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Herpes Simplex Virus Gene Expression in Neurons: Viral DNA Synthesis Is a Critical Regulatory Event in the Branch Point between the Lytic and Latent Pathways

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Herpes simplex virus establishes a latent infection in peripheral neurons. We examined viral gene expression in rat peripheral neurons in vitro and determined that viral gene expression is attenuated and delayed in these neurons compared with that in Vero cells. In addition, using pharmacologic and genetic blocks to viral DNA synthesis, we found that viral α and β gene expression was upregulated by viral DNA synthesis. Although maximal gene expression in neurons requires viral DNA synthetic activity, activation of viral gene expression was seen even in the presence of herpes simplex virus DNA polymerase inhibitors, but not in the absence of the origin-binding protein. Initiation of viral DNA synthesis is apparently a key regulatory event in the balance between the lytic and latent pathways in peripheral neurons.

Alphaherpesviruses, such as herpes simplex virus types 1 and 2 (HSV-1 and HSV-2, respectively) establish a latent infection in neurons of the peripheral nervous system. The mechanism of establishment of a latent infection remains poorly understood. In vivo, HSV infects the neuron at its nerve terminal, travels up the axon, and, upon reaching the cell body, pursues one of two pathways: it either enters a lytic cycle and produces infectious progeny, or it establishes a latent infection in which the viral genome remains transcriptionally quiescent in an apparently healthy neuron (for reviews, see references 11 and 34). Under certain conditions, such as axotomy, UV light, hyperthermia, elevations in cyclic AMP (cAMP) levels, or nerve growth factor deprivation, HSV can be stimulated to reactivate from latency and can enter a lytic cycle (1, 2, 27, 31, 32, 35, 37, 38, 39). The ability of HSV to enter into a latent state in a sensory ganglion and then repeatedly reactivate and spread to other hosts is a primary reason that HSV is such a prevalent human pathogen.

The fact that the virus can enter into the latent state only in neurons suggests that virus-cell interactions are fundamentally different in neurons compared with those in other cell types that the virus infects. Several differences have been noted between infection of neurons and nonneuronal cells, and these differences may provide important clues to understanding how a latent infection is established. First, certain HSV mutants with lesions in genes that encode viral DNA replication enzymes (e.g., ribonucleotide reductase and thymidine kinase) and that replicate to near wild-type levels in dividing cells, do not replicate in neurons (6, 20, 23, 36). This is likely to be due to limiting concentrations of deoxyribonucleoside triphosphates in neurons, which cause a greater dependence on virus-encoded enzymes to provide the precursors for viral DNA synthesis. Second, neurons are relatively nonpermissive for HSV; several studies suggest that this is a result of attenuated α gene expression (19, 20, 22). Finally, peripheral neurons do not exhibit a virion host shutoff effect after infection with HSV (25).

A thorough characterization of viral gene expression and DNA replication in neurons has not been done. However, viral gene expression has been well characterized as an ordered cascade in continuous cell lines such as Vero cells (14a–18, 33). α gene expression occurs first and does not require expression of other viral genes. Several of the α gene products then transactivate the expression of β genes, whose products are required for viral DNA synthesis. γ*1* genes are expressed at a low level prior to viral DNA replication, but maximal expression of γ*1* genes and expression of γ*2* genes require viral DNA replication. Blocking of viral DNA replication results in a lack of expression of γ*2* gene products but may lead to a slight increase in α and β gene products (14, 29).

Several observations suggest that viral gene expression may be regulated differently in peripheral neurons and, specifically, that viral DNA replication may have a critical regulatory role in this process. Inhibition of viral DNA synthesis with acyclovir (ACV) during infection of dissociated sympathetic neurons from rat superior cervical ganglia (SCG) allows the establishment of latency at high multiplicities of infection (MOI; e.g., MOI of 5) (38). It was speculated that ACV acted by inhibiting viral gene expression and thus prevented the onset of a lytic infection in these cultures. In addition, in studies with a viral mutant (*hr*R3) which carries a *lacZ* expression cassette inserted into the gene (*UL39* or β*6*) encoding the large subunit of viral ribonucleotide reductase (ICP6), *lacZ* expression was reduced in neurons after infection with *hr*R3 virus in the presence of ACV (6). Since *lacZ* expression is driven by a β gene promoter, the data suggested that DNA synthesis is involved in the regulation of viral β gene expression in neurons.

Further support for the idea that viral DNA synthesis regulates viral gene expression in neurons was provided by Kosz-Vnenchak et al., who conducted studies of viral gene expression in trigeminal ganglia in vivo by in situ hybridization (20, 21). They observed that a virus with a deletion mutation in the gene encoding thymidine kinase (*tk*) displays dramatically re-

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duced expression of both α and β genes in trigeminal ganglion neurons during an acute infection in vivo. In addition, during reactivation of explanted trigeminal ganglion neurons latently infected with wild-type virus, decreased α and β gene transcripts were observed in the presence of inhibitors of viral DNA synthesis. Kosz-Vnenchak et al. proposed a model to explain their results, whereby infection of the neuron is followed by very low levels of α and β gene expression. After β gene products are synthesized, a very small amount of viral DNA is replicated, and this serves as a signal to increase viral gene expression. They offered several possible reasons to explain this effect. Viral DNA synthesis may simply increase the genome copy number, which could then titrate out any putative DNA-binding factors that are inhibitors of viral gene expression. Alternatively, they proposed that viral DNA synthesis may physically alter the viral DNA template; this would, in some way, serve to increase transcription of α and β genes, which, in turn, would promote the lytic cycle. Regardless, according to their model, if DNA synthesis does not occur, a latent infection is established.

To further understand the relationship between viral DNA replication and α and β gene expression in neurons, we analyzed viral gene expression in HSV-infected rat SCG neurons by using an experimental paradigm derived from the previously reported in vitro model of HSV latency (37). We found that the duration of the lytic cycle is longer in SCG neurons than in Vero cells. In addition, inhibition of viral DNA synthesis with ACV dramatically reduced expression of α and β genes in these neurons but not that in Vero cells. Our data provide direct evidence that viral gene expression is regulated differently in neurons compared with that in nonneuronal cells and suggest that initiation of viral DNA synthesis is one of the key regulatory events that determine whether HSV infection of neurons follows a lytic or latent course.

MATERIALS AND METHODS

Culture of sympathetic neurons. Primary dissociated cultures of sympathetic neurons were prepared from the superior cervical ganglia of embryonic rats on day 20 (E-20) (Harlan Sprague-Dawley, Indianapolis, Ind.). Cells were preplated as described previously (9). In brief, superior cervical ganglia were dissected from E-20 rats. Ganglia were enzymatically digested for 35 min with collagenase (Sigma, St. Louis, Mo.), followed by a 35-min digestion with trypsin (Sigma). The cells were mechanically dissociated through a flame-polished Pasteur pipette. To reduce nonneuronal cells to less than 2% of the total cell population, cells were preplated in S medium (10% fetal bovine serum; Life Technologies, Grand Island, N.Y.) or in minimal essential medium (Sigma) on a 100-mm-diameter petri dish for 90 min. Nonadherent neurons were washed off with S medium and collected in silanized, conical glass tubes by centrifugation at 500 × *g* for 10 min. Cells were resuspended in S medium plus nerve growth factor (50 ng/ml, 2.5S; prepared by the method of Bocchini and Angeletti [3]) and FdU (20 μM uridine, 20 μM fluorodeoxyuridine [Sigma]) (AM-50) and plated in a small drop of AM-50 for 4 h at a density of 2,500 to 5,500 neurons per well on 24-well, collagen-coated plates (Falcon, Lincoln Park, N.J.). Thereafter, the cells were fed 400 μl of AM-50 per well. Cells were fed every third day. For the first 7 days, the cells were fed with AM-50. Thereafter, the cells were fed neuronal medium lacking FdU (F-50). Cultures were incubated at 35°C with 5% CO_2.

Culture of Vero cells. Vero cells (African green monkey kidney cells) were obtained from American Type Culture Collection. Cells were harvested with 0.05% trypsin–0.02% EDTA in phosphate-buffered saline (PBS), digested for 5 min, and counted under a hemacytometer. Prior to infection, they were plated in 24-well tissue culture plates at a density of approximately 100,000 cells per well in Vero medium (Dulbecco's minimal essential medium plus 10% fetal bovine serum and 1% penicillin [100 μg/ml]–streptomycin [100 U/ml] [P/S; Life Technologies]). Cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Viruses. Wild-type HSV-1 (KOS strain) was generously provided by Mark Challberg (National Institutes of Health, Bethesda, Md.). The *hr*R3 and *hr*94 HSV-1 mutants were generously provided by Sandra Weller (University of Connecticut, Farmington, Conn.) and have been previously described (12, 24). UL26icp6gluc mutant virus was generously provided by Paul Hippenmeir (Monsanto Corp., St. Louis, Mo.) and is identical in phenotype to another *UL26* null mutant virus described previously (10). The titer of *hr*R3 was determined on Vero cells, that of *hr*94 was determined on S22 cells (a complementing Vero cell line), and that of UL26icp6gluc was determined on BHK/UL26 cells.

Growth and purification of viruses. Viruses were grown on semiconfluent monolayers of Vero cells in T-225 tissue culture flasks (Corning Corp., Corning, N.Y.). Cells were inoculated with 0.001 PFU of virus per cell. The virus was allowed to propagate for 3 to 4 days in the flask or until the monolayer was completely destroyed. Medium and cells from infected flasks were pooled into sterile centrifuge bottles and spun at 3,000 × *g* for 10 min (spin 1). The supernatant was poured into new bottles and spun for 2 h at 9,000 × *g* (spin 2). The pellet from spin 1 was resuspended in 1 to 2 ml of sterile minimal essential medium, freeze-thawed three times, and then spun at 5,000 × *g* for 10 min (spin 3). The supernatant from spin 3 was used to suspend the viral pellet from spin 2 (suspension 1). Suspension 1 was layered over 0.8 ml of 25% sucrose–PBS (Ca^{2+} Mg^{2+} free) in a 3-ml ultracentrifuge tube. The tube was centrifuged at 55,000 × *g* for 90 min. After this, the supernatant was aspirated and the pellet was resuspended in 200 to 500 μl of S medium by being vortexed for 10 s (suspension 2). The titer of suspension 2 was determined by plaque assay on cell monolayers of the appropriate cell line (Vero cells for the KOS strain) in the presence of 0.2% pooled human gamma globulin (Armour Pharmaceutical Company, Kankakee, Ill.).

Infection of neurons and Vero cells. Neuronal and Vero cell cultures were prepared as described above. Prior to infection, the number of neurons and Vero cells per well was determined by cell counts. To facilitate counting of neurons, grids were scored with a razor blade into the bottoms of 24-well plates from a single dissection. Cell numbers per well were determined for four randomly selected wells. A mean number of cells per well was determined. Standard deviations were routinely less than 8% of the mean. The number of Vero cells per well was determined by treatment of four randomly selected wells with 200 μl of 0.05% trypsin–0.2% EDTA in PBS for 5 min at 37°C. The cells were resuspended, and a 10-μl aliquot from each well was counted on a hemacytometer. A mean number of cells was determined for a single plating, and the standard deviations were routinely less than 5%.

Infection of neurons was performed 11 to 14 days after dissection. Medium overlying the cultures was reduced to a volume of 200 μl, and the appropriate amount of virus was brought up in a volume of 10 μl of cognate media and added to the cultures to minimize the effects of physical perturbation of the cells. Inoculum was spread evenly over the cultures by gentle rocking at low speed during incubation at 37°C. After 1 h, the infectious medium was removed and the cells were fed with prewarmed F-50 or S medium in the presence of 0.2% pooled human gamma globulin (Armour Pharmaceutical Company).

Single-step growth analysis of HSV in SCG neurons or Vero cells. After infection, the cells were washed three times with their respective medium to remove excess virus. The cell cultures were then maintained in their respective medium in the absence of pooled human gamma globulin. At various times after infection, the medium overlying the cells was removed and replenished with fresh medium. To detect and quantitate viral progeny, the titer of progeny in 50 μl of conditioned medium was determined on Vero cells.

RT-PCR. One of the problems encountered with studying the expression of any gene (cellular or viral) either in vivo or in vitro is that many of the current techniques (Northern [RNA] blots and RNA protection assays) require relatively large numbers of cells to obtain semiquantitative data. Since large, pure populations of neurons are difficult to procure, the effort required to gather enough cells to analyze many transcripts by Northern blotting or RNA protection assay is not a reasonable option. In situ hybridization permits examination of the expression of a gene in an individual cell, but this assay is not quantitative and the same tissue cannot be probed for multiple genes. The reverse transcriptase PCR (RT-PCR) methodology has been used extensively by our laboratory to identify genes that are induced during programmed cell death in neurons (8, 9). We used this method to characterize the cascade of HSV-1 gene expression in neurons at various times after infection. Cells were infected, and at various times after infection, the cells were lysed, total RNA was harvested, and polyadenylated RNA was selected with an oligo(dT) column. The mRNA was then converted to cDNA by reverse transcription with random hexamers as primers. PCR with specific primers was then performed on an aliquot of each cDNA sample. Thus, the time courses of expression of several genes were determined with identical samples.

RNA isolation and cDNA preparation. Total mRNA was prepared by the acid guanidinium isothiocyanate-phenol technique from wells containing a predetermined number of cells. The cells were lysed in 400 μl of lysate buffer, and one-half of the lysate was enriched for mRNA by oligo(dT) affinity column chromatography (Pharmacia-QuickPrep Micro mRNA purification kit). The mRNA was concentrated by coprecipitation with glycogen. The mRNA was resuspended in 20 μl of RNase-free H_2O. The mRNA was reverse transcribed by using the Moloney murine leukemia virus RT (Superscript; Bethesda Research Laboratories, Inc.) and random hexamers as primers (Bethesda Research Laboratories, Inc.). The final reaction mixture consisted of 200 U of Superscript, 500 μM deoxynucleoside triphosphates (dNTPs), and 40 U of RNasin (Promega). This solution was incubated at 20°C for 10 min and then 42°C for 50 min, and the Superscript was then inactivated by being heated to 95°C for 2 min. The resultant cDNA was sufficient for 15 to 35 PCR analyses. Each sample was brought up in a volume of distilled water so that 10 μl of water contained the cDNA equivalent of the mRNA from 100 cells.

PCR amplification. PCR mixtures were prepared on ice and contained 50 μM dCTP; 100 μM dGTP, dATP, and dTTP; 20 μCi of [α-^{32}P]dCTP (3,000 Ci/

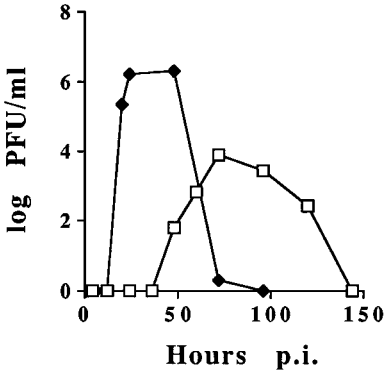


FIG. 1. Time course of detection of viral progeny in cultured SCG neurons (□) and Vero cells (◆). SCG neurons were plated at a density of 3,000 cells per well. Cells were infected at an MOI of 5 for 1 h and washed three times with medium, and then culture continued in the presence of F-50 medium for the duration of the experiment. Vero cells were plated at a density of 140,000 cells per well. Cells were infected at an MOI of 1 for 1 h and washed three times with medium, and then culture continued in the presence of S medium for the duration of the experiment. Medium was removed from the cultures at the indicated time points, and the titer of progeny virus was determined by plaque assay. Each datum point represents the mean of four individual samples. p.i., postinfection.

mmol); 1.5 mM $MgCl_2$; 5× *Taq* reaction buffer (Promega); 1 μM (each) primer; 1 U of *Taq* polymerase (Promega); and 1/50 of the cDNA synthesized from the previous step in a final volume of 50 μl. The reaction mixtures were prepared in 500-μl microcentrifuge tubes, covered with a drop of mineral oil, and subjected to various numbers of cycles of PCR amplification. Typical reaction conditions were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. The following primer sets were used for the various messages: tyrosine hydroxylase (*toh*), 5′ TTC.AGA .AAG.GCC.CTC.TCA.GA, 3′ CCG.CTG.CTG.CTG.CAG.CT; α*0*, 5′ ACG.GAC .ACG.GAA.CTG.TTC.GAG.A, 3′ TGT.TGC.GCA.ATT.GCA.TCC.AGG.T; α*27*, 5′ TTC.TCC.AGT.GCT.ACC.TGA.AG, 3′ TCA.ACT.CGC.AGA.CAC.GAC.T GG.A; β*6* 5′ GCC.ATC.GAA.AAC.TAC.GTG.CGA, 3′ ACT.CAC.AGA.TCG.T TG.ACG.AC; γ*B*, 5′ ATT.CTC.CTC.CGA.CGC.CAT.ATC, 3′ AAA.GCC.CCC .ATT.GGC.CTG.GTA; and γ*C*, 5′ TCT.GTC.TCG.TCT.CCT.CTT.CTC, 3′ TTA .TTA.TTT.TGG.GTC.CGT.GCC. To confirm that the primer sets amplified the appropriate messages, PCR products were run on an acrylamide gel with standard molecular weight markers. Viral gene primer sets were run in reaction mixtures containing cDNAs from uninfected cells to confirm that cellular homologs were not being amplified. After amplification, the cDNAs were separated by polyacrylamide gel electrophoresis and visualized by autoradiography of the dried gels on a PhosphorImager (Molecular Dynamics). The intensity of individual bands was determined by the PhosphorImager and normalized for the number of cells per sample, as determined by cell counts prior to infection. Normalization was verified in neurons over the first 24 h of infection by RT-PCR for *toh* message levels in each sample. The number of PCR cycles required to achieve a linear relationship between input cDNA and intensity of gene expression per number of cells was determined by separating a 50-μl reaction mixture for each gene into three 14-μl aliquots. The aliquots were subjected to various numbers of PCR cycles to document the range of cycle numbers over which a linear relationship between cDNA and signal intensity was achieved.

β-Galactosidase and β-glucuronidase histochemistry. Cells were washed twice with PBS and fixed for 5 min in a solution of 2% formaldehyde–0.4% gluteraldehyde (Fisher Scientific) in PBS. Cells were then rinsed twice with PBS and histologically stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside or 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (Sigma) overnight (30). Both cells that were positive for staining and cells that were negative for staining were counted under a microscope.

[^{3}H]thymidine labeling. The components of the thymidine labeling medium for neurons were identical to those described previously with the inclusion of [^{3}H]thymidine. Fifty microcuries of [^{3}H]thymidine (Amersham, Arlington Heights, Ill.) per well was added 24 h prior to infection, and the [^{3}H]thymidine was maintained in the medium until the cultures were harvested at 4, 6, 8, 12, or 30 h postinfection. Labeling medium was removed, and cells were washed twice with 0.5 ml of PBS. Cells were lysed in 200 μl of 10 mM Tris-HCl–1 mM EDTA–1% sodium dodecyl sulfate (SDS [pH 7.4]). Lysate was triturated five times in the well of the dish. Fifty microliters of each sample was precipitated in 2 ml of 10% trichloroacetic acid (TCA) (Sigma) for 8 h at 4°C. Precipitated samples were filtered over a 0.2-μm-pore-diameter nitrocellulose membrane (Midwest Scientific, Valley Park, Mo.) under a vacuum and washed with another 4 ml of 10% TCA solution. Samples were dried overnight and placed in scintillation vials with 7 ml of scintillation fluid (Beckman, Fullerton, Calif.) and

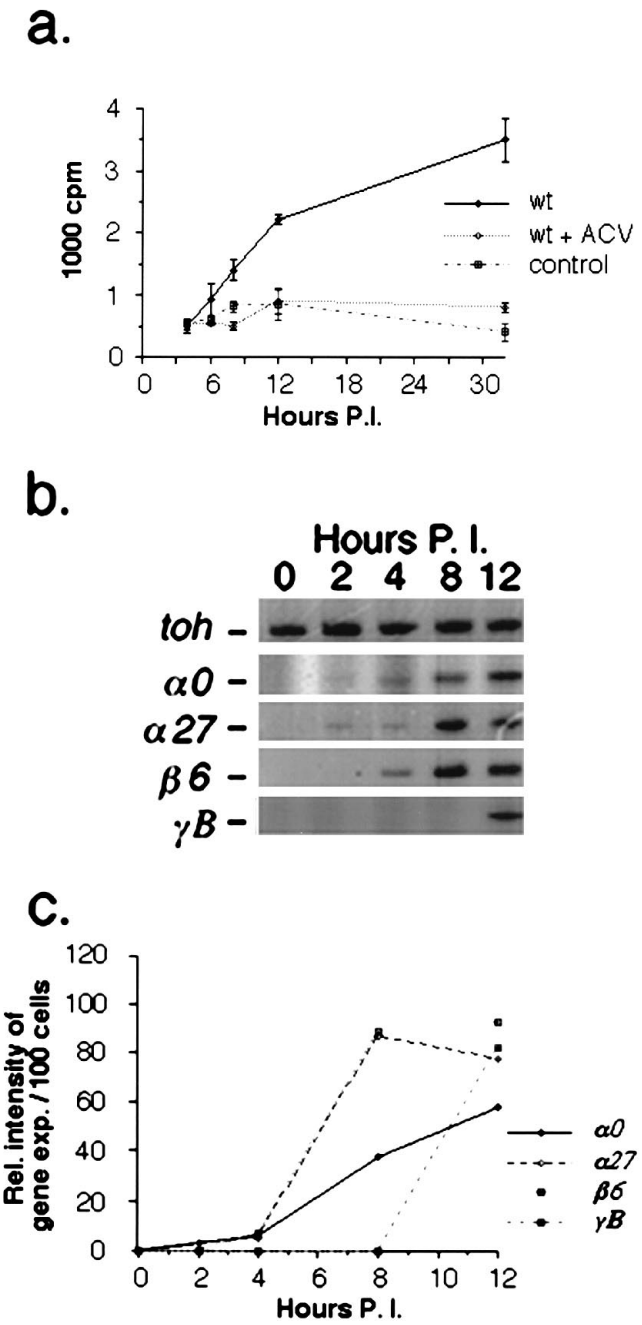


FIG. 2. Time course of viral DNA synthesis and gene expression in HSV-infected SCG neurons. (a) Viral DNA synthesis in HSV-infected SCG neurons. SCG neurons were plated at a density of 3,000 per well. Eleven days after plating, the cells were labeled in the presence of [^{3}H]thymidine as described in Materials and Methods. Neurons were infected with wild-type (wt) HSV-1 (KOS) at an MOI of 20, harvested at various times postinfection (P.I.), and assayed for the amount of TCA-precipitable tritium. (b) Viral gene expression in HSV-infected neurons. Neurons were plated at a density of 3,500 per well. Eleven days after plating, the cells were infected with wild-type HSV-1 (MOI, 5). Cells were harvested at the indicated times after infection for mRNA. RT-PCR was performed for a cellular transcript (*toh*) and four viral transcripts (α*0*, α*27*, β*6* and γ*B*). Products were run on a 10% polyacrylamide gel, dried, and exposed to a PhosphorImaging plate. This experiment was repeated twice with similar results. (c) Graphic representation of the time course of viral gene expression (exp.) in HSV-infected neurons. Intensity as given on the *y* axis is a measure of the relative (rel.) intensity of the bands compared with that of a background region of the gel without bands present.

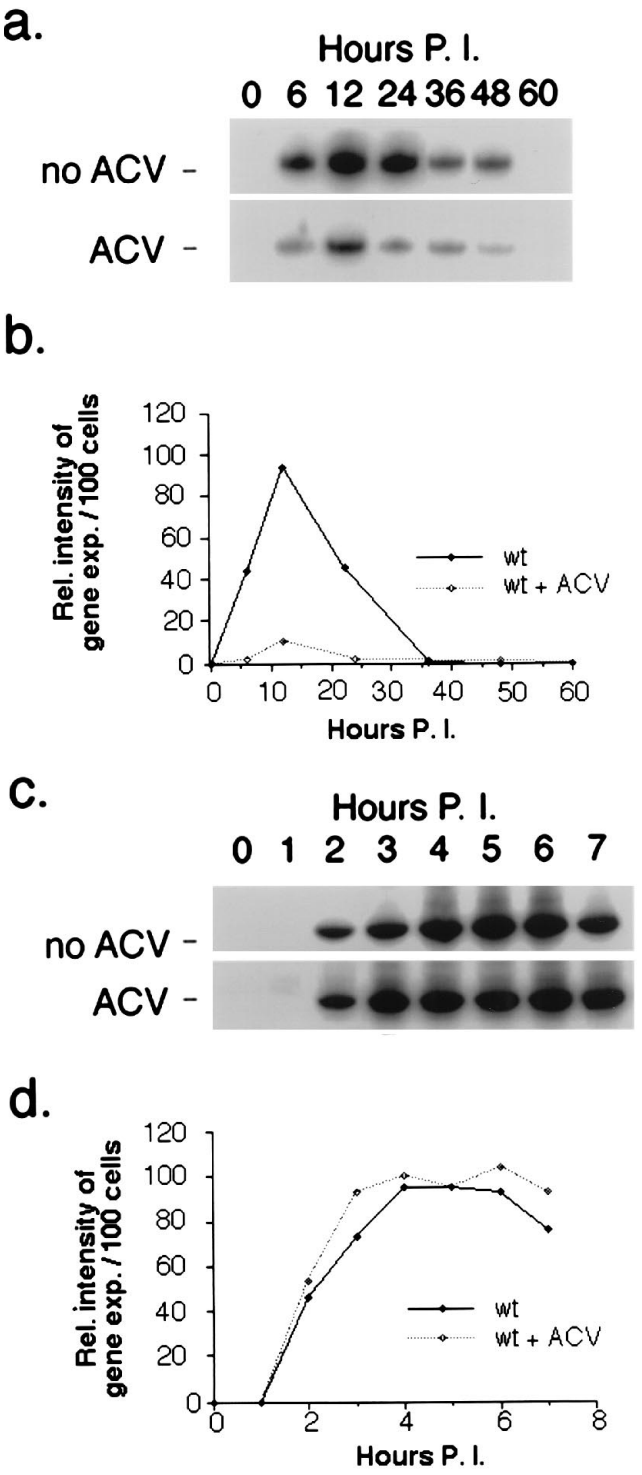


FIG. 3. Analysis of the effect of ACV on the steady-state levels of α*0* transcripts in HSV-infected neurons and Vero cells. (a) PhosphorImage of RT-PCR products demonstrating α*0* expression in HSV-infected neurons. Neurons were plated at a density of 5,500 per well. Eleven days after plating, the cells were infected with wild-type HSV-1 (MOI, 5) in the presence or absence of ACV. Cells were harvested at the indicated times after infection for mRNA. RT-PCR was performed to detect the α*0* transcript. Reaction samples were run on a 10% polyacrylamide gel, dried, and exposed to a PhosphorImaging plate. This experiment was repeated three times with similar results. (b) Graphic display of α*0* expression in HSV-infected neurons. Data are plotted as the relative (rel.) intensity of expression (exp.) compared with the signal of the gel background over time. wt, wild type. (c) PhosphorImage of RT-PCR products demonstrating α*0* expression in HSV-infected Vero cells. Vero cells were plated overnight at a density of 50,000 per well. The cells were infected with wild-type HSV-1 (MOI, 5) in the presence or absence of ACV. Cells were harvested at the indicated times after infection for mRNA. RT-PCR was performed to examine α*0* expression. Reaction samples were run on a 10% polyacrylamide gel, dried, and exposed to a PhosphorImaging plate. This experiment was repeated twice with similar results. (d) Graphic display of α*0* expression in HSV-infected Vero cells. Data are plotted as the intensity of expression compared with the gel background signal.

allowed to equilibrate overnight. Samples were counted for 1 min in a Beckman 1801 scintillation counter, and the values were subtracted from the background counts generated in culture dishes lacking cells.

RESULTS

Single-step growth of HSV in rat SCG neurons and Vero cells. Experiments were performed to determine the length of time required for HSV-1 to replicate in neurons compared with that in Vero cells. Rat SCG neurons and Vero cells were cultured as described in Materials and Methods and infected with HSV-1 (KOS strain) at an MOI of 5 (neurons) or 1 (Vero cell). At various times after infection, medium from the cultures was removed for detection of virus by plaque assay, the sensitivity of which was determined to be 12 PFU/ml (data not shown). We detected no viral progeny (i.e., fewer than 12 PFU/ml) in cultured neurons until 48 h after infection, whereas viral progeny were detected as early as 16 h after the infection of Vero cells (Fig. 1). Use of higher viral inocula on both cell types resulted in a similar time course of virus production (data not shown). Thus, the HSV-1 replicate cycle was at least three times longer in rat SCG neurons than in Vero cells, which suggested that the cascade of viral gene expression was delayed in these cells.

Viral DNA replication in HSV-infected neurons. To characterize the HSV replication cycle in neurons, we measured viral DNA synthesis in HSV-infected neurons. Neurons were cultured in the presence of [^{3}H]thymidine (50 μCi per well) beginning 24 h prior to infection. Twelve hours prior to infection, one-third of the cultures were treated with ACV (50 μM). Two thirds of the SCG neuron cultures were infected with HSV-1 (MOI of 20) and harvested at various times after infection by lysis in SDS-containing buffer, and the amount of TCA-precipitable tritium was determined. Uninfected cells incorporated very little tritium into TCA-precipitable material. Infected neurons displayed significant increases in the incorporation of [^{3}H]thymidine beginning 8 to 12 h after infection and for the duration of the experiment. Incorporation was reduced to mock-infected levels by treatment of the infected cells with ACV (Fig. 2a).

Analysis of HSV-1 gene expression in neurons. Previous studies with the HSV-1 mutant *hr*R3, which has the promoter of a β gene (β*6* or *UL39*) controlling *lacZ*, demonstrated that β-galactosidase activity peaked 36 h after infection in rat SCG neurons, whereas β-galactosidase activity peaked 6 to 8 h after infection in Vero cells (6, 6a). This suggested that the cascade of viral gene expression required for the production of viral progeny is significantly altered in neurons. To directly analyze viral gene expression in neurons, RT-PCR was performed with mRNA from infected neurons.

Cultures of SCG neurons or Vero cells were infected with HSV (KOS strain) at an MOI of 5 for 1 h and, after being washed twice with F-50 or Vero medium, the cells were maintained in their respective medium containing pooled human gamma globulin. RNA was harvested and RT-PCR was performed 2, 4, 8, or 12 h after infection (Fig. 2b and c). Levels of the transcript from the neuron-specific tyrosine hydroxylase

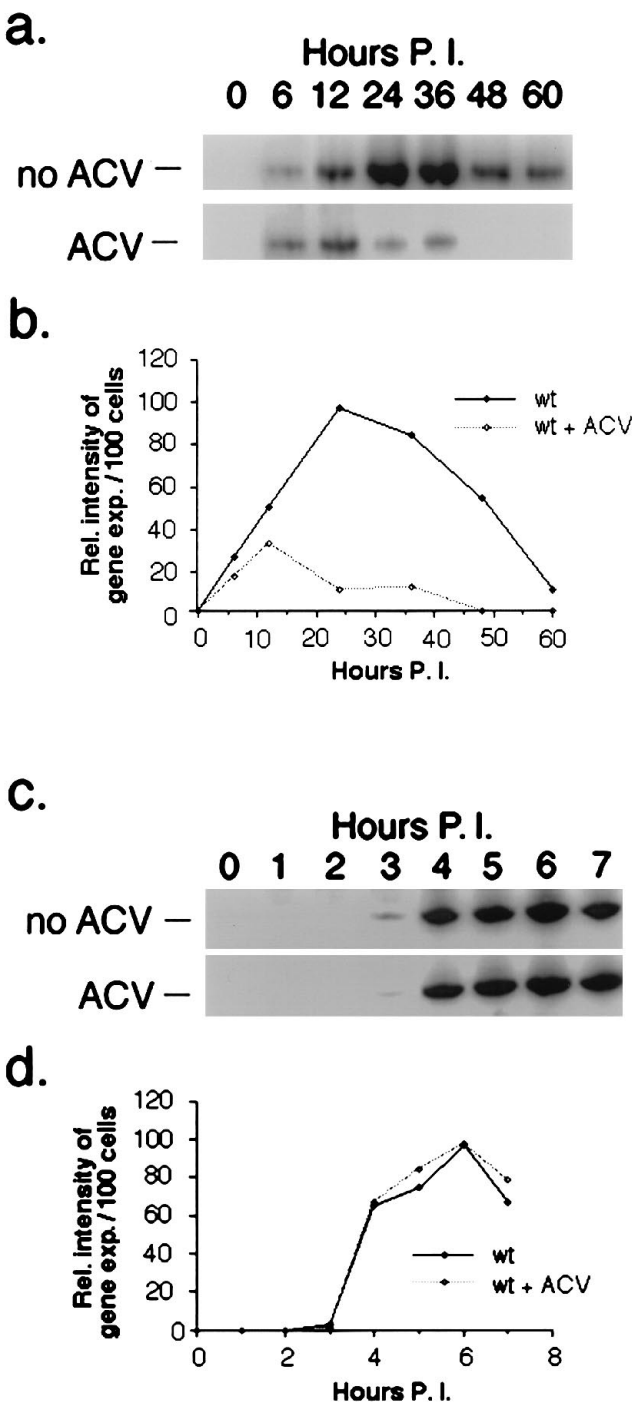


FIG. 4. Analysis of the effect of ACV on the steady-state levels of β*6* transcripts in HSV-infected neurons and Vero cells. (a) PhosphorImage of RT-PCR products demonstrating β*6* expression in HSV-infected neurons. Neurons were plated at a density of 5,500 per well. Eleven days after plating, the cells were infected with wild-type (wt) HSV-1 (MOI, 5) in the presence or absence of ACV. Cells were harvested at the indicated times postinfection (P.I.) for mRNA. RT-PCR was performed to examine β*6* expression. Reaction samples were run on a 10% polyacrylamide gel, dried, and exposed to a PhosphorImaging plate. This experiment was repeated twice with similar results. (b) Graphic display of β*6* expression in HSV-infected neurons. ACV mediates a 10-fold reduction in β*6* expression. β*6* expression in neurons is presented in graph form. Data are plotted as the relative (rel.) intensity of expression (exp.) compared with the background signal. (c) PhosphorImage of RT-PCR products demonstrating β*6* expression in HSV-infected Vero cells. Vero cells were plated overnight at a density of 50,000 per well. The cells were infected with wild-type HSV-1 (MOI, 5) in the presence or absence of ACV. Cells were harvested at the indicated times after infection for mRNA. RT-PCR was performed to examine β*6* expression. Reaction samples were run on a 10% polyacrylamide gel, dried, and exposed to a PhosphorImaging plate. This experiment was repeated twice with similar results. (d) Graphic display of β*6* expression in HSV-infected Vero cells. β*6* expression in Vero cells is presented in graph form. Data are plotted as the relative intensity of expression compared with the background signal.

gene (*toh*) were measured to evaluate the effect of infection on cellular transcription and as a methodological control to verify that equal amounts of cDNA were derived at the various time points. Infection with HSV had no effect on the level of *toh* message in the neurons during the first 24 h after infection. α*0* was expressed as early as 2 h after infection in both neurons and Vero cells (Fig. 2b and c and 3c and d). *UL39* (β*6*) was not detected until 4 to 6 h after infection in neurons, whereas in Vero cells it was detected by 3 h after infection (Fig. 2b and c and 4c and d). γ*B* was not detected in neurons until 12 h after infection (Fig. 2b and c). In contrast, γ*B* was detectable in Vero cells by 4 h after infection (data not shown). Thus, the onset of viral β and γ gene expression in neurons was delayed relative to that of Vero cells, and this difference was most dramatic with γ genes (Fig. 5). Appreciable levels of viral DNA synthesis occur in neurons after expression of the α gene, α*0*, and the β gene, *UL39* (compare Fig. 2a and b). The expression of these genes was sustained until 12 and 24 h after infection, during which time viral DNA synthesis was ongoing (compare Fig. 2a and b and 3a). As expected, γ gene expression was not detectable until after viral DNA synthesis had occurred (compare Fig. 2a and b and 2c). These results demonstrate that viral gene expression in neurons was markedly delayed relative to expression in Vero cells.

ACV inhibits the expression of viral α and β genes in neurons but not in Vero cells. To determine the effect of viral DNA synthesis on viral gene expression, the cells were incubated in the presence or absence of ACV (50 μM) for 12 h prior to infection. At various times after infection, RNA was harvested and converted to cDNA, and aliquots of each cDNA sample were subjected to PCR. Levels of the transcript from the neuron-specific *toh* gene were also measured. Infection with HSV in the presence or absence of ACV had little or no effect on the level of *toh* message detectable in these cells within the first 24 h after infection (data not shown). This is consistent with our previous finding that neurons do not undergo virion host shutoff within the first 24 h after viral infection and again verifies that the amounts of input mRNA and cDNA at each time point are equivalent (25). At later time points, there was a decrease in the level of *toh* RNA in cells infected in the absence of ACV but not in the presence of ACV (data not shown).

Using the same cDNA samples, we next evaluated levels of cDNA of viral transcripts. Peak expression of the α gene, α*0*, was at about 12 h after infection of neurons (Fig. 3a and b). Peak expression of α*0* in Vero cells was between 4 and 6 h postinfection (Fig. 3c and d). Pretreatment of the cells with ACV markedly reduced this peak in neurons (Fig. 3a and b) but either had no effect or slightly enhanced the expression of α*0* in Vero cells (Fig. 3c and d). Likewise, ACV had no effect on the expression of α*27* in Vero cells but reduced the expression of this α gene in neurons (data not shown). Expression of the β gene β*6* message in neurons was maximal by 24 h but was dramatically reduced by treatment with ACV (Fig. 4a and b). In Vero cells, expression of the β*6* message was maximal by 6 h after infection; however, treatment with ACV had no effect on β*6* mRNA levels (Fig. 4c and d). An inhibitor of viral DNA synthesis, therefore, dramatically reduced the expression of α

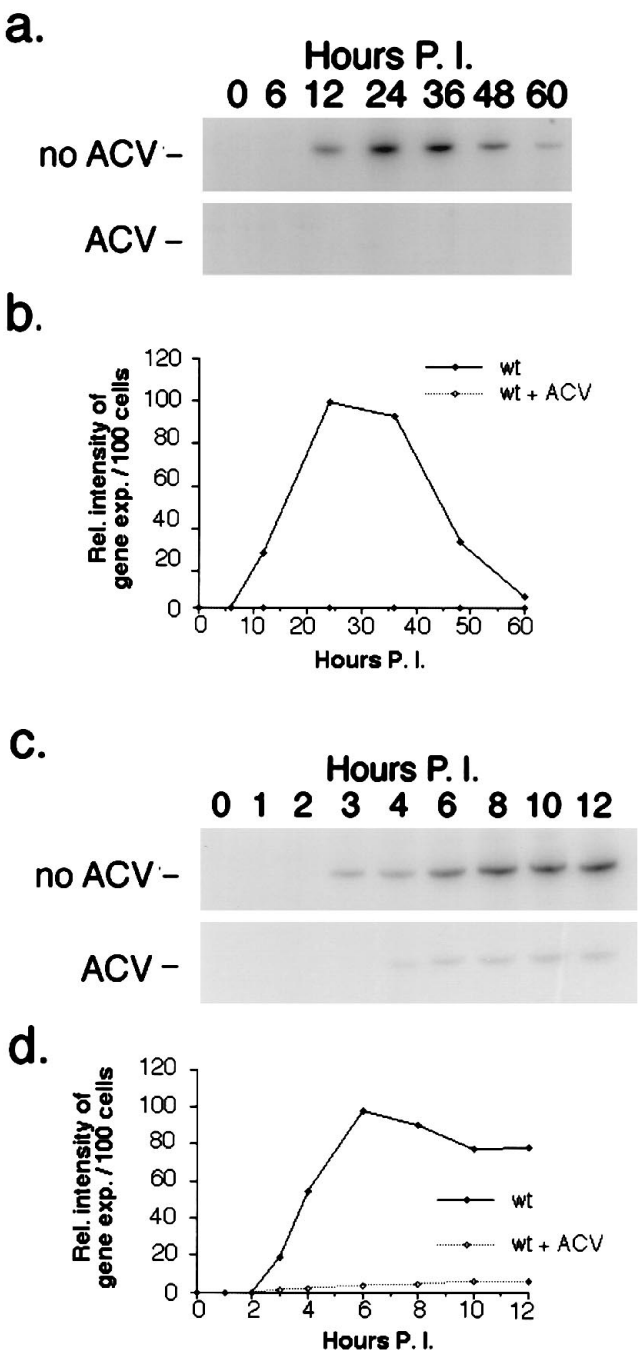


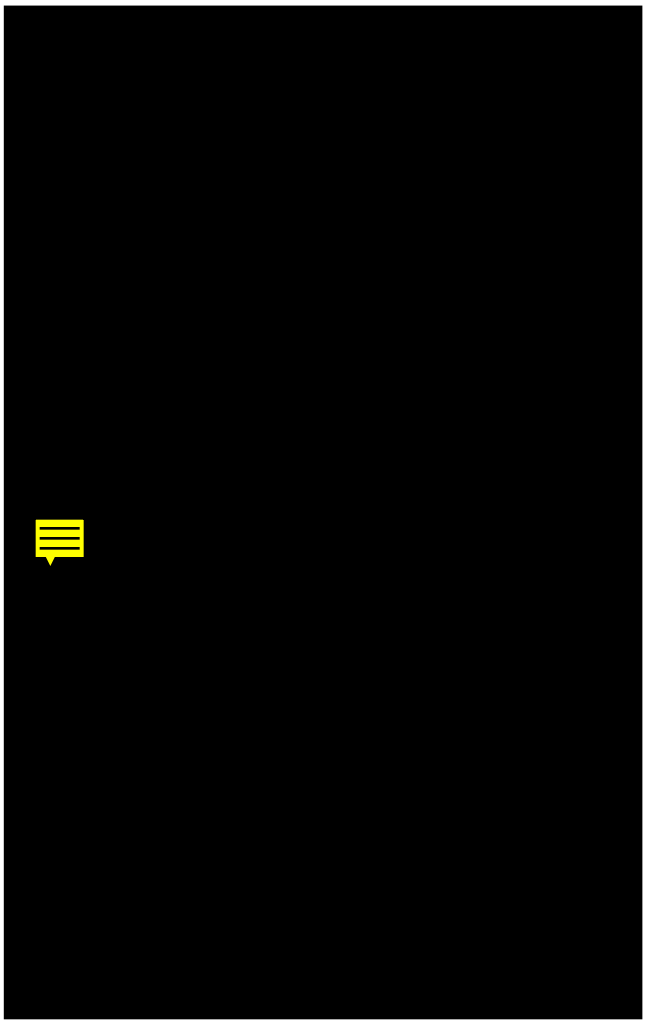
FIG. 5. Analysis of the effect of ACV on the steady-state levels of γ*C* transcripts in HSV-infected neurons and Vero cells. (a) PhosphorImage of RT-PCR products demonstrating γ*C* expression in HSV-infected neurons. Neurons were plated at a density of 5,500 per well. Eleven days after plating, the cells were infected with wild-type HSV-1 (MOI, 5) in the presence or absence of ACV. Cells were harvested at the indicated times postinfection (P.I.) for mRNA. RT-PCR was performed to examine γ*C* expression. Reaction samples were run on a 10% polyacrylamide gel, dried, and exposed to a PhosphorImaging plate. This experiment was repeated twice with similar results. (b) Graphic display of γ*C* expression in HSV-infected neurons. γ*C* expression in neurons is presented in graph form. Data are plotted as the relative (rel.) intensity of expression (exp.) compared with the background signal. wt, wild type. (c) PhosphorImage of RT-PCR products demonstrating γ*C* expression in HSV-infected Vero cells. Vero cells were plated overnight at a density of 50,000 per well. The cells were infected with wild-type HSV-1 (MOI, 5) in the presence or absence of ACV. Cells were harvested at the indicated times after infection for mRNA. RT-PCR was performed to examine γ*C* expression. Reaction samples were run on a 10% polyacrylamide gel, dried, and exposed to a PhosphorImaging plate. This experiment was repeated twice with similar results. (d) Graphic display of γ*C* expression in HSV-infected Vero cells. γ*C* expression in Vero cells is presented in graph form. Data are plotted as the relative intensity of expression compared with the background signal.

and β gene expression in neurons but, as expected, had little effect on expression of these viral genes in Vero cells.

The expression of the γ*2* gene, γ*C*, was similarly evaluated. γ*C* mRNA was first detected in neurons 12 h after infection, and as expected, ACV completely blocks its expression (Fig. 5a). The peak expression for γ*C* mRNA in neurons was between 24 and 36 h postinfection. In comparison, γ*C* was first expressed in Vero cells by 3 to 4 h and peaked by 10 h postinfection (Fig. 5a and b). The difference in the peak expression of γ*C* in the Vero cells (10 h) and in the neurons (24 h) in the absence of ACV demonstrates that the viral cascade was temporally different in the two cell types.

Inhibition of viral DNA synthesis reduces viral gene expression in neurons during a single infectious cycle. It is a formal possibility that multiple rounds of viral replication are required for accumulation of detectable levels of viral transcripts in the neurons and that ACV acts by blocking viral replication and secondary spread in the cultures, thus indirectly reducing the level of viral transcripts we observe. To compensate for this, after infection, the cells in the previous experiments were cultured in medium containing human gamma globulin, which contains HSV-neutralizing antibodies. This step, however, would not prevent direct cell-to-cell spread of virus. We set out, therefore, to establish definitively that the effect of ACV on α and β gene expression in neurons occurred during a single infectious cycle and was not due to the drug preventing multiple cycles of infection. We accomplished this by using a mutant virus (UL26icp6gluc) with a defect in a gene (*UL26*) which encodes the protein assemblin (see Materials and Methods) (10). Assemblin is essential for packaging infectious virus particles in infected cells, and in noncomplementing cells, this mutant expresses all lytic genes (except *UL26*) and synthesizes normal levels of viral DNA. In addition, since UL26icp6gluc has a β*6* promoter (β-glucuronidase cassette in the *UL26* open reading frame), infected cells express β-glucuronidase with the kinetics of a β gene. As expected, in UL26icp6gluc-infected Vero cells, ACV and phosphonoacetic acid (PAA) had no effect on β-glucuronidase activity (data not shown). SCG neurons were cultured in the presence or absence of ACV or PAA, infected with UL26icp6gluc over a range of MOIs, and fixed and histochemically stained for β-glucuronidase activity 48 h after infection. Each well of neurons was counted for blue and white cells, and the data are presented as the percentage of blue cells at a given MOI (Fig. 6b). Photomicrographs of the neurons are shown in Fig. 6a. As can be seen, ACV and PAA treatment reduced expression of β-glucuronidase in neurons. These results demonstrate definitively that the maximal expression of HSV genes in neurons during a single infectious cycle requires viral DNA synthesis.

Initiation of viral DNA synthesis is necessary for maximal viral gene expression in neurons. The requirement for viral DNA synthesis for HSV α and β gene expression in neurons could be explained by an increase in the copy number of the viral DNA and/or a change in the physical state of the DNA (e.g., the formation of a replication fork). To distinguish between these possibilities, we used two mutant viruses, *hr*R3 and *hr*94. *hr*R3 carries a mutation in the viral gene (*UL39*) which encodes the large subunit of ribonucleotide reductase (12). In *hr*R3-infected neurons, viral DNA synthesis is initiated, but



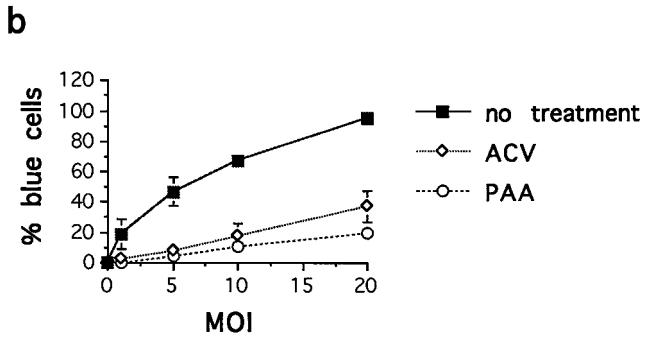


FIG. 6. Histochemical analysis of the effect of inhibitors of viral DNA synthesis on β-glucuronidase expression in UL26icp6gluc-infected neurons. (a) Photomicrographs of UL26icp6gluc-infected neurons. Neurons were plated at a density of 3,000 per well. Eleven days after plating, the cells were infected over a range of MOIs with the HSV-1 mutant virus UL26ICP6gluc in the presence or absence of ACV. Cells were fixed and histochemically stained 48 h postinfection for β-glucuronidase. (b) Graph of β-glucuronidase expression in UL26icp6gluc-infected neurons. Cells that were positive and cells that were negative by staining were counted in each well. β-Glucuronidase-expressing (blue) cells are represented as a percentage of the total cells per well.

because of a deficiency of deoxyribonucleotide pools, the elongation step of viral DNA synthesis is impaired compared with that of the wild-type virus. As a result, incorporation of [^{3}H]thymidine into TCA-precipitable material is not detectable above background levels in *hr*R3-infected neurons (6, 6a). *hr*94 has a null mutation in the essential DNA synthesis gene (*UL9*), which encodes the viral origin-binding protein, and in *hr*94-infected cells, viral DNA synthesis is not initiated (24). Both viruses have a β*6* promoter (*lacZ* cassette in the genome), and, therefore, β-galactosidase activity can be used as a marker of β gene expression.

When Vero cells were infected with either *hr*R3 or *hr*94 and histochemically stained for β-galactosidase activity 8 h after infection, the number of blue cells correlated with the number of PFU inoculated, and was unaffected by the presence of ACV (Fig. 7) or phosphonoacetic acid (data not shown). When neurons were infected separately with either virus, ACV reduced the number of blue cells in *hr*R3-infected neuronal cultures by about 50 to 75% (Fig. 8a and c). This suggests that a low but undetectable level of viral DNA synthesis occurs in *hr*R3-infected neurons. The percentage of blue-staining neurons in cultures infected with *hr*94 was far lower (5- to 20-fold lower) than that in cultures infected with *hr*R3 in the presence of ACV (Fig. 8b and c). Therefore, inhibition of DNA synthesis with agents (e.g., ACV) that act by a chain termination mechanism and that permit initiation and partial elongation of viral DNA synthesis still allowed some degree of viral gene transactivation. ACV had no effect on the number of blue-staining cells in *hr*94-infected neurons (Fig. 8c). These results indicate that the effect of the block in the initiation of viral DNA synthesis (e.g., in the *hr*94-infected cells) on viral gene expression cannot be overcome by increasing the number of viral genomes delivered to the cell. Moreover, these results

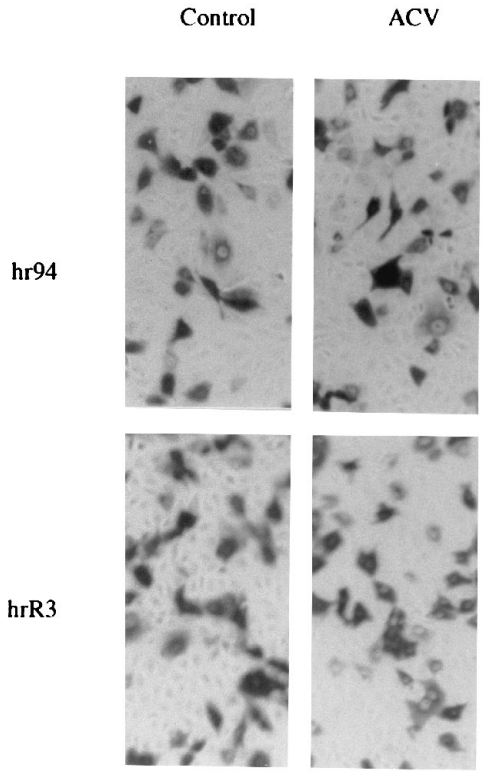
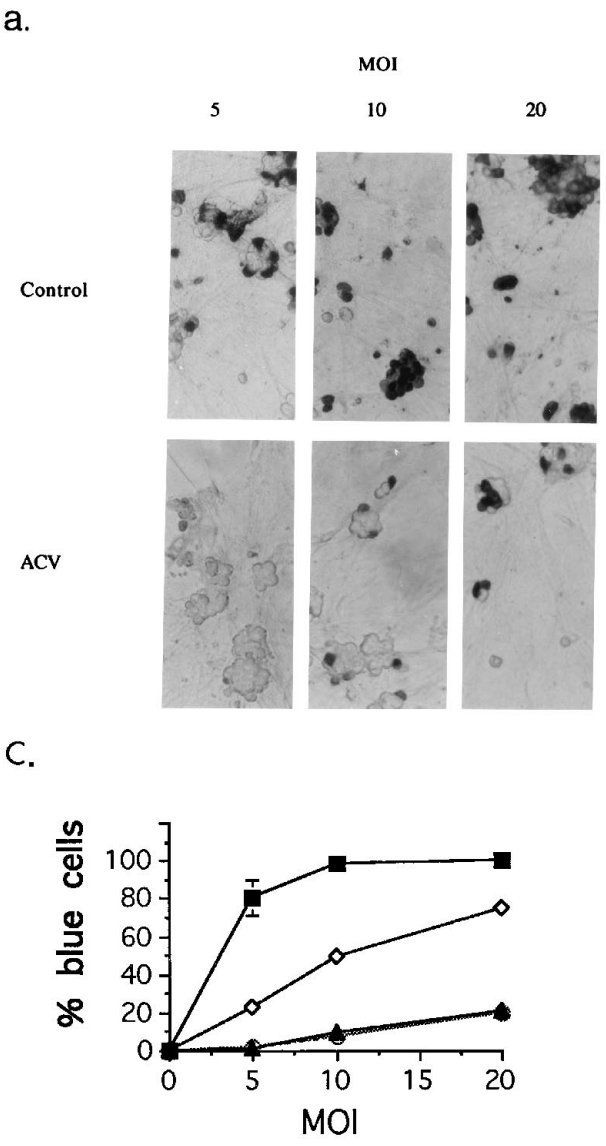


FIG. 7. Photomicrograph of Vero cells histochemically stained for β-galactosidase after infection with *hr*R3 or *hr*94 in the presence and absence of ACV. Vero cells were plated overnight at 50,000 per well. The cells were cultured prior to and during infection in the presence or absence (control) of ACV. Cells were infected with either *hr*R3 or *hr*94 at an MOI of 0.5. Eight hours after infection, the cells were fixed and stained for β-galactosidase activity.



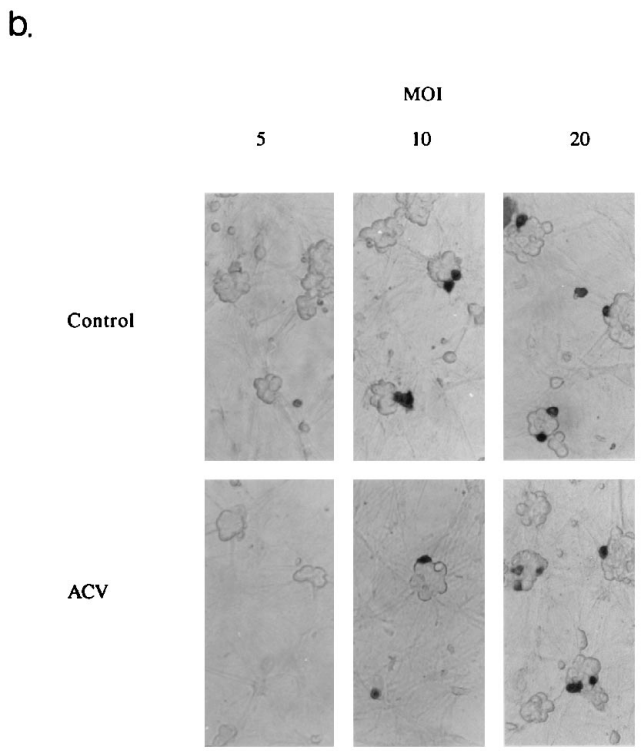


FIG. 8. Histochemical analysis of the effect of ACV on β-galactosidase expression in *hr*R3-infected and *hr*94-infected SCG neurons. Neurons were plated at a density of 3,000 per well. Eleven days after plating, the cells were cultured in the presence or absence of ACV and then infected with either *hr*R3 or *hr*94 over the indicated range of MOIs. The cells were fixed and stained for β-galactosidase activity 48 h after infection. (a) Photomicrograph of *hr*R3-infected neurons. (b) Photomicrograph of *hr*94-infected neurons. (c) Graphic display of the percentage of β-galactosidase-positive neurons after infection with *hr*R3 (■ and ◇) and *hr*94 (▲ and ○) in the presence (◇ and ○) and absence (■ and ▲) of ACV. Blue and white cells were counted from each well ($n = 3$). Bars indicate standard deviation from the mean.

strongly suggest that transactivation of α and β gene expression from the viral genome requires initiation of replication and formation of a replication fork during viral DNA synthesis.

DISCUSSION

In this report, we demonstrate that maximal expression of all classes of HSV lytic genes in infected SCG neurons requires viral DNA synthesis. Furthermore, the primary mechanism by which viral DNA replication drives viral α and β gene expression involves the processes of initiation and elongation of DNA synthesis and is not simply the result of amplification of the viral genome. Expression of α and β genes in neurons was inhibited dramatically when viral DNA synthesis was blocked by treatment of the cells with ACV prior to infection. In contrast, and as expected, treatment with ACV had no inhibitory effect on α and β gene expression in Vero cells. Our results were not due to an effect of ACV on secondary spread of virus in culture, because inhibitors of viral DNA synthesis reduced the level of reporter gene activity in neurons infected with a mutant virus (UL26icp6gluc) incapable of egressing from cells.

To further examine the role of viral DNA replication in the regulation of α and β gene expression in neurons, we compared β-galactosidase histochemical stainings of neurons infected with one of two *lacZ*-expressing viral mutants that are impaired for viral DNA synthesis at different points in the replication process. The two mutant viruses contain identical reporter cassettes: a β*6* promoter driving *lacZ*. Vero cells infected with each of these mutants displayed no difference in the number of blue cells, and ACV had no effect on the number of *lacZ*-positive cells (Fig. 7). ACV did reduce the percentage of *lacZ*-positive neurons in SCG cultures infected with *hr*R3 (a ribonucleotide reductase null mutant) (Fig. 8a). This result is consistent with previous findings that ACV treatment allowed the establishment of latency in *hr*R3-infected neurons, and we interpret this as indicating that there is a low but undetectable level of viral DNA synthesis in *hr*R3-infected neurons (6). Nevertheless, the number of *lacZ*-positive cells was much greater in neuronal cultures infected with *hr*R3, even in the presence of DNA synthesis inhibitors, than in neuronal cultures infected with *hr*94 (an origin-binding protein null mutant which is unable to initiate viral DNA replication) (Fig. 8c). This result strongly suggests that very early events during viral DNA synthesis activate α and β gene expression in neurons.

Several lines of evidence demonstrate that the decrease in HSV α and β gene expression we observed in neurons in the presence of inhibitors of viral DNA synthesis is actually due to inhibition of viral DNA synthesis. First, ACV treatment of

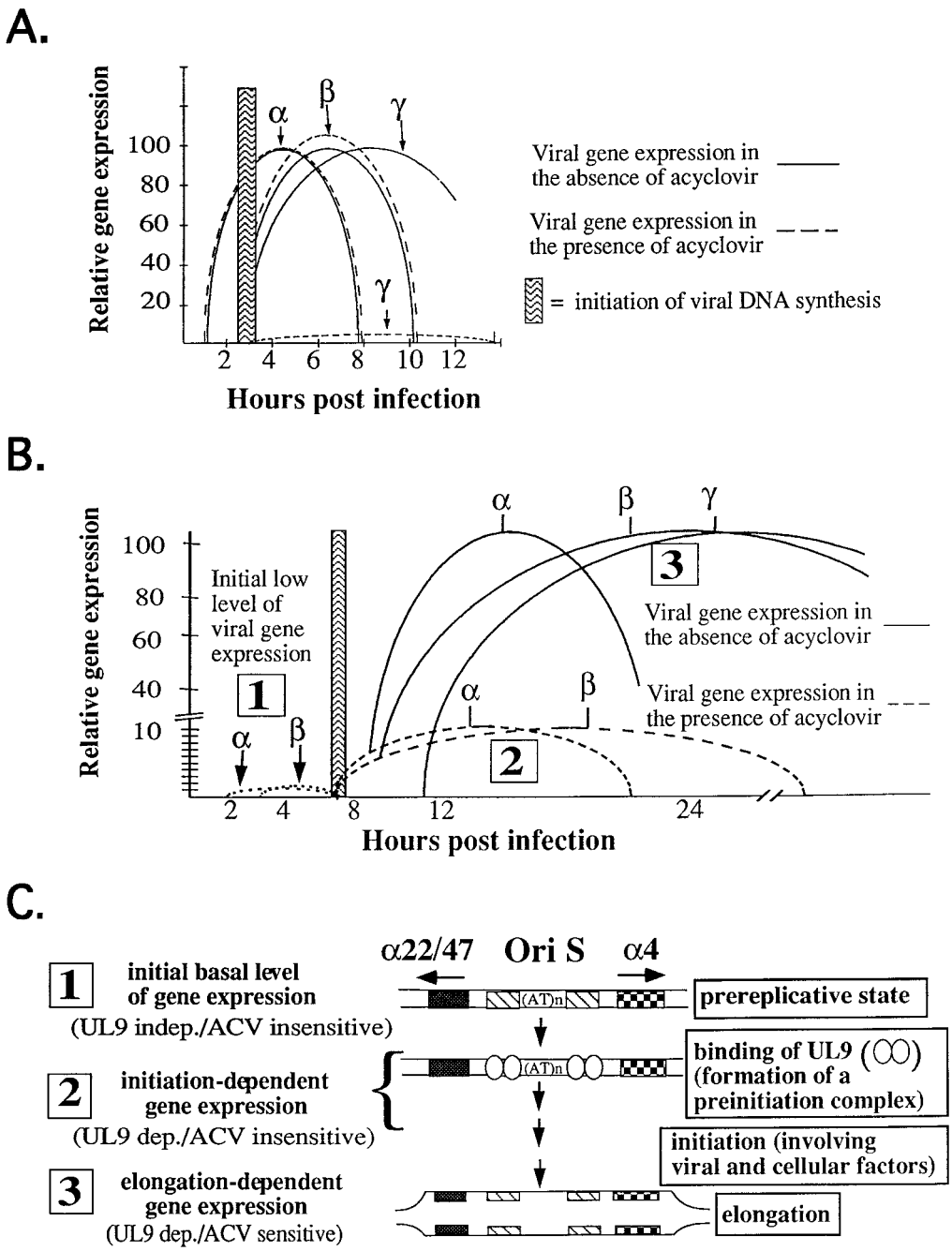


FIG. 9. Schematic representation of the cascade of HSV gene expression in Vero cells (A) and neurons (B) and a model which proposes that the regulation of viral gene expression in neurons is dependent on UL9-mediated initiation of viral DNA synthesis (C). dep., dependent; indep., independent. (A) Cascade of viral gene expression in Vero cells. The solid lines indicate the pattern of viral gene expression when viral DNA synthesis occurs; the dashed lines represent the pattern of viral gene expression when viral DNA synthesis is blocked. Only γ gene expression is reduced by inhibition of DNA synthesis. (B) Cascade of viral gene expression in neurons. Early after infection of peripheral neurons, a low level of α gene expression occurs, which promotes a low level of β gene expression (dotted lines [part 1]). The dashed lines represent the pattern of viral gene expression when viral DNA synthesis is blocked by ACV (part 2). The solid lines indicate the pattern of viral gene expression when viral DNA synthesis is allowed to occur in the absence of ACV (part 3). (C) Model of UL9-mediated activation of α and β gene expression in neurons. Early after infection of peripheral neurons, a low level of α gene expression occurs, which promotes a low level of β gene expression (part 1). The products of the β genes include proteins essential for viral DNA synthesis, such as the origin-binding protein (UL9) (OO), which is critical for the initiation of viral DNA synthesis at the origins of DNA replication. UL9 binding at *oriS* and subsequent events during the preinitiation process induce the expression level of the *α4* gene, which encodes the viral transcriptional transactivator ICP4 (part 2). The increase in the level of ICP4, coupled with advancement of the replication process at the origins, further elevates the expression of α and β genes. Finally, extensive elongation then fully activates expression of β and γ genes (part 3).

infected neurons had the same effect on both α and β promoters. Second, both ACV and PAA, two inhibitors of viral DNA synthesis with pharmacologically distinct mechanisms, had the same effect on expression of a reporter gene (β-glucuronidase) driven by a β gene promoter (β*6*) in infected neurons and yet had no such effect on Vero cells. Third, ACV had the expected effect of inhibiting viral DNA replication in neurons infected with wild-type virus. Finally, ACV had no effect on expression of *lacZ* from a β promoter in neurons infected with a virus incapable of viral DNA synthesis (*hr*94), thus ruling out the possibility of a drug effect that is independent of viral DNA synthesis.

Overall, our results provide direct evidence for and extend the model proposed by Kosz-Vnenchak et al. (21). Figure 9 is a schematic representation of the cascade of viral gene expression in neurons compared with that in Vero cells and shows a proposed model of how initiation might induce viral gene activation. Unlike the situation in Vero cells, in infected neurons, the level of expression of α genes is extremely low. The reason for the low level of α gene expression in peripheral neurons is not clear, but it is thought to be due to either limiting amounts of cellular transcription factors required for α gene transactivation, the presence of an α gene repressor, or a combination of both which prevents VP16-mediated α gene induction (13, 19). Regardless, this low level of α gene expression drives a low level of β gene expression (Fig. 9B, part 1). The viral gene expression during this phase would be similar to that seen in *hr*94-infected neurons (Fig. 8b and c). The resulting gene products initiate the synthesis of DNA (which is indicated by the hatched bar in Fig. 9B). Initiation of viral DNA synthesis most likely begins with the binding of the *UL9* gene product to the viral origins of DNA replication (Fig. 9C, part 2). The initiation process, which is likely to involve a conformational change of the viral DNA at the origins, then transactivates α gene expression, particularly α*4*. Expression of α*4*, possibly in combination with further initiation events but independent of elongation, then upregulates α and β gene expression (Fig. 9B, part 2). This phase of the transactivation process is resistant to ACV and PAA but is dependent on an intact *UL9* gene. Evidence for this phase is presented in Fig. 8a and c. The percentage of *lacZ*-expressing neurons in cultures infected with *hr*R3 (a virus which can initiate DNA synthesis) in the presence of ACV is higher than that in cultures infected with *hr*94 (a virus which cannot initiate DNA synthesis) (compare *hr*R3 plus ACV with either *hr*94 group in Fig. 8c). Thus, initiation of viral DNA synthesis, even when elongation is blocked, is sufficient to significantly enhance viral gene expression over basal levels in neurons.

In the absence of viral DNA synthesis inhibitors, elongation of nascent viral DNA occurs, and this further stimulates both α and β gene expression (Fig. 9B, solid lines and part 3). This phase of the activation process is sensitive to agents (ACV and PAA) which terminate elongation (compare *hr*R3 and *hr*R3 plus ACV in Fig. 8c). According to the model, elongation of nascent viral DNA leads to activation of other viral promoters and further enhancement of gene expression (Fig. 9C, part 3). We believe that elongation and not an increase in the copy number of a given gene is primarily responsible for the induction of gene expression during this phase. We base this belief on the observation that, in spite of the fact that *hr*R3 does not replicate in neurons and we are unable to detect viral DNA synthesis, gene expression in *hr*R3-infected neurons has an ACV-sensitive phase (Fig. 8c) (6). This suggests that very low levels of viral DNA synthesis, as probably occur in *hr*R3-infected neurons, are sufficient to activate viral gene expression. In addition, increasing the amount of *hr*94 virus used to infect the neurons resulted in only a small increase in the number of *lacZ*-positive cells (Fig. 8b and c). Thus, an elevation in the genome copy number was not as effective a stimulus of viral gene expression as initiation of viral DNA synthesis. These results suggest that template amplification is not the primary stimulus of activation of viral gene expression.

In contrast to the situation in neurons, maximal viral α and β gene expression in Vero cells is achieved without initiation (Fig. 7, compare *hr*R3 and *hr*94) or elongation (Fig. 3d, 4d, 7, and 9A). In fact, pharmacological or genetic blocks in viral DNA synthesis have been reported to lead to an induction of β genes (Fig. 9A) (14). However, inhibition of viral DNA synthesis will lead to a reduced expression of γ genes in Vero cells, as seen in Fig. 5d. This includes a substantial reduction in γ*1* genes and a nearly complete inhibition of γ*2* genes, which are represented collectively as γ genes in Fig. 9A.

This model predicts that the HSV origin-binding protein (UL9) is required for maximal viral gene expression in neurons because it is likely to be critical during the initiation of DNA replication at the viral origins (5). The UL9 gene product, in addition to its origin-binding activity, is a DNA helicase, and although UL9-dependent origin unwinding has not been demonstrated directly, it is likely to be the critical event during initiation of HSV DNA replication (4, 26). The details of HSV DNA replication remain to be elucidated, however, and a role for UL9 during elongation has not been ruled out. In fact, the helicase activity of UL9 may be important during fork propagation. However, we favor the concept that UL9-mediated DNA events at the origin during formation of a preinitiation complex and during initiation itself stimulate transcription from adjacent viral promoters. This may involve a conformational change and/or unwinding of DNA at the viral origins in the presence of other viral replication factors (e.g., ICP8), possibly in combination with other viral and cellular factors and transcriptional transactivators. According to this idea, genes most proximal to the origins are likely to be the first to be activated by this mechanism, and it is perhaps no coincidence that each copy of α*4* (which encodes an essential transactivator of both α and β genes) is adjacent to an *oriS*. Furthermore, all five α genes are clustered around *oriS* 1 and 2. In addition, *UL29* and *UL30*, which encode the viral DNA polymerase and major single-stranded DNA binding protein (ICP8), respectively, flank *oriL*, although *oriL*-mediated activation of these genes is probably not essential, since an *oriL* mutant virus has been shown to be able to reactivate (28). *UL9*, the gene which encodes the origin-binding protein, however, is not proximal to any of the origins. It is located at a distance from the origins which may serve to selectively control when viral DNA synthesis-dependent activation of viral transcription occurs. Since this process may be critical for reactivation from latency, expression of *UL9* may be regulated differently in neurons than in other cell types. In fact, one would predict that events which trigger reactivation of the virus may alter the regulation of this gene.

There is indirect evidence that suggests that this prediction is correct. First, *UL9* is one of several viral genes that contain a cAMP response element in their promoters. Second, dibutyryl cAMP has been shown to induce the *UL9* promoter in a rat pheochromocytoma cell line (PC12) but not in other cell lines of nonneuronal lineage (7). Finally, elevations in cAMP levels have been shown to trigger reactivation of latent HSV (32). One might predict that expression of *UL9* during reactivation from latency may be altered so that it either precedes or is concomitant with expression of α genes such as α*4*. We are conducting experiments to determine if this is the case.

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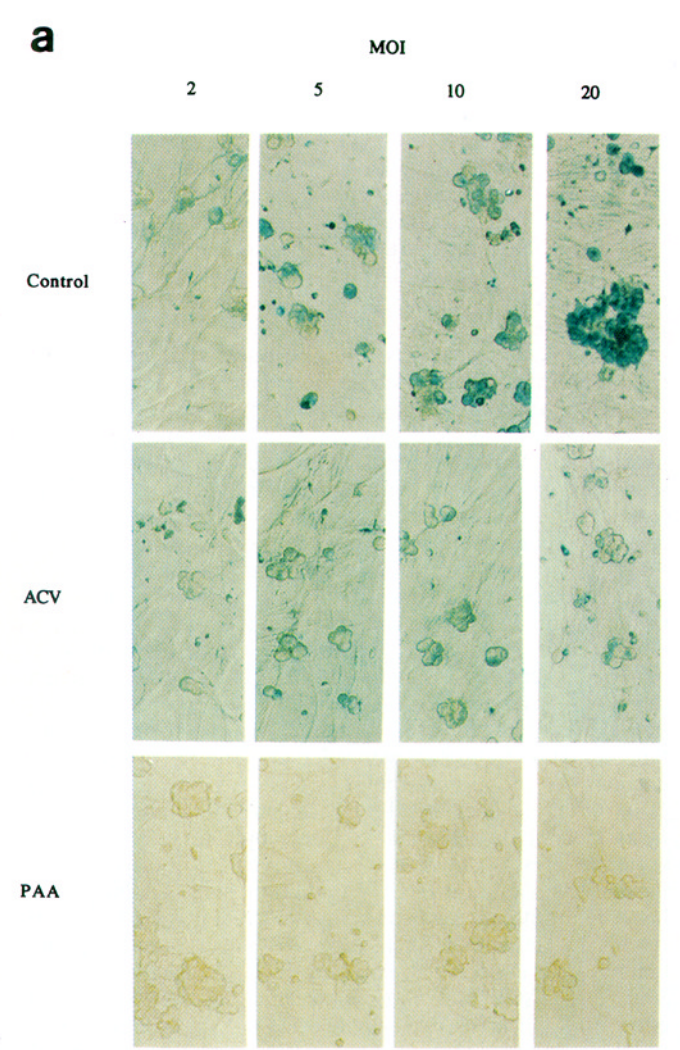
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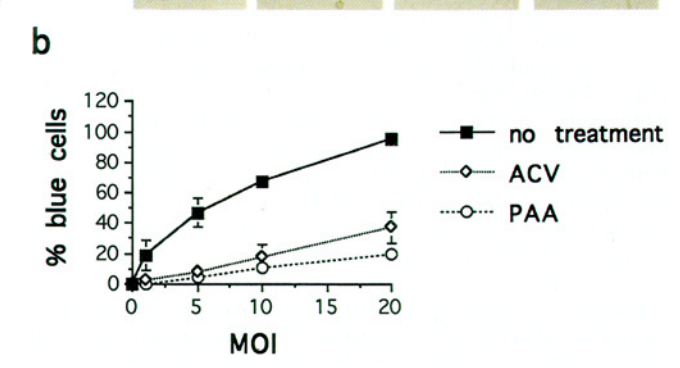
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