3 cells is unknown, having

identified a region of the ICP0 promoter that responds to the cellular activity, we should now be able to fine map individual response elements, isolate the effector proteins that bind to these elements, and clone the genes that encode the effector proteins.

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