JOURNAL OF VIROLOGY, July 2006, p. 6810–6821 Vol. 80, No. 14
0022-538X/06/$08.00+0 doi:10.1128/JVI.00334-06

ICP0 Gene Expression Is a Herpes Simplex Virus Type 1 Apoptotic Trigger

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Received 15 February 2006/Accepted 25 April 2006

Apoptosis is a highly regulated programmed cell death process which is activated during normal development and by various stimuli, such as viral infection, which disturb cellular metabolism and physiology. That herpes simplex virus type 1 (HSV-1) induces apoptosis but then prevents its killing of infected cells is well-established. However, little is known about the viral factor/event which triggers the apoptotic process. We previously reported that infections with either (i) a temperature-sensitive virus at its nonpermissive temperature which does not inject viral DNA into nuclei or (ii) various UV-inactivated wild-type viruses do not result in the induction of apoptosis (C. M. Sanfilippo, F. N. W. Chirimuuta, and J. A. Blaho, J. Virol. 78:224–239, 2004). This indicates that virus receptor binding/attachment to cells, membrane fusion, virion disassembly/tegument dispersal, virion RNAs, and capsid translocation to nuclei are not responsible for induction and implicates viral immediate-early (IE) gene expression in the process. Here, we systematically evaluated the contribution of each IE gene to the stimulation of apoptosis. Using a series of viruses individually deleted for α27, α4, and α22, we determined that these genes are not required for apoptosis induction but rather that their products play roles in its prevention, likely through regulatory effects. Sole expression of α0 acted as an "apoptoxin" that was necessary and sufficient to trigger the cell death cascade. Importantly, results using a recombinant virus which contains a stop codon in α0 showed that it was not the ICP0 protein which acted as the apoptotic inducer. Based on these findings, we propose that α0 gene expression acts as an initial inducer of apoptosis during HSV-1 infection. This represents the first description of apoptosis induction in infected cells triggered as a result of expression of a single viral gene. Expression of apoptotic viral genes is a unique mechanism through which human pathogens may modulate interactions with their host cells.

Abnormalities in apoptotic control mechanisms contribute to the development of an assortment of human diseases, including cancer and autoimmunity. Due to the cell's innate ability to self-destruct, apoptosis is also an important mechanism of host response to viral infection. Human herpes simplex virus type 1 (HSV-1) is a large DNA virus (25) whose infection is characterized by the establishment of latency in neuronal cells (60) and the recurrence of lytic infection in epithelial cells (reviewed in reference 52). While productive HSV-1 replication induces major biochemical changes in infected cells, collectively referred to as cytopathic effect, it is now recognized that the virus also triggers apoptosis in transformed or tumor cells, not primary cells (3), but the subsequent synthesis of infected cell proteins during an apoptotic-prevention window (3 to 6 hours postinfection [hpi]) delays the cell death process from killing the infected cells (2, 4, 24, 33, 35). Neither the association of the virus with its cell surface receptor, a tumor necrosis factor receptor 1 family member (37), nor events immediately following virion entry into cells are sufficient to trigger apoptosis per se (4). Lytic HSV-1 replication occurs in a highly regulated cascade involving four phases of gene expression: immediate early (IE), early, leaky late, and true late (reviewed in reference 52). Induction of apoptosis by HSV-1 requires expression of the first class of viral genes (IE genes) (57). Thus, potential preformed RNA molecules, packaged inside incoming virion particles (9), are not responsible for apoptosis induction. In spite of these recent advances, the specific herpesviral component necessary for triggering apoptosis in human cells, termed the viral "apoptoxin," has remained unidentified.

Depending on the nature of the apoptotic death signal, transduction cascades which involve the proteolytic activation of caspases, a family of aspartate-specific cysteinyl proteases, are initiated. Whether the apoptotic stimulus initiates from a cell surface death receptor (extrinsic) or from the mitochondria (intrinsic), downstream executioner caspases, such as caspase-3, are common to both signaling pathways. Caspase activation leads to the processing of various cytoplasmic and nuclear substrates, such as the DNA repair enzyme poly(ADP-ribose) polymerase, or PARP, a 116-kDa protein which generates an 85-kDa product upon processing (4). These biochemical changes are responsible for the morphological manifestations characteristic of apoptotic cells, including cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation (reviewed in reference 32). Infection with HSV-1 triggers the activation of caspase-3 (loss of pro-caspase-3) without subsequent cleavage of PARP (4), unless viral protein synthesis is prohibited (2, 24, 34). Thus, while the process of apoptosis induction by HSV-1 has been clearly demonstrated, the viral factor responsible for triggering apoptosis remains unknown. Here, we show that a single viral RNA molecule, α0, expressed with immediate-early kinetics, triggers apoptosis during viral infection of human cells.

Furthermore, sole expression of this RNA apoptotic trigger is both necessary and sufficient to induce the apoptotic cell death cascade. Taken together, these findings raise the intrigu-

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ing possibility that HSV-1 may indeed benefit from the existence of such an apoptoxin.

MATERIALS AND METHODS

Cell lines and viruses. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum. Human epithelial (HEp-2) and monkey kidney (Vero) cells were obtained from the American Type Culture Collection (Rockville, Md.). Vero 2.2 is a derivative Vero cell line expressing ICP27 under its own promoter (59). FO6 is a derivative Vero cell line expressing ICP4, ICP27, and ICP0 under their own promoters (53). L7 is a derivative Vero cell line expressing ICP0 under its own promoter (provided by Priscilla Schaffer, Harvard Medical School) (53). HSV-1(KOS), HSV-1(17) (provided by Betsy Herold, Mount Sinai), and HSV-1(F) are the strains of wild-type HSV-1 used in this study. The KOS derivative vBSΔ27 (59), termed Δ27, contains a replacement of the α27 gene with the *Escherichia coli lacZ* gene; this virus was propagated and titers were determined on Vero 2.2 cells. HSV-1(*d*109), termed *d*109, is a KOS-derived mutant virus deleted for all five IE genes which contains the green fluorescent protein (GFP) gene under the control of the human cytomegalovirus (HCMV) IE promoter (53); this virus was propagated and titers were determined on FO6 cells. The strain 17 derivative HSV-1(CgalΔ3), termed CgalΔ3, contains the *E. coli lacZ* gene under the HCMV IE promoter inserted into an intergenic site in the U_S portion of a mutant genome deleted for 3.6 kb of the ICP4 coding region (31); this virus was propagated and titers were determined on FO6 cells. The strain F derivative HSV-1(R7802) (obtained from Bernard Roizman, University of Chicago), termed R7802, lacks the coding domain of ICP22 (44); this virus was propagated and titers were determined on Vero cells. The KOS derivatives HSV-1(7134), termed 7134, which contains the *E. coli lacZ* gene in place of the ICP0 coding region (11), and HSV-1(*n*212), termed *n*212, which contains a nonsense mutation (stop codons in all three frames) at ICP0 amino acid 212 (11) (gifts of Priscilla Schaffer, Harvard Medical School), were propagated and titers were determined on L7 cells. HSV-1(*d*106) (gift of Neal DeLuca, University of Pittsburgh), termed *d*106, is a KOS-derived mutant virus expressing the α0 gene, but inactivated for the remaining four IE genes, which contains the GFP gene under the control of the HCMV IE promoter (53); this virus was propagated and titers were determined on FO6 cells. All viral titers were determined at 48 hpi by standard dilution techniques, and all values are the means from duplicate infections. Direct comparisons between a series of viruses (as shown in figure panels) were made by determining titers on the relevant cells to ensure consistency. Levels of incoming virion-derived VP22 were measured in each case to confirm that particle-to-PFU ratios were effectively identical. In all experiments, cell monolayers were infected with multiplicities of infection (MOI) of 5 PFU per cell and the infections were maintained at 37°C in DMEM containing 5% newborn-calf serum (NBCS) for 24 h. All tissue culture reagents were purchased from Life Technologies.

Protein synthesis inhibition by CHX. To inhibit de novo protein synthesis and, therefore, allow apoptosis in wild-type-virus-infected cells (2, 33), cycloheximide (CHX) (Sigma) was added to the medium of HEp-2 monolayers at a final concentration of 10 μg/ml. This concentration of CHX was previously shown to be sufficient to completely block viral protein synthesis in HSV-1(KOS)-infected HEp-2 cells (2). Cells were pretreated with CHX for 1 h at 37°C prior to infection, and CHX was maintained in the medium until 24 hpi, at which time whole-cell extracts were prepared as described below.

Transfection and subsequent infection. Plasmid pSH (11), which contains the entire 3.2-kb ICP0 coding region (including three exons and two introns) as well as 1.0-kb 5′ and 0.4-kb 3′ flanking sequences cloned into pUC8, was generously provided by Priscilla Schaffer. Plasmid pUC19 (New England Biolabs) was the control empty vector used in this study. HEp-2 cells were seeded at 8×10^5 cells/10-mm-diameter dish in DMEM containing 5% NBCS and transfected 20 to 24 h later (60 to 70% confluence) by using a calcium phosphate coprecipitate transfection method as follows. Purified plasmid DNA (0.625 to 7.5 μg) in a final volume of 100 μl was mixed with 100 μl of 2× HEPES-buffered saline, pH 7.05, and 10 μl of 2.5 M $CaCl_2$ and incubated for 15 min at 25°C. Mixtures were then added to 10-mm-diameter dishes of HEp-2 cells containing 2 ml of fresh DMEM plus 5% NBCS and incubated at 37°C. After 3 h, the cells were washed with serum-free DMEM, incubated at room temperature for 1 min with 1 ml of a 15% glycerol–serum-free DMEM solution, washed with serum-free DMEM, replaced with fresh DMEM plus 5% NBCS, and incubated at 37°C for 24 h. At 24 h posttransfection, cells were mock infected or infected with HSV-1(*d*109) and incubated at 37°C for 24 h, at which time whole-cell extracts were prepared.

Preparation of infected-cell extracts, denaturing gel electrophoresis, and transblotting. Whole extracts of infected cells were obtained at 24 hpi as previously described (57). The protein concentrations of all cell extracts were determined by a modified Bradford assay (Bio-Rad) (7a) as recommended by the vendor. Equal amounts of infected cell proteins (50 μg) were separated in denaturing 15% *N,N′*-diallyltartardiamide–acrylamide gels and electrically transferred to nitrocellulose membranes in a tank apparatus (Bio-Rad) prior to immunoblotting with various primary antibodies. Unless otherwise noted in the text, all biochemical reagents were obtained from Sigma. Nitrocellulose was obtained directly from Schleicher & Schuell. Prestained protein molecular size markers were purchased from Life Technologies.

Immunological reagents. The following primary antibodies were used to detect viral proteins: (i) 1113, mouse anti-ICP27 monoclonal antibody (Goodwin Institute for Cancer Research, Plantation, Fla.); (ii) 1114, mouse anti-ICP4 monoclonal antibody (Goodwin); (iii) 1112, mouse anti-ICP0 monoclonal antibody (Goodwin); (iv) 1104, mouse anti-gC monoclonal antibody (Goodwin); (v) RGST22, rabbit polyclonal antibody directed against the ICP22 protein (2); and (vi) RGST49, rabbit polyclonal antibody directed against the VP22 protein (57). Immunoblotting experiments were performed to detect cellular apoptotic proteins by using mouse anti-caspase-3 monoclonal antibody (Transduction Laboratories, Inc.) and mouse anti-PARP monoclonal antibody (Pharmingen). Previous studies showed that PARP processing is first detected at 11 hpi and reaches maximum levels at 18 to 24 hpi (well within an HSV single-step growth) following infection with apoptotic viruses (4, 5). Thus, for comparisons between viruses, we looked specifically at 24 hpi. Secondary goat anti-rabbit and goat anti-mouse antibodies conjugated with alkaline phosphatase were purchased from Southern Biotechnology (Birmingham, AL).

Microscopy and quantitation of apoptotic cells with condensed chromatin. The phenotypes of the infected cells were documented by fluorescence and phase-contrast light microscopy. The numbers of apoptotic cells were determined as previously described (57). For analyses of chromatin condensation, cells were incubated at 23 hpi with the DNA dye Hoechst 33258 (Sigma) at a final concentration of 0.05 μg/ml for 15 min. Live cells were observed with an Olympus IX70/IX-FLA inverted fluorescent microscope, and images were acquired using a Sony DKC-5000 digital photo camera at a resolution of 600 to 800 dots per inch linked to a PowerMac and processed through Adobe Photoshop. The exact numbers of cells with condensed chromatin and fragmented nuclei (2), as well as the total numbers of cells in representative fields, were counted, and the percentages of apoptotic cells were calculated as follows: (number of apoptotic cells/total number of cells) × 100.

RESULTS

We previously reported that infections with either (i) a temperature-sensitive virus at its nonpermissive temperature which does not inject viral DNA into nuclei or (ii) various UV-inactivated wild-type viruses do not result in the induction of apoptosis (57). These findings imply that virus receptor binding/attachment to cells, membrane fusion, virion disassembly/tegument dispersal, virion RNA, and capsid translocation to nuclei are not responsible for the induction of apoptosis by HSV. The goal of this study is to define the initial trigger of HSV-dependent apoptosis.

IE gene expression and not protein synthesis is the initial trigger of HSV-1-dependent apoptosis. To confirm the necessity of IE gene expression in the induction of HSV-1-dependent apoptosis, human HEp-2 epithelial cells were mock, HSV-1(KOS), HSV-1(*d*109), or HSV-1(vBSΔ27) infected in the presence or absence of CHX. At 24 hpi, whole infected-cell extracts were prepared, separated on a denaturing gel, transferred to nitrocellulose, and probed with anti-ICP4, -PARP, and -caspase-3 antibodies as described in Materials and Methods. The results (Fig. 1) were as follows.

Infection of HEp-2 cells with wild-type KOS virus in the presence of CHX resulted in significant caspase-3 and PARP processing (Fig. 1, lane 4). This experiment demonstrates that de novo protein synthesis is not necessary for the initial induction step. Of course, it does not preclude potential viral proteins being responsible for later apoptosis progression under

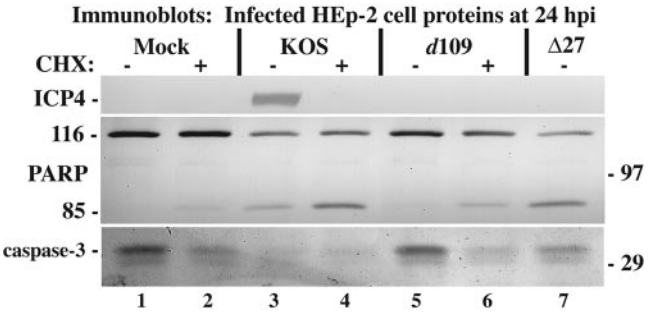

FIG. 1. HSV-1 IE gene expression, but not the ICP27 gene, is required for apoptosis induction. Mock-, HSV-1(KOS)-, *d*109-, or Δ27-infected (MOI of 5) HEp-2 cell extracts at 24 hpi, untreated (−) or treated with CHX (+), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antibodies to viral IE ICP4 and cellular (PARP and caspase-3) proteins as described in Materials and Methods. The anti-caspase-3 antibody used recognizes pro-caspase-3; loss of immune reactivity indicates caspase-3 activation. Under these CHX treatment conditions, no newly synthesized viral proteins were detected, with the exception of the previously described three slow-migrating IE ICP27 (triplet) forms (58). CHX treatment of uninfected cells activates caspase-3, but PARP remains predominantly uncleaved (4); thus, PARP processing during virus infection above that observed with mock infection plus CHX indicates apoptosis. "116" and "85" denote full-length (116,000-molecular-weight) and processed (85,000-molecular-weight) PARP, respectively. Locations of prestained molecular size markers (in kDa) are indicated on the right.

all conditions. Infection with Δ27, which contains a deletion of the IE ICP27 gene and cannot produce late viral proteins (59), also results in PARP and caspase-3 cleavage (Fig. 1, lane 7). In contrast, infection with *d*109, a recombinant virus devoid of all IE genes but expressing GFP from a heterologous promoter (53), yields results similar to those of mock infection (Fig. 1, compare lane 5 with lane 1), demonstrating the requirement of viral IE genes for triggering apoptosis (57). The amount of PARP cleavage with *d*109 in the absence of CHX is lower than that seen with mock infection in the presence of CHX (Fig. 1, compare lane 5 with lane 2). Slight amounts of cleaved PARP observed with *d*109 plus CHX may be due to an antiviral response, described earlier, which occurs with virus under conditions when IE proteins are not produced (19, 28, 38, 39, 42, 50, 57). Because *d*109 expresses GFP but not viral protein, events prior to viral IE gene expression, including receptor binding, membrane fusion, and capsid/tegument entry into the cytoplasm, are not sufficient to trigger apoptosis. It is important to note that both the *d*109 and Δ27 viruses possess deletions of the ICP27 gene. Detailed analyses of a series of recombinant viruses containing mutations throughout ICP27 led to the conclusion that ICP27's antiapoptotic function resides in its necessity for the synthesis of later antiapoptotic viral gene products (5). Since the Δ27 virus is capable of inducing apoptosis, these findings eliminate the ICP27 gene as being responsible for triggering apoptosis during HSV-1 infection.

HSV-1 IE ICP4 and ICP22 play roles in apoptosis prevention, rather than induction. To identify the HSV-1 apoptoxin, we systematically evaluated the contribution of each individual IE gene to stimulation of the cell death process. We initially analyzed the IE ICP4 and ICP22 deletion viruses, HSV-1(CgalΔ3) (31) and HSV-1(R7802) (44), respectively, for their abilities to induce cellular death factor processing, membrane blebbing, and chromatin condensation, as described in Materials and Methods. In the first set of experiments (Fig. 2), infection with CgalΔ3 induced more PARP processing than infection with its parental wild-type strain, HSV-1(17), in the absence of CHX (Fig. 2B, compare lane 5 with lane 3) and CgalΔ3 had significantly more cells with condensed chromatin (49% compared to 4%) (Fig. 2C). Addition of CHX to these infections resulted in enhanced PARP processing (Fig. 2B) and chromatin condensation (Fig. 2C). As expected, CHX addition precluded all viral protein synthesis except for previously described (58) ICP27 triplet forms (Fig. 2A, lanes 4 and 6). These results imply that ICP4 is involved in apoptosis prevention. Based on a detailed analysis of ICP27 mutant viruses, we previously concluded that ICP27 indirectly blocks apoptosis, likely by its regulatory functions (5). It is predicted that ICP4-null viruses would behave in a similar manner.

In the second set of experiments (Fig. 3), we observed that infection with R7802 induced more processed PARP (Fig. 3B, compare lane 5 with lane 3) and cells with condensed chromatin (37% compared to 4%) (Fig. 3C) than infection with its parental strain, HSV-1(F). Comparison of these results with those shown in Fig. 2 seems to indicate that infection with the ICP22-null virus yields the same amount of apoptosis as infection with a virus in which both copies of ICP4 are deleted (CgalΔ3). Since the levels of apoptosis detected with the parental viruses, HSV-1(17) and HSV-1(F), are equally negligible without CHX (e.g., compare Fig. 2B, lane 3, with Fig. 3B, lane 3), strain differences do not likely influence the results. R7802-infected cells had more condensed chromatin (64% compared to 37%) and showed significantly more cleaved PARP in the presence of CHX than in its absence (Fig. 3B, compare lane 6 with lane 5). Again, these findings imply that ICP22 is also involved in apoptosis prevention.

Consistent with these findings are the observations that infection with viruses either double deleted for ICP4 and the viral protein kinase U_S3 (36) or possessing a carboxy-terminal truncation of ICP22 (4) are defective in blocking apoptosis. While it was shown that ICP22 mutant viruses underexpress ICP0 (44), ICP4 mutant viruses overexpress some genes, including ICP0 (17, 18). However, since apoptosis occurred in CgalΔ3- and R7802-infected cells upon CHX treatment (Fig. 2 and 3), the ICP4 and ICP22 genes are not required for apoptosis induction during HSV-1 infection. Together, these results lead us to the conclusion that expression of one of the remaining viral IE genes must be responsible for viral induction of apoptotic cell death.

A recombinant virus devoid of IE ICP0 does not induce apoptosis. We next assessed the role of the viral IE α0 gene, which encodes ICP0, in the apoptotic triggering process. The ICP0 protein is a highly modified, multifunctional protein which plays a key role in the interactions of HSV-1 with its host cells (reviewed in references 20 and 26). ICP0 has been studied intensely in recent years, due in part to the discoveries of its (i) ability to rearrange PML/ND10 (21, 22), (ii) ubiquitin E3 ligase activity (7, 23, 27, 63), (iii) role in blocking the cellular antiviral response (19, 28, 38, 39, 42), and (iv) involvement in viral genome circularization (29), while certain issues in the ICP0 field are somewhat controversial (20, 61). HEp-2 cells were mock, HSV-1(KOS), HSV-1(7134), or HSV-1(vBSΔ27)

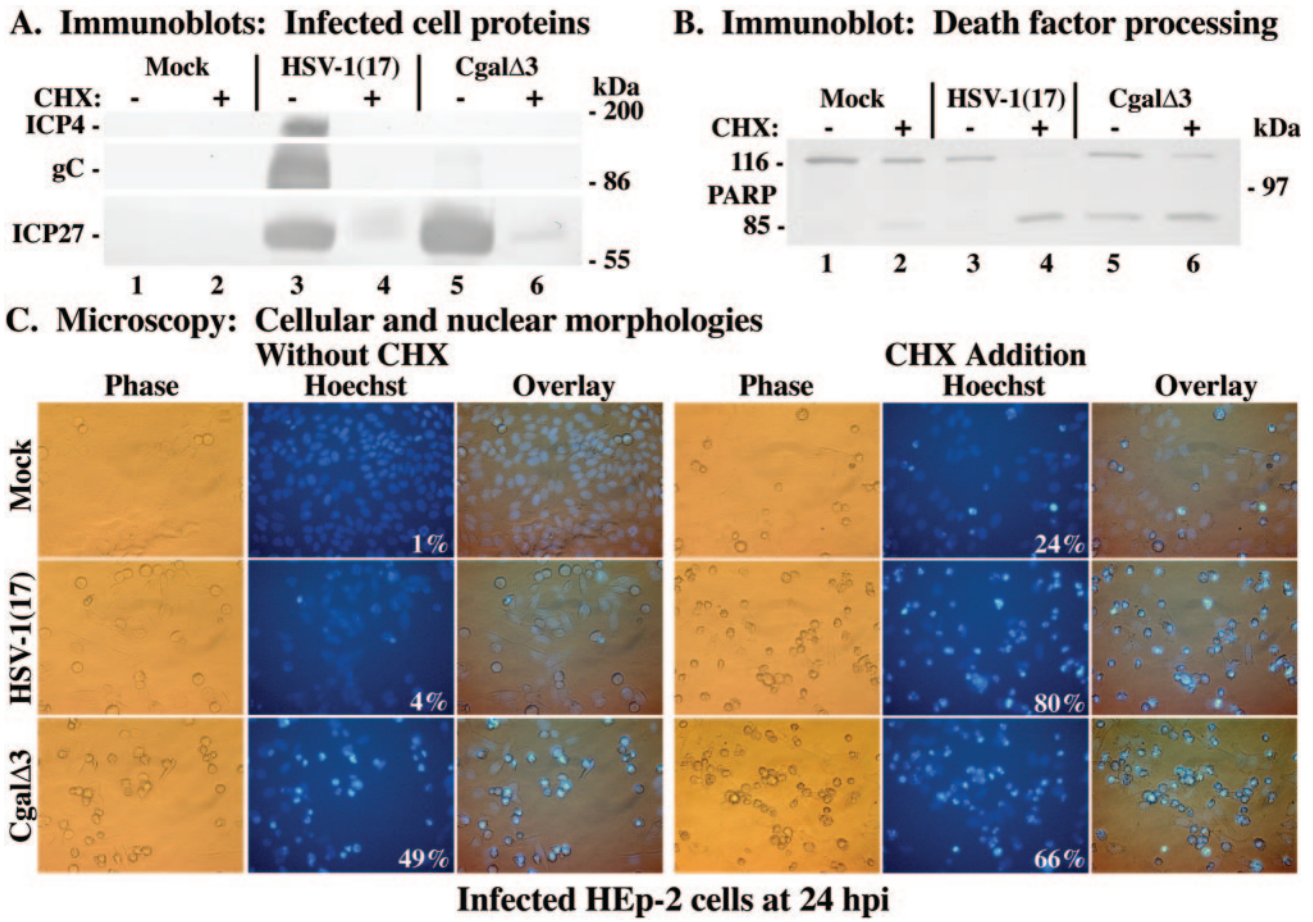

FIG. 2. The HSV-1 IE ICP4 gene is not required for apoptosis induction. Mock-, HSV-1(17)-, or CgalΔ3-infected (MOI of 5) HEp-2 cells, untreated (−) or treated with CHX (+), at 24 hpi were extracted, separated in denaturing gels, transferred to nitrocellulose, and reacted with antibodies to viral (IE ICP4, true-late gC, and IE ICP27) (A) and cellular (PARP) (B) proteins. Locations of prestained molecular size markers (in kDa) are indicated on the right. "116" and "85" denote full-length (116,000-molecular-weight) and processed (85,000-molecular-weight) PARP, respectively. Prior to extraction, cells were stained with Hoechst DNA dye and visualized by phase-contrast (Phase) and fluorescence (Hoechst) microscopy (C) and the percentages of cells with condensed chromatin were determined as described in Materials and Methods. Corresponding Phase and Hoechst panels were overlaid to produce merged images (Overlay). Original magnification, ×40.

infected in the presence or absence of CHX. At 24 hpi, cells were stained with Hoechst and visualized by microscopy, and whole infected-cell extracts were prepared, separated on a denaturing gel, transferred to nitrocellulose, and probed with anti-ICP4, -ICP0, -ICP27, -VP22, -PARP, and -caspase-3 antibodies, as described in Materials and Methods. Viruses which lack ICP0 can synthesize viral polypeptides at levels similar to those for wild-type virus at high MOI (Fig. 4A, compare lane 5 with lane 3), consistent with previous results (10), while viral protein synthesis and virus yields are reduced at low MOI (10, 11, 26).

Infection with the IE ICP0 deletion virus HSV-1(7134) (11) in the presence of CHX induced levels of PARP and caspase-3 processing similar to those observed for HSV-1(KOS)-infected cells without CHX (Fig. 4B, compare lane 6 with lane 3). Importantly, PARP processing in cells infected with 7134 plus CHX was greatly reduced compared to that in cells infected with KOS plus CHX (Fig. 4B, compare lane 6 with lane 4). As was observed with *d*109 (Fig. 1, lane 6), it is conceivable that the slight amount of cleaved PARP observed with 7134 plus CHX may be due to an antiviral response, described earlier, which is detected when IE gene expression is precluded (19, 28, 38, 39, 42, 50, 57). These levels were significantly lower than that seen with cells infected with the wild-type KOS virus under the same treatment conditions or with Δ27 alone. The lack of detection of ICP4 and ICP0 in apoptotic Δ27-infected cells (Fig. 4A, lane 7) is consistent with earlier time course studies (2). Microscopic examination of cells infected in the presence of CHX revealed that 69% of the wild-type-KOS-infected cells had characteristic apoptotic morphologies (membrane blebbing, cell shrinkage, and condensed chromatin), while only 29% of the 7134-infected cells displayed these features (Fig. 4C). These results suggest that transcription of the α0 gene is necessary for apoptosis induction by HSV-1. In addition, our findings may explain, in part, earlier reports that ICP0 appears toxic to cells (53, 54).

Expression of the IE ICP0 gene is both necessary and sufficient for the induction of apoptosis during HSV-1 infection. To confirm that α0 expression is sufficient for the induction of apoptosis, we used the recombinant virus HSV-1(*d*106) (53),

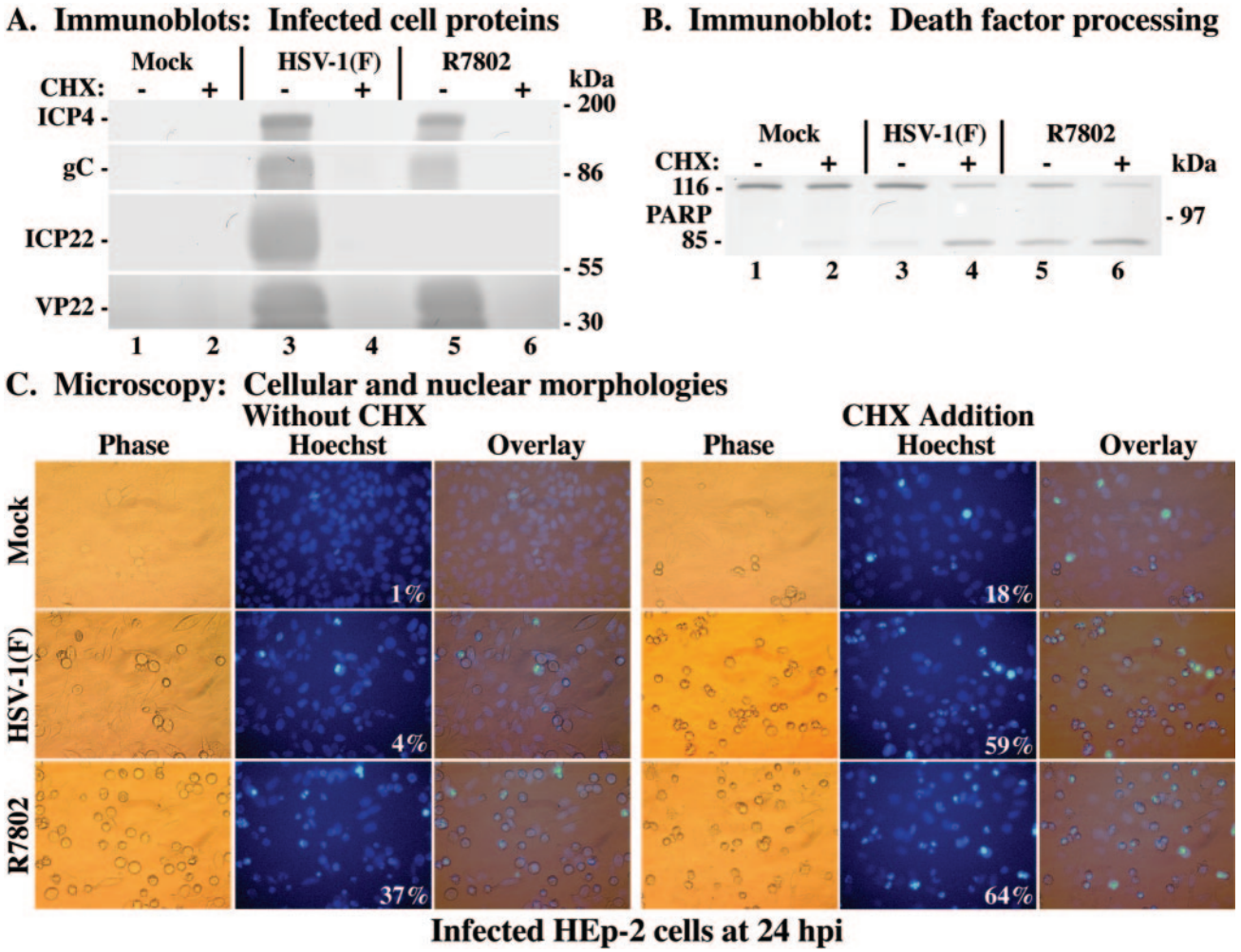

FIG. 3. The HSV-1 IE ICP22 gene is not required for apoptosis induction. Mock-, HSV-1(F)-, or R7802-infected (MOI of 5) HEp-2 cells, untreated (−) or treated with CHX (+), at 24 hpi were extracted, separated in denaturing gels, transferred to nitrocellulose, and reacted with antibodies to viral (IE ICP4, true-late gC, IE ICP22, and leaky-late VP22) (A) and cellular (PARP) (B) proteins. Locations of prestained molecular size markers (in kDa) are indicated on the right. "116" and "85" denote full-length (116,000-molecular-weight) and processed (85,000-molecular-weight) PARP, respectively. Prior to extraction, cells were stained with Hoechst DNA dye and visualized by phase-contrast (Phase) and fluorescence (Hoechst) microscopy (C) and the percentages of cells with condensed chromatin were determined as described in Materials and Methods. Corresponding Phase and Hoechst panels were overlaid to produce merged images (Overlay). Original magnification, ×40.

which produces only ICP0 (Fig. 5A, lane 7) and a GFP reporter gene (Fig. 5C). Infection with *d*106 in the absence of CHX yielded more PARP and caspase-3 processing than infection with *d*109 (Fig. 5B, compare lane 5 with lane 9). That there was more full-length PARP and pro-caspase-3 observed with *d*109 than with the ICP0-only *d*106 implies that *d*106 triggers apoptosis. Interestingly, the levels of death factor processing with *d*106 did not appear as high as those with the positive apoptotic control, Δ27 (Fig. 5B, compare lane 5 with lane 7). We also observed that 60% of *d*106-infected cells exhibited apoptotic morphological features, compared to 68% of the Δ27-infected cells (Fig. 5D). Since Δ27 does not accumulate ICP0 protein like *d*106 (Fig. 5A, compare lane 9 with lane 7), this result raises the intriguing possibility that the ICP0 protein may participate in preventing the apoptotic process from killing infected cells. Recent evidence of a role for ICP0 in ubiquitinating p53 (6) is consistent with our finding.

Importantly, we detected complete cellular death factor processing with *d*106 upon CHX treatment, which was identical to that observed with Δ27 (Fig. 5B, compare lane 6 with lane 8). In the presence of CHX, the number of *d*106-infected cells (Fig. 5D) exhibiting apoptotic morphologies (43%) exceeded those of the control mock- and *d*109-infected cells (11% and 17%, respectively) and approached the values for KOS- and Δ27-infected cells (60% and 59%, respectively), which also express the α0 gene. These results indicate that the de novo expression of the α0 gene is both necessary and sufficient to induce apoptosis in HSV-1-infected human cells.

To determine whether the α0 gene in the absence of other viral factors triggers apoptosis, we made use of the pSH plasmid, which contains the entire ICP0 coding region as well as 1.0-kb 5′ and 0.4-kb 3′ flanking sequences, as described previously (11). Since the *d*109 virus has lost its ability to induce apoptosis (Fig. 1) (57), the rationale of this experiment was to

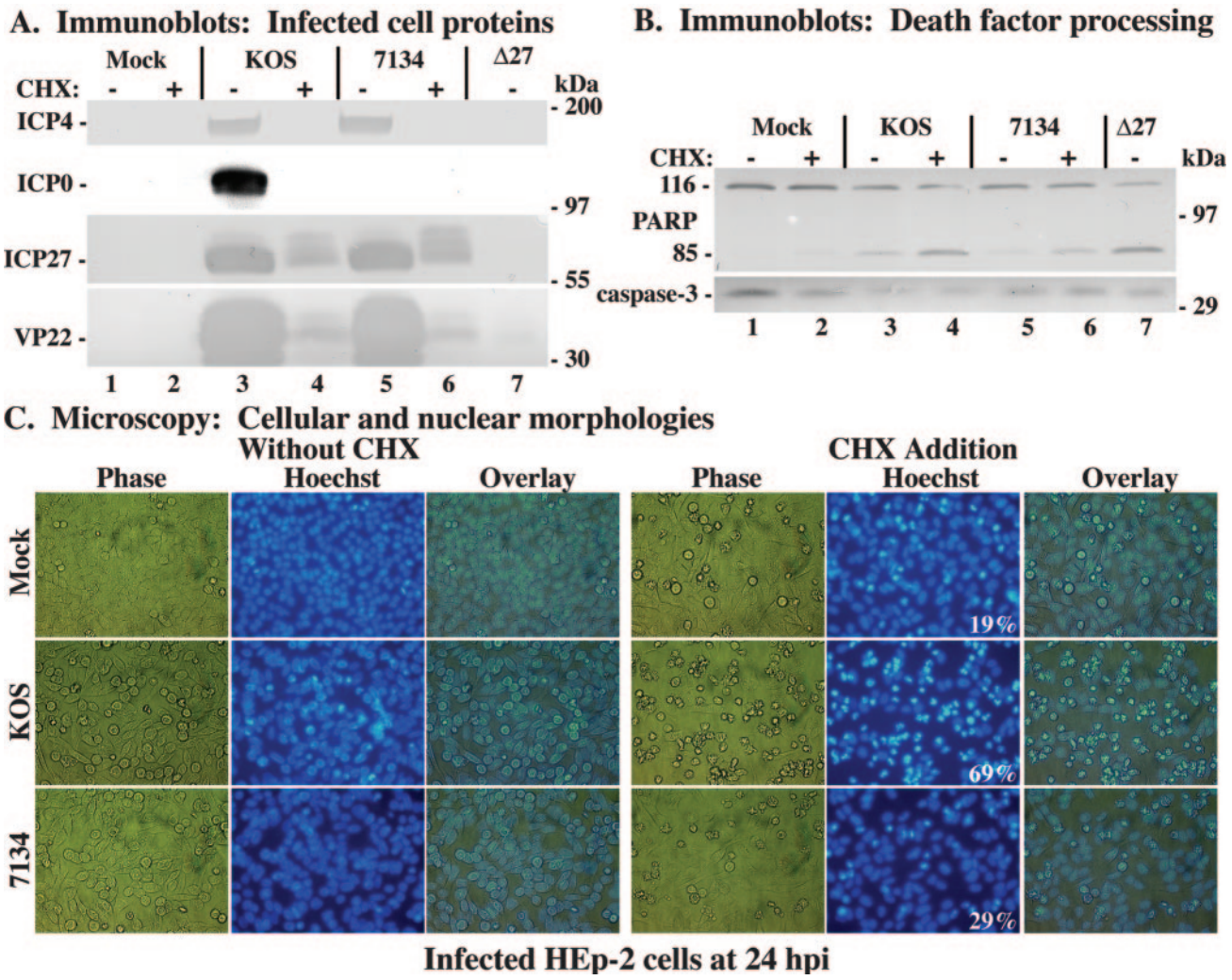

FIG. 4. Expression of the IE ICP0 gene is necessary for the induction of apoptosis during infection by HSV-1. (A) Cell extracts at 24 hpi from mock-, HSV-1(KOS)-, 7134-, or Δ27-infected HEp-2 cells, untreated (−) or treated with CHX (+), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antibodies to viral (IE ICP4, IE ICP0, IE ICP27, and leaky-late VP22) proteins. Slight amounts of incoming virion VP22 were detected in the presence of CHX. (B) Mock-, HSV-1(KOS)-, 7134-, or Δ27-infected cell proteins were immunoblotted with antibodies to cellular (PARP and pro-caspase-3) proteins. Locations of prestained molecular size markers (in kDa) are indicated on the right. "116" and "85" denote full-length (116,000-molecular-weight) and processed (85,000-molecular-weight) PARP, respectively. (C) The cells used to prepare the extracts shown in panels A and B were also examined by phase-contrast microscopy (Phase) to observe morphological changes. Staining of nuclear chromatin with Hoechst 33258 was visualized by fluorescence microscopy (Hoechst). Corresponding Phase and Hoechst panels were overlaid to produce merged images (Overlay). The exact numbers of cells with condensed chromatin and fragmented nuclei (2) were determined as described in Materials and Methods. Original magnification, ×40.

complement the defect in *d*109 and enable it to trigger apoptosis. HEp-2 cells were transfected at 60 to 70% confluence with 2.5 μg of the pSH plasmid (containing α0) or the empty vector control, plasmid pUC19, as described in Materials and Methods. At 24 h posttransfection, cells were mock infected or infected with *d*109 for 24 h, at which time whole-cell extracts were prepared for use in immunoblotting analyses with anti-ICP0, -actin (loading control), and -PARP antibodies (Fig. 6).

High levels of the viral ICP0 protein were detected in mock-infected cells transfected with pSH, and these were significantly increased in pSH-transfected cells infected with *d*109 due to transactivation by incoming virion VP16 (12, 49). No ICP0 was detected in cells transfected with pUC19, as expected. In pUC19-transfected cells, no PARP processing was observed. Interestingly, cells transfected with pSH showed detectable levels of processed PARP, with or without *d*109 infection. These levels of cleaved PARP were similar for both mock- and *d*109-infected cells transfected with pSH, even though increased amounts of ICP0 were detected following *d*109 infection. Thus, these results imply that α0 expression by itself is capable of inducing apoptosis in the absence of viral infection and suggest that the amount of PARP processing is limited by the transfection efficiency.

To determine whether the PARP cleavage seen with pSH-transfected cells was dependent upon transfection efficiency, we performed a titration experiment using various concentrations of the α0-containing plasmid DNA. HEp-2 cells were transfected at 60 to 70% confluence with 0.625, 1.25, 2.5, 5.0,

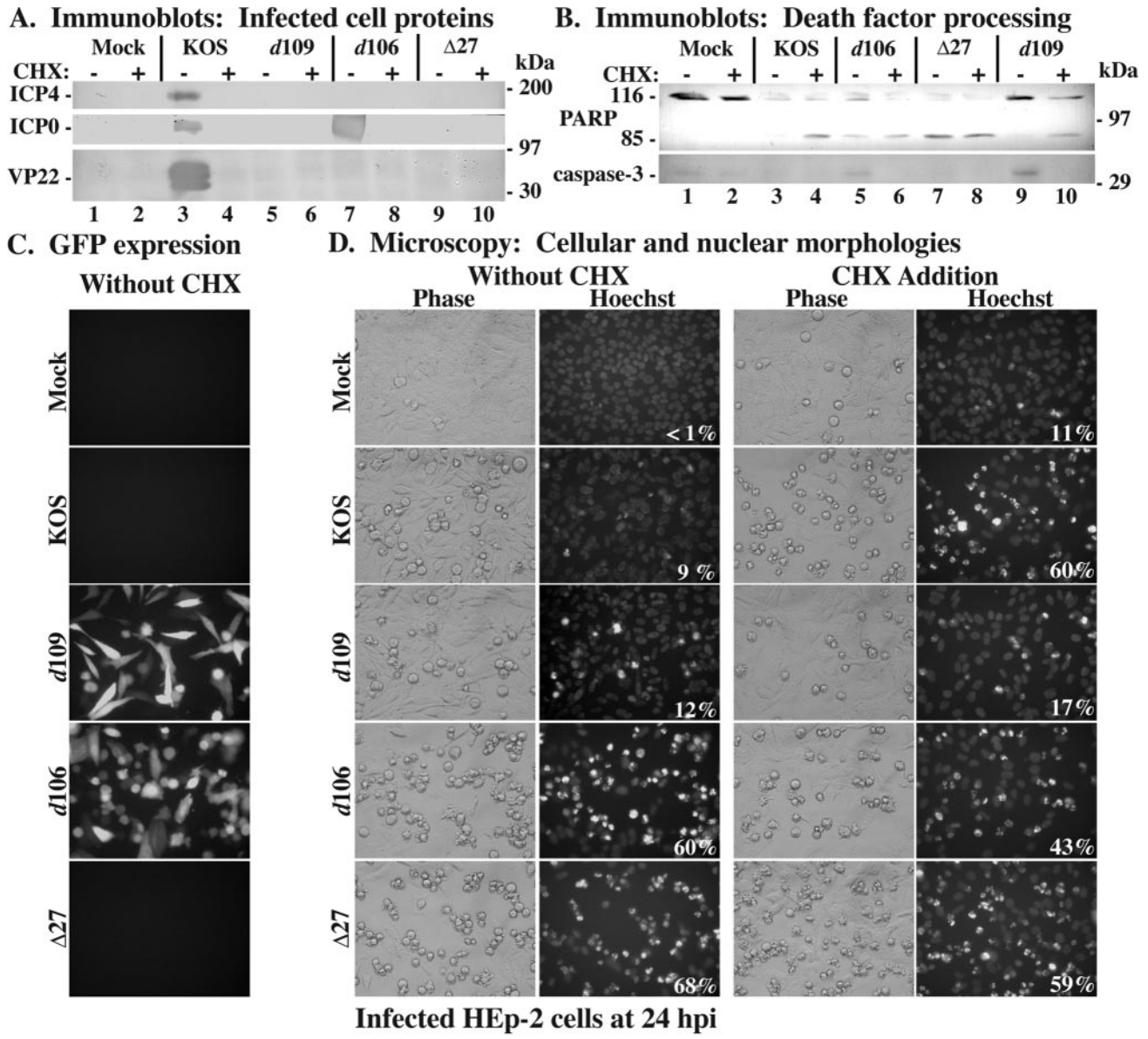

FIG. 5. Sole expression of the IE ICP0 gene during HSV-1 infection is sufficient to induce apoptosis. (A) Cell extracts at 24 hpi from mock-, HSV-1(KOS)-, *d*109-, *d*106-, or Δ27-infected HEp-2 cells, untreated (−) or treated with CHX (+), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antibodies to viral (IE ICP4, IE ICP0, and leaky-late VP22) proteins. (B) Mock-, HSV-1(KOS)-, *d*109-, *d*106-, or Δ27-infected cell extracts were reacted with antibodies to cellular (PARP and pro-caspase-3) proteins. Locations of prestained molecular size markers (in kDa) are indicated on the right. "116" and "85" denote full-length (116,000-molecular-weight) and processed (85,000-molecular-weight) PARP, respectively. (C) Expression of GFP in the infected cells (Without CHX) used to prepare the extracts shown in panels A and B was visualized by fluorescence microscopy. Since both *d*109 and *d*106 synthesize GFP, but only *d*106 triggers apoptosis, GFP expression does not trigger apoptosis. (D) The cells used to prepare the extracts shown in panels A and B were examined by phase-contrast microscopy (Phase) to observe morphological changes. Staining of nuclear chromatin with Hoechst 33258 was visualized by fluorescence microscopy (Hoechst). The exact numbers of cells with condensed chromatin and fragmented nuclei (2) were determined as described in Materials and Methods. Original magnification, ×40.

or 7.5 μg of pSH or pUC19. At 24 h posttransfection, cells were stained with Hoechst DNA dye and examined by phase-contrast and fluorescence microscopy, and whole-cell extracts were prepared for immunoblotting with anti-ICP0 antibody. The results (Fig. 7A) showed that progressively increasing amounts of transfected pSH DNA up to 5.0 μg correlated with increasing levels of ICP0 detected; ICP0 levels in cells transfected with 7.5 μg of pSH were slightly decreased compared to levels in cells transfected with 5.0 μg of pSH. In cells transfected with 0.625 to 5.0 μg of pSH, 4 to 25% of cells presented apoptotic features (Fig. 7B), which correlated with increasing amounts of transfected pSH DNA. While 25% of cells transfected with 5.0 μg of pSH showed hallmarks of apoptosis, only 15% of cells transfected with 7.5 μg of pSH did. Thus, α0

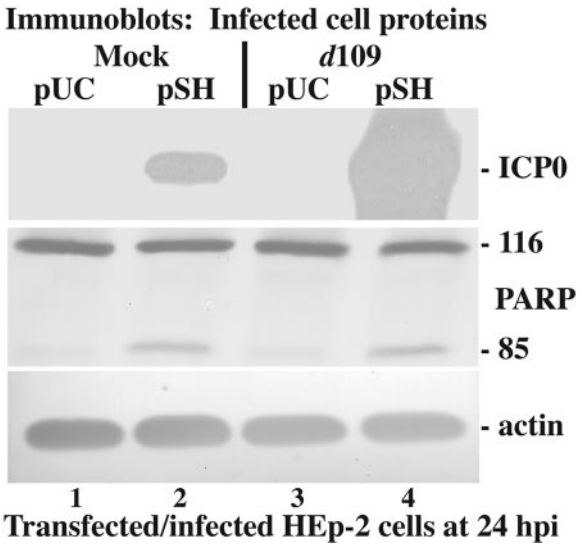

FIG. 6. Apoptosis in ICP0-transfected, *d*109-infected HEp-2 cells. HEp-2 cells were transfected with 2.5 μg of pUC (empty vector) or pSH (ICP0) plasmid and mock infected or infected with HSV-1(*d*109) at 24 h posttransfection. Whole-cell extracts were prepared at 24 hpi, separated in a denaturing gel, transferred to nitrocellulose, and probed with anti-ICP0, -actin, and -PARP antibodies as described in Materials and Methods. "116" and "85" denote full-length (116,000-molecular-weight) and processed (85,000-molecular-weight) PARP, respectively.

expression correlates with the percentage of cells displaying apoptotic morphologies.

HSV-1(*n*212), which contains a stop codon in both copies of the ICP0 gene, induced apoptosis when protein synthesis was inhibited. Our apoptosis results with the ICP0-only virus *d*106 in the presence of CHX strongly suggested that it is α0 gene expression that acts as the apoptotic trigger. The previous transfection studies (Fig. 6 and 7) showed a correlation between α0 gene dosage and cellular apoptotic features (chromatin condensation and membrane blebbing). However, due to the limitation of the transfection efficiency in these studies, we were unable to apply metabolic inhibitors to this transient expression system to convincingly assess the role of α0 gene expression (data not shown). Therefore, to confirm that expression of the α0 gene is required for the induction of apoptosis in HEp-2 cells, we made use of the recombinant virus HSV-1(*n*212), which contains a nonsense (stop) mutation in the α0 gene at codon 212 (11). The rationale of this experiment is that cells infected with *n*212 will express the complete α0 transcript but will not produce a full-length ICP0 protein. HEp-2 cells were mock infected or infected with HSV-1(KOS), HSV-1(7134), or HSV-1(*n*212) in the absence or presence of CHX. Whole-cell extracts were prepared at 24 hpi, and immunoblotting assays were performed using anti-ICP4, -ICP0, and -PARP antibodies. Prior to the preparation of whole-cell extracts, infected cells were stained with Hoechst DNA dye and cell morphologies were documented by phase-contrast and fluorescence microscopy, as described in Materials and Methods. The results (Fig. 8) were as follows.

Similar levels of the viral ICP4 protein were detected in KOS-, 7134-, and *n*212-infected cells in the absence of CHX, and no ICP0 was observed with 7134 and *n*212, as expected (Fig. 8A, lanes 3, 5, and 7). No viral proteins were detected in KOS-, 7134-, or *n*212-infected cells in the presence of CHX (Fig. 8A, lanes 4, 6, and 8). These findings indicate that the *n*212 virus is deficient in producing full-length ICP0 protein but otherwise synthesizes levels of ICP4 comparable to levels found in wild-type KOS. We observed a slight level of PARP cleavage in cells infected with KOS without CHX but not in cells infected with 7134 or *n*212 in the absence of CHX (Fig. 8B, compare lane 3 with lanes 5 and 7).

In the presence of CHX, cells infected with KOS showed almost complete PARP processing, while little to no PARP cleavage was observed with 7134-infected cells (Fig. 8B, compare lane 6 with lane 4). The level of PARP processing in CHX-treated cells infected with *n*212 was higher than that observed with 7134-infected cells treated with CHX (Fig. 8B, compare lane 8 with lane 6). This result suggests that the *n*212 virus induced apoptosis when protein synthesis was inhibited. With CHX addition, 80% of KOS-infected cells presented apoptotic hallmarks, compared to 39% of cells infected with *n*212 (Fig. 8C). In contrast, 7134-infected cells exhibited the same level of apoptotic morphologies as cells that were mock infected (23% and 24%, respectively). Thus, while both 7134 and *n*212 were unable to produce ICP0 protein (Fig. 8A), only *n*212 retained the ability to trigger the cell death process. Based on these results, we conclude that transcription of the α0 gene is required for induction of apoptosis during HSV-1 infection.

DISCUSSION

Based on our results, we propose that α0 gene expression acts as an initial inducer of apoptosis during HSV infection. To our knowledge, this is the first description of the induction of apoptosis in infected cells triggered as a result of transcription of a single defined viral gene. While the mechanism through which this RNA-mediated triggering occurs remains unclear, our findings may represent an important new process by which viruses mediate their interactions with host cells. This by no means excludes potential roles of other viral genes or gene products in the apoptosis induction process. That the levels of apoptosis observed with the α0 gene-expressing viruses *d*106 and *n*212 did not reach the levels of that with vBSΔ27 or KOS plus CHX seems to imply the need for an apoptosis progression factor. In fact, our recent discovery that optimal apoptosis in HSV-1-infected Vero cells requires the de novo synthesis of infected cell protein (41) strongly argues a role for additional facilitators of apoptosis progression beyond the initial α0 trigger. In addition, differences in apoptosis induction observed between HSV-1 and HSV-2 (64) are likely due to the involvement of such facilitators of apoptosis progression.

An alternative explanation for why *n*212 did not appear capable of inducing apoptosis as efficiently as KOS plus CHX also exists. The presence of the stop codon-containing linker insertion in the *n*212 mutant may alter the stability of the resultant α0 transcript. Consistent with this idea is the fact that the nonsense-mediated decay pathway protects cells from mutations or errors in transcription, which might produce truncated, potentially hazardous proteins, by the rapid degradation of mRNAs containing premature stop codons (14, 40, 62, 65).

One consistent (57) observation in our studies is that during

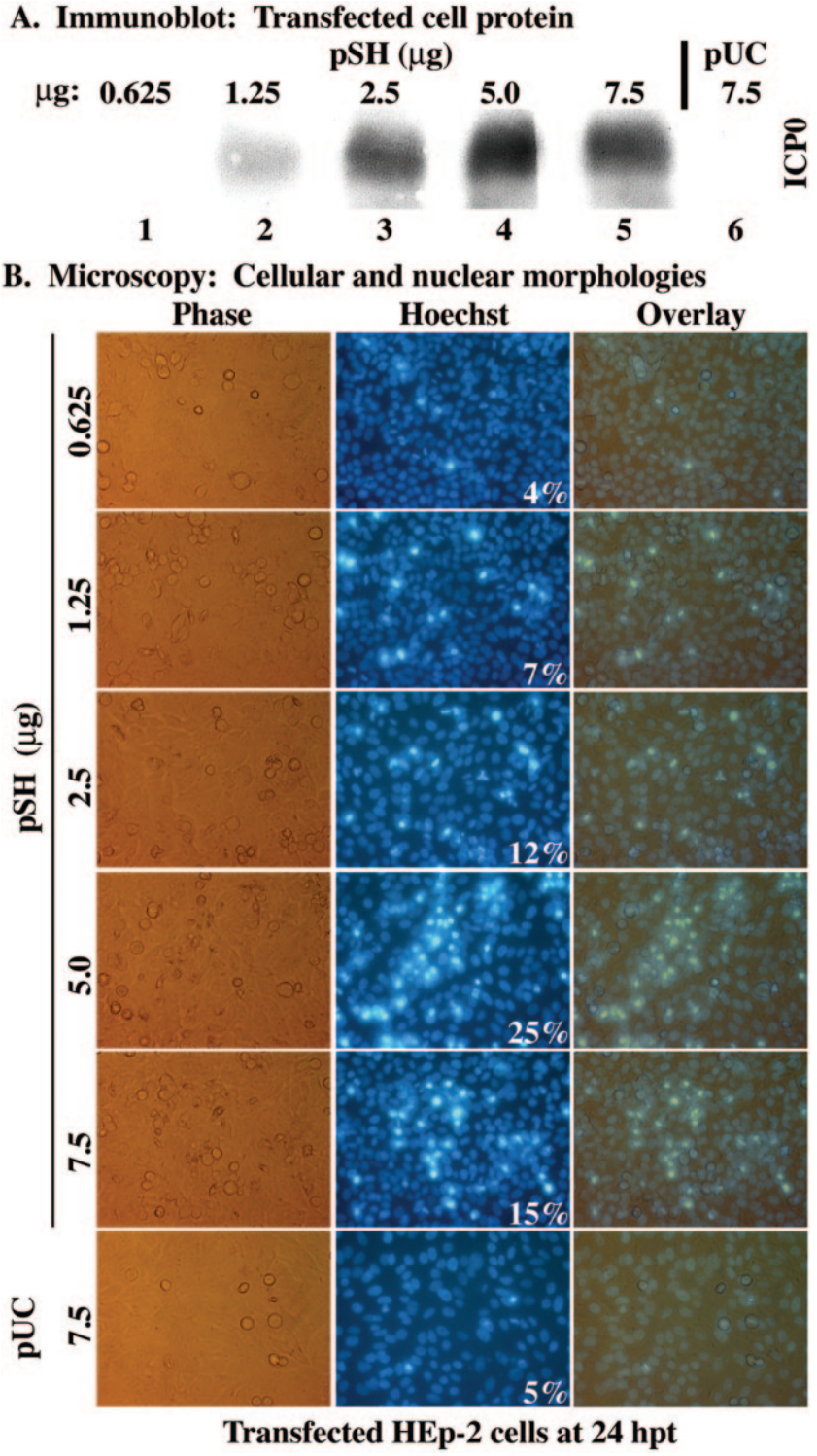

FIG. 7. Apoptosis in ICP0-transfected HEp-2 cells. (A) Immune reactivities of transfected cell proteins and (B) cell morphologies in HEp-2 cells transfected with 0.625, 1.25, 2.5, 5.0, or 7.5 μg of pSH (ICP0) or 7.5 μg of pUC (empty vector) plasmid. Whole-cell extracts were prepared at 24 h posttransfection, separated in a denaturing gel, transferred to nitrocellulose, and probed with an anti-ICP0 antibody (A) as described in Materials and Methods. (B) Phase-contrast (Phase), corresponding fluorescence (Hoechst), and merged (Overlay) images of pSH- and pUC-transfected cells were visualized at 24 h posttransfection as described in Materials and Methods. These cells were used to prepare the whole-cell extracts shown in panel A. The percentages of cells showing apoptotic condensed chromatin are shown. hpt, hours posttransfection. Original magnification, ×40.

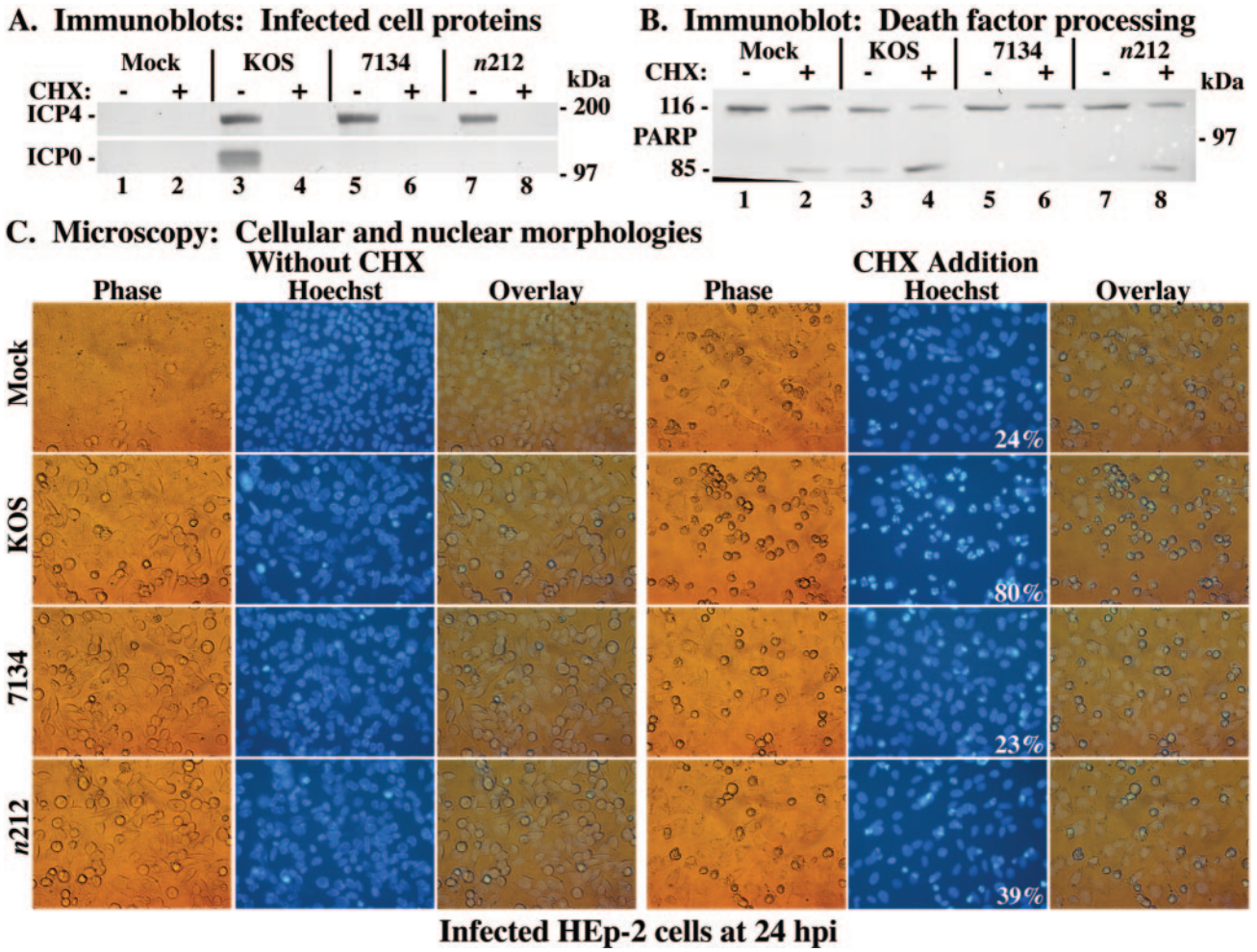

FIG. 8. Expression of the IE ICP0 gene, not the ICP0 protein, is necessary for the induction of apoptosis during infection by HSV-1. (A) Cell extracts at 24 hpi from mock-, HSV-1(KOS)-, 7134-, or *n*212-infected HEp-2 cells, untreated (−) or treated with CHX (+), were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antibodies to viral (IE ICP4 and IE ICP0) and cellular (PARP) (B) proteins. Locations of prestained molecular size markers (in kDa) are indicated on the right. "116" and "85" denote full-length (116,000-molecular-weight) and processed (85,000-molecular-weight) PARP, respectively. (C) The cells used to prepare the extracts shown in panels A and B were also examined by phase-contrast microscopy (Phase) to observe morphological changes. Staining of nuclear chromatin with Hoechst 33258 was visualized by fluorescence microscopy (Hoechst). Corresponding Phase and Hoechst panels were overlaid to produce merged images (Overlay). The exact numbers of cells with condensed chromatin and fragmented nuclei (2) were determined as described in Materials and Methods. Original magnification, ×40.

infections under conditions in which IE proteins are not produced and the α0 gene is not expressed (i.e., ICP0-null 7134 plus CHX and IE-null *d*109 infections), a slight amount of apoptosis is seen. While the basis of this minimal but detectable apoptosis is unknown, an obvious explanation exists. Numerous researchers have described an antiviral response which is initiated prior to IE gene expression and appears to require the ICP0 protein to block its effect (19, 28, 38, 39, 42, 50, 57). The key question here is what exactly the effect of this antiviral response is. The expectation is that if this antiviral response is not precluded it will eventually lead to a decrease in protein translation. Such a response would be expected to affect the level of cellular antiapoptotic factors, perhaps resulting in the slight levels of apoptotic features we could detect without α0 gene expression. An intriguing corollary to this theory comes from close inspection of the cells in question. Cells which are *d*109 infected contain GFP (readily seen in Fig. 5C) and mostly retain an elongated, flat phenotype. It actually appears that most of the cells exposed to *d*109 which possess condensed chromatin, membrane blebbing, and more-rounded, shrunken features do not contain GFP (compare Fig. 5C and D). Now, either these altered cells are so far gone that GFP is lost/not produced or the actual effects of the induced antiviral state are elicited in a paracrine manner on surrounding (perhaps yet uninfected) cells. Clearly, additional studies are needed to discriminate between these two possibilities.

Synthesis of a single HSV-1 RNA molecule, the latency-associated transcript, was suggested as a mechanism of neuronal cell survival during latent infection (1, 8, 45). An example of a defined proapoptotic viral protein is the virion protein number 3 (apoptin) of the single-stranded DNA chicken anemia virus, whose localization to nuclei correlates with apopto-

sis induction (43). At least two possible mechanisms exist to explain how the ICP0 RNA molecule may function proapoptotically. Molecular modeling studies using the mfold (http://www.bioinfo.rpi.edu/applications/mfold) algorithm (30) predict that potential hairpin formations exist in the transcribed α0 RNA molecule, as a consequence of the high G+C content of the HSV-1 genome (52), which might be recognized by cellular proteins and result in apoptosis induction. Because HSV-1 encodes an antagonist of the activated protein kinase R (15, 16), the α0 transcript could potentially play a role in the formation of double-stranded RNA and subsequent apoptosis induction. The recent discovery that human herpesvirus 4 (Epstein-Barr virus) and human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) encode microRNA molecules (47, 55) bolsters a functional role for viral double-stranded RNA in triggering apoptosis. It is important to note that recent computational algorithms have identified potential microRNA structures in the α0 gene (46), but their potential role in viral apoptosis remains unknown. The structure of the double-stranded DNA at the ends of the adeno-associated virus genome was also predicted to function in a proapoptotic manner (51).

That the induction of apoptosis is unique to ICP0 RNA suggests, however, a specific rather than a general response. Thus, pre-mRNA splicing events within the α0 transcript should also be considered. Few HSV-1 mRNAs are spliced, and α0 is the only spliced viral gene which contains two intron sequences (26), one of which may serve a regulatory function (48). It is conceivable that these intronic regions, whose excised lariat structures (56) can accumulate in infected-cell cytoplasm (13), may be sensed by the host cell machinery and ultimately lead to apoptosis induction. Other potential ICP0 RNA-based apoptotic induction mechanisms may also exist, including initiation, elongation, and termination of transcription; nuclear export events; and initial interactions with the host cell translational machinery. The development of appropriate biochemical and molecular genetic systems is required to define the mechanism by which α0 gene expression triggers the apoptotic cascade.

In summary, HSV-1 and perhaps other large genome DNA viruses induce apoptosis in infected cells through an RNA metabolic event associated with the transcription of a single viral gene. The existence of a viral RNA apoptotic trigger synthesized with IE kinetics implies that triggering apoptosis may serve a beneficial function for the virus during infection. The repertoire of tools that pathogenic human viruses utilize as they modulate the apoptotic cell death process to their advantage includes defined viral RNA molecules.

ACKNOWLEDGMENTS

We thank Elise Morton for expert technical assistance in growing the HSV-1(*d*109) virus and proofreading the manuscript, Neal Deluca for generously providing the HSV-1(*d*106) virus, and Priscilla Schaffer for generously providing HSV-1(7134), HSV-1(*n*212), and pSH.

These studies were supported in part by grants from the United States Public Health Service (AI 38873 and AI 48582 to J.A.B). C.M.S. is a predoctoral trainee and was supported in part by a United States Public Health Service Institutional Research Training Award (AI 07647).

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