UL31 of Herpes Simplex Virus 1 Is Necessary for Optimal NF-κB Activation and Expression of Viral Gene Products

Kari L. Roberts and Joel D. Baines*

Department of Microbiology and Immunology, Cornell University, Ithaca, New York 14853

Received 11 January 2011/Accepted 2 March 2011

Previous results suggested that the UL31 gene of herpes simplex virus 1 (HSV-1) is required for envelopment of nucleocapsids at the inner nuclear membrane and optimal viral DNA synthesis and DNA packaging. In the current study, viral gene expression and NF-κB and c-Jun N-terminal kinase (JNK) activation of a herpes simplex virus mutant lacking the UL31 gene, designated ΔUL31, and its genetic repair construct, designated ΔUL31-R, were studied in various cell lines. In Hep2 and Vero cells infected with ΔUL31, expression of the immediate-early protein ICP4, early protein ICP8, and late protein glycoprotein C (gC) were delayed significantly. In Hep2 cells, expression of these proteins failed to reach levels seen in cells infected with ΔUL31-R or wild-type HSV-1(F) even after 18 h. The defect in protein accumulation correlated with poor or no activation of NF-κB and JNK upon infection with ΔUL31 compared to wild-type virus infection. The protein expression defects of the UL31 deletion mutant were not explainable by a failure to enter nonpermissive cells and were not complemented in an ICP27-expressing cell line. These data suggest that pUL31 facilitates initiation of infection and/or accelerates the onset of viral gene expression in a manner that correlates with NF-κB activation and is independent of the transactivator ICP27. The effects on very early events in expression are surprising in light of the fact that UL31 is designated a late gene and pUL31 is not a virion component. We show herein that while most pUL31 is expressed late in infection, low levels of pUL31 are detectable as early as 2 h postinfection, consistent with an early role in HSV-1 infection.

The herpes simplex virus type 1 (HSV-1) virion, like that of all herpesviruses, consists of an envelope surrounding an icosahedral capsid shell which contains a double-stranded linear DNA genome. Between the proteaceous capsid and lipid envelope lies an assemblage of more than 20 viral proteins termed the tegument. Upon entry, some tegument proteins are released into the cytosol to help prime the cell for infection. For example, the virion host shutoff (vhs) protein degrades mRNA to favor viral gene expression (20, 26), and VP16 (viral protein 16, also designated α-TIF) redirects host transactivators to viral promoters (19, 25). Once the virus has entered the cell, the cytoplasmic DNA-containing capsid traffics toward the host nucleus by using the microtubule motor dynein (33). Upon engaging a nuclear pore, the HSV-1 genome exits the cell cytoplasmic DNA-containing capsid and enters the nucleoplasm (3, 34). Expression of viral genes is temporally regulated, starting with immediate-early and early genes (16, 17). Most α genes encode regulatory proteins. These include ICP4 (infected cell protein 4), which transcriptionally activates other genes, and ICP27, which augments viral gene expression at both pre- and posttranscriptional levels. Gene products from the β class (e.g., ICP8, an essential DNA binding protein) are involved in DNA replication, whereas the γ genes encode structural proteins, like the major capsid protein VP5. Viral DNA (vDNA) replication occurs during the transition from β to γ gene expression; the γ genes can be further subdivided into γ1 (leaky late) and γ2 (true late). This subdivision stems from a dependence on vDNA synthesis. If vDNA replication is blocked, such as with the use of the DNA polymerase inhibitor phosphonoacetic acid (PAA), γ1 gene expression is diminished and γ2 gene expression is precluded.

As the virus enters the cell, cellular signaling events mediate a transition in host cell functionality that favors viral propagation. For example, interaction of viral glycoprotein D (gD) with a tumor necrosis factor (TNF) receptor known as herpesvirus entry mediator (HVEM; also known as HveA and TNFRSF14) induces a transient activation of NF-κB (nuclear factor κB) (32). This induction, lasting approximately 2 h postinfection (hpi), is likely stimulated through the TNF receptor-associated factor (TRAF) signal transduction pathway and is dependent on cell type (e.g., the cell must express the appropriate receptor). It has also been reported that the tegument protein UL37 activates NF-κB through an interaction with TRAF6 (22). In addition to this transient activation of NF-κB, there is a second wave of NF-κB activation that requires de novo HSV-1 gene expression (2). This wave of NF-κB activation initiates at approximately 6 hpi and has been shown to require the α gene product ICP27 (9). NF-κB is a transcriptional regulator found in almost every cell type and is normally activated in response to cell stress, such as inflammation or viral infection (reviewed in references 11, 12, and 18). While some viruses block NF-κB activation (for a review, see reference 14), HSV-1 requires activation of NF-κB for efficient infection (24). This requirement has been demonstrated by use of dominant negative repressors (1, 8, 24), knockout cell lines (8), and inhibitory drugs (6).

This study focuses on UL31 of HSV-1. Deletion of UL31 decreases viral titers to various degrees in different cell lines: up to 1,000-fold in Vero cells, and at least 10-fold in rabbit skin...
cells (RSC) (21). pU31 (the gene product of U31) plays an important role in nuclear egress, because viral capsids largely fail to bud through the inner nuclear membrane in cells infected with U31 deletion mutants (4, 28). A similar phenotype is observed upon deletion of pU31’s interaction partner, pU34 (31), an integral membrane protein that localizes to the inner nuclear membrane in wild-type virus infections. Nuclear rim targeting of pU31 is dependent on expression of pU34. Without this binding partner, the bulk of pU31 localizes in the nucleoplasm (28). Both U31 and U34 are also required for alteration of the nuclear lamina (27), which is composed largely of a meshwork of intermediate filaments lining the inner surface of the inner nuclear membrane. The nuclear lamina is required for maintaining the structure of the nucleus, and its pU31/pU34-dependent perforation during HSV infection is believed to promote access of nucleocapsids to the inner nuclear membrane for envelopment. Both pU34 and pU31 are incorporated into the virion during budding through the inner nuclear membrane into the perinuclear space. The envelope of this perinuclear virion is lost upon fusion with the outer nuclear membrane as the capsid enters the cytosol; thus, pU31 is not a component of the final extracellular virion (7, 23, 29). Deletion of U31 also diminishes vDNA replication and packaging (4).

In the current study, U31 expression was found to be required for efficient NF-κB activation and optimal viral protein expression. The latter observation may help explain pU31’s effects on DNA packaging, which require late viral gene products. The effects on gene expression were shown to be independent of pU31’s interaction partner pU34. Although U31 has been designated a true late gene based on sensitivity to quench autofluorescence. The cells were permeabilized with 0.1% Triton X-100 for 2 min followed by a 10-min block in 10% human serum in PBS. Mouse anti-ICP4, Rumbaugh-Goodwin Institute for Cancer Research, Plantation, FL), rabbit anti-phospho-Jun N-terminal kinase (p-JNK; 1:1,000; Santa Cruz Biotechnology sc7929). Diluted primary antibodies were reacted with blocked membrane for either 1 h at room temperature or overnight at 4°C with gentle agitation. After extensive washing in PBS, bound primary antibody was detected by addition of anti-mouse (1:2,000; Santa Cruz Biotechnology) or anti-rabbit (1:2,000; Santa Cruz Biotechnology) horseradish peroxidase-conjugated secondary antibody diluted in 5% milk–TBST (1:2,000) for 15 min at room temperature. The following primary antibodies were diluted 1:100 in 1% BSA–PBS: mouse IgG (Jackson Laboratories) diluted 1:100 in 1% BSA–PBS. The coverslips were then washed 3 times in PBS and dipped in double-distilled H2O before mounting on glass slides with mowiol plus 2.5% DABCO to prevent photo-bleaching. All digital images were taken using a Zeiss Axio Imager M1 fluorescence microscope and were processed with Adobe Photoshop software.

Drug treatments. In some experiments, Hep2 cells in a six-well dish were pretreated with 10 μM cycloheximide (CHX) for 1 h at 37°C, 5% CO2 or left untreated. After 1 h, cells were washed with versene (PBS plus 0.5 mM EDTA) and infected with wild-type HSV-1(F) at a multiplicity of infection (MOI) of 10 PFU/cell or mock infected. Ten μM CHX was included with the added virus for the appropriate wells, and the cells were rocked slowly for 1 h at 37°C to allow for viral attachment and entry. After the 1-h incubation, the virus or mock overlay was removed, the cells were washed with versene, and fresh 199V was added back to the cells (containing 10 μM CHX where appropriate). At time zero the cells were returned to the 37°C 5% CO2 incubator until collected.

For experiments using PAAs, Hep2 cells in a six-well dish were infected with HSV-1(F) at an MOI of 10 PFU/cell or mock infected. At the start of infection, 200 μg/ml PAA was added to the appropriate wells. Cells were washed 12 h later, and total cell lysates were collected. For all drug treatment experiments, total cell lysates were collected as described in “Immunoblot analyses” above.

MATERIALS AND METHODS

Viruses and cells. Wild-type HSV-1 strain F [HSV-1(F)], ΔU31, and ΔU31-R were provided by Bernard Roizman and have been described previously (4, 5). ΔU34 was provided by Richard Roller and has been described previously (31). d27-1, the ICP27-null virus, was provided by the Goodwin Institute for Cancer Research, Plantation, FL, and total cell lysates were collected. For all drug treatment experiments, total cell lysates were collected as described in “Immunoblot analyses” above.

RESULTS

Infections with ΔU31 but not ΔU34 are delayed for α, β, and γ gene expression. It has been reported that ΔU31 infections result in decreased viral DNA accumulation, cleavage, and packaging (4). The current studies were initiated to determine how pU31 might contribute to these activities, with the hypothesis that U31 might augment viral gene expression. Because pU31 interacts with pU34, we included a ΔU34 virus in the experiment to determine if any detected phenotype was dependent upon the pU31-pU34 interaction. Hep2 cells were mock infected or infected with wild-type HSV-1(F), ΔU31, or ΔU34 viruses at an MOI of 10 PFU per cell. At 4 and 18 hpi, total cell lysates were collected and proteins were separated by SDS-PAGE as described in Materials and Methods. The results are shown in Fig. 1. For this and subsequent immunoblot assays, quantification was done using Image J. Similar results were noted in Hep2 cells infected with the U31 deletion virus at up to 50 PFU per cell (data not shown).

Surprisingly, immunoblotting (Fig. 1) showed that essen-
mock-infected cells, whereas ΔU_{131}-infected cell lysates contained protein levels of IκBa at 85% of the mock level. These data indicate that U_{131} plays a role in ensuring elimination of IκBa in infected cells, and thus activation of NF-κB.

We also examined relative levels of activated p-JNK and found that at 18 hpi the ΔU_{131} virus induced levels of p-JNK that amounted to only 28% of the level detected from the wild-type HSV-1(F) infection. In contrast, levels of total JNK were similar in all lanes. Taken together, the results indicate that in addition to a viral protein expression deficiency, the ΔU_{131} virus exhibits deficiencies in NF-κB and JNK activation that are normally seen during HSV-1 infection.

NF-κB and JNK activation are downregulated in ΔU_{131} infections of different cell types. To determine if NF-κB and JNK activation seen in the ΔU_{131}-infected Hep2 cells (Fig. 1) occurred in other cell types, RSC, Vero, HeLa, and Hep2 cells, as well as the ΔU_{131}-complementing cell line, clone 7 (an RSC derivative) were mock infected or infected with the ΔU_{131}, HSV-1(F), or ΔU_{131}-R (repair) viruses. All cell types were infected at an MOI of 10 PFU per cell, and total cell lysates were collected at 4, 8, 12, and 18 hpi as described in Materials and Methods. Sample loading was assessed by probing with an antibody directed against lamin A/C. Although the amounts loaded in each lane were consistent within samples from a given cell line, they varied somewhat between cell lines. Nevertheless, the consistency of loading between samples from each cell type enabled comparisons of protein expression by the different viruses in each cell type examined.

Overall, the results indicated a positive correlation between defects in protein synthesis in ΔU_{131} infections (Fig. 1) and both decreased JNK and NF-κB activation in the various cell lines (Fig. 2) as follows.

In the nonpermissive Hep2 and HeLa cell lines, IκBa levels were similar upon infection with all viruses at 4 hpi, whereas at later time points IκBa was downregulated to 36% and 34% of mock-infected cell levels in HSV-1(F) and ΔU_{131}-R, respectively. However, in cells infected with ΔU_{131}, IκBa was downregulated to only 78% of mock levels. The differences were accentuated at 18 hpi, when IκBa was downregulated to 15% and 7% of mock-infected cell levels for HSV-1(F) and ΔU_{131}-R, respectively, but downregulated to only 76% of mock levels in the ΔU_{131} infection.

In HeLa cells, activated JNK was first detected at 8 hpi in cells infected with HSV-1(F) and ΔU_{131}-R, but in cells infected with ΔU_{131} the p-JNK level was only 8% of that in cells infected with ΔU_{131}-R. Even at 18 hpi, the p-JNK level in ΔU_{131}-infected cells was only 15% of that in the ΔU_{131}-R infection. Although activated JNK was never abundant in Hep2 cells, it was first detected at 8 hpi with the wild-type virus. p-JNK levels were significantly lower at later time points (8%, 3%, and 0.4% of p-JNK levels in cells infected with ΔU_{131}-R at 8, 12, and 18 hpi, respectively).

Vero cells, unlike Hep2 and HeLa cells, exhibited easily detectable phosphorylated JNK as early as 4 hpi, although levels in cells infected with ΔU_{131} were 46% of the level observed upon infection with ΔU_{131}-R. The differences in

FIG. 1. Delayed protein expression in Hep2 cells infected with ΔU_{131} but not ΔU_{34} viruses. Hep2 cells were infected with 10 PFU per cell of HSV-1(F), ΔU_{31}, or ΔU_{34} or were mock infected. Total cell lysates were collected at 4 and 18 hpi. Proteins were denatured and separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with antibodies against ICP4, ICP8, gC, p-JNK, total JNK, IκBa, and lamin A/C, which was used as a loading control. Bound antibodies were revealed by reaction with appropriately conjugated antibodies, followed by chemiluminescence and exposure to X-ray film as described in Materials and Methods.
activated JNK levels decreased over time, such that by 18 hpi, the level of JNK activation in Vero cells infected with ΔUL31 was 92% of that in Vero cells infected with ΔUL31-R. In general, IκBα levels in Vero cells were inversely correlated with those of activated JNK. Although the IκBα level was higher by 8 hpi with ΔUL31 (22% of mock) than upon infection with the repair virus (0.9% of mock), differences among the different viral infections were negligible by 12 hpi (8% for F, 61% for ΔUL31, and 47% for ΔUL31-R relative to mock). We conclude that Vero cells infected with ΔUL31 are more permissive with respect to NF-κB and JNK activation than either Hep2 or HeLa cells infected with this virus.

Immunoblot signals of IκBα were not as strong in RSC as in the other cell lines examined. Nevertheless, this cell line contained more IκBα when mock infected or infected with ΔUL31 than in cells infected with the wild-type HSV-1(F) or ΔUL31-R. Compared to mock infection levels, IκBα levels in HSV-1(F), ΔUL31, and ΔUL31-R were 19%, 167%, and 21%, respectively. The differences in IκBα levels in the different virus infections were largely eliminated by 12 hpi, as the percentage of detectable IκBα relative to the mock level were 3%, 16%, and 2% for HSV-1(F), ΔUL31, and ΔUL31-R, respectively. Very little activated JNK (p-JNK) was detected in RSC at any time point. Thus, rabbit skin cells were more permissive with respect to NF-κB and JNK activation in the context of ΔUL31 than either Hep2 or HeLa cells.

In clone 7 cells, which partially rescue the UL31 deletion virus (21), less activated JNK and more IκBα were observed after infection with ΔUL31 than with the other viruses. Specifically, the IκBα levels were only 11% relative to mock levels in infections with the ΔUL31-R virus, but 63% with ΔUL31 at 8 hpi. At 4 and 8 hpi, p-JNK levels in cells infected by ΔUL31 were 16% and 45%, respectively, compared to levels in infections with the repair virus. Relevant to this observation, we have noted a delay in viral protein expression in clone 7 cells infected with ΔUL31 (data not shown) and an inability of these cells to restore replication to the level seen upon infection with ΔUL31-R (21). Thus, the incomplete rescue of ΔUL31 replication and protein expression in this cell line correlates with poor JNK and NF-κB activation.

ICP27 provided in trans does not rescue the observed defects of ΔUL31-infected Vero cells. Previous reports suggested that loss of ICP27 could produce similar defects to those we have observed in characterization of the UL31 deletion mutant (9, 10). We therefore examined NF-κB and JNK activation and also gC expression in an ICP27-expressing cell line (V27) infected with ΔUL31, HSV-1(F), ΔUL31-R, or d27-1 (ICP27-null) viruses. Vero cells were used as a control, because V27 is derived from this cell type (30). The results are presented in Fig. 3.

In both Vero and V27 cells, JNK activation was decreased in the ΔUL31 infection relative to wild-type HSV-1(F) levels. The presence of ICP27 provided in trans (V27 cells) partially rescued JNK activation in both the ΔUL31 and ICP27-null virus (d27-1). Specifically, p-JNK protein levels in the ΔUL31 and d27-1 infections increased from 15% and 31% (respectively) of wild-type levels at 4 hpi to 50% and 67% (respectively) of wild-type levels at 8 hpi. In contrast, p-JNK protein levels in Vero cells remained at similar levels in cells infected with ΔUL31 compared to wild-type infection at 4 and 8 hpi (66% and 59% of wild-type levels, respectively).

ICP4 expression in ΔUL31-infected Vero and V27 cells was dramatically reduced compared to wild-type infections in these cell types. At 4 and 8 hpi, ICP4 protein levels in the ΔUL31 Vero infections were 0.6% and 54% of wild type, respectively.
The presence of ICP27 provided in \textit{trans} failed to rescue ICP4 expression for both ΔU131 and ICP27-null (d27-1) infections by 8 hpi. In ΔU131 and d27-1 infections in V27 cells, ICP4 protein levels were only 17% and 8% of wild-type infection levels (respectively) at 4 hpi and increased to only 23% and 11% of wild-type infection (respectively) at 8 hpi.

Lysates from ΔU131-infected Vero and V27 cells contained readily detectable protein levels of IxBo at 8 hpi (70% and 119% of mock infection levels, respectively), whereas wild-type infections resulted in IxBo levels that were only 32% and 16% of those observed in mock infection. This is an indication that NF-κB was not activated normally in the ΔU131 infections. ICP27 provided in \textit{trans} was sufficient to rescue NF-κB activation in the ICP27-null virus by 8 hpi, since the IxBo protein level from the d27-1 infection was only 10% of the mock level. We conclude that ΔU131's defects in protein expression and NF-κB and activation cannot be complemented in an ICP27-expressing cell line, and JNK activation is only partially rescued. This suggests that U131's contribution to activation of NF-κB and JNK is mostly independent of ICP27.

**Infection with ΔU131 leads to delayed expression of ICP4 but not failure to initiate infection.** One possible explanation of the current results is that the ΔU131 virus is unable to efficiently enter or otherwise infect cells. In this scenario, the observed phenotypes might simply reflect a large number of uninfected cells in the population. To address this possibility, we infected the restrictive Hep2 cell line with the ΔU131 or ΔU131-R viruses at an MOI of 10 PFU per cell. Cell lysates were collected at 4 and 8 hpi, and the denatured proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with antibodies against ICP4, ICP27, p-JNK, and IxBo. Lamin A/C was used as a loading control.

The order in lanes 1 to 4 was modified from the original blot to match the order of loaded samples run previously. Bound antibodies were revealed by reaction with appropriately conjugated antibodies, followed by chemiluminescence and exposure to X-ray film as described in Materials and Methods.

**FIG. 4. Infection with ΔU131 leads to delayed expression of ICP4 but not failure to initiate infection.** (A) Hep2 cells were infected at an MOI of 10 PFU per cell of either ΔU131 or the genetic repair construct, ΔU131-R, and were fixed at 1, 6, or 10 hpi in PFA, permeabilized, and stained with antibody to ICP4 (green) to visualize infected cells. The cells were counterstained with Hoechst dye (blue) to visualize cellular nuclei. (B) Quantification of the ratio of ICP4-expressing cells to total cells infected with ΔU131-R or ΔU131 (MOI, 10 PFU per cell) at 6 and 10 hpi. Student’s \( t \) test showed a statistically significant difference in ICP4 expression in the ΔU131-R and ΔU131-infected cells at 6 hpi (\( P < 0.001 \), indicated by the asterisk) but not 10 hpi (\( P > 0.05 \)). The same statistical test showed a statistically significant difference in ICP4 expression in ΔU131-infected cells fixed at the 6- and 10-hpi time points (\( P < 0.01 \), indicated by the asterisk). ΔU131-R infected cells exhibited ICP4-specific immunofluorescence, whereas only a few cells infected with ΔU131 displayed such immunostaining. At 10 hpi, many ICP4-positive cells were found throughout the ΔU131-R- and ΔU131-infected cell monolayers. These observations are consistent with the data presented above, indicating that cells infected with wild-type viruses express high levels of ICP4 at early time points, whereas ICP4 expression in ΔU131-infected cells starts at very low levels that increase with time postinfection. The gradual increase in ICP4-expressing cells upon infection with the U131 deletion virus cannot be a consequence of cell-to-cell spread within the culture because (i) this would not be expected at 6 hpi and (ii) the U131 deletion virus cannot exit noncomplementing cells. A quantification of infection efficiency at 6 hpi and 10 hpi is shown in Fig. 4B. Student’s \( t \) test demonstrated that the difference in the number of ICP4-expressing cells of total cells was statistically significant (\( P < 0.001 \)), whereas this difference was no longer statistically different at 10 hpi (\( P > 0.05 \)). The difference at the 6- and 10-hpi time points in ΔU131-infected cells was also statistically dif-

**FIG. 3. ICP27 in the V27 cell line does not rescue the protein expression deficits of ΔU131 at early times after infection.** Vero cells or the Vero-derived ICP27-expressing cell line V27 were infected with HSV-1(F), ΔU131, ΔU131, or d27-1 or mock infected at an MOI of 10 PFU per cell. Total cell lysates were collected at 4 and 8 hpi, and the denatured proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with antibodies against ICP4, ICP27, p-JNK, and IxBo. Lamin A/C was used as a loading control. The order in lanes 1 to 4 was modified from the original blot to match the order of loaded samples run previously. Bound antibodies were revealed by reaction with appropriately conjugated antibodies, followed by chemiluminescence and exposure to X-ray film as described in Materials and Methods.
different (P < 0.01), indicating that a significant number of cells transitioned from not expressing ICP4 at detectable levels to detectable expression over the course of 4 h. We conclude that the defects in ICP4 expression observed in cells infected with the U_{31} deletion were due to a delay in the onset of expression, rather than a failure of the virus to enter and infect a subset of cells.

**U_{31} expression is detectable early in infection.** The effects of pU_{31} in early protein expression were seemingly incongruous with its sole expression late in infection. To test whether or not U_{31} is only expressed late in infection, Hep2 cells were treated with 10 μM CHX or left untreated for 1 h. The cells were then infected with wild-type HSV-1 (MOI, 10) or mock infected. For CHX-treated cells, the CHX level was maintained at all steps of infection. Total cell lysates were collected at 1, 2, 3, and 5 h postinfection, and proteins were separated by SDS-PAGE. The immunoblot was reacted with antibody to pU_{31}, ICP4, or lamin A/C. Bound antibodies were revealed by reaction with appropriately conjugated antibodies, followed by chemiluminescence and exposure to X-ray film as described in Materials and Methods.

The data indicate that U_{31} is expressed at detectable levels as early as 2 hpi. The kinetics of pU_{31} expression early in infection correlated with those of ICP4 expression, whereas at 5 hpi, the amount of pU_{31} increased more dramatically than those of ICP4. Importantly, little or no protein was visible in the CHX-treated lysates, indicating that, as expected, the early detection of pU_{31} is due to active transcription/translation and was not a consequence of its presence within the inoculum. These data indicate that U_{31}, previously thought to be expressed only at late times after infection, has low-level expression at early times after infection.

**U_{31} expression late in infection is PAA sensitive.** Since true late genes are sensitive to DNA polymerase inhibitors, we asked whether pU_{31} expression late in infection was dependent on vDNA synthesis (i.e., sensitive to the DNA polymerase inhibitor PAA). To test this possibility, Hep2 cells were infected with wild-type HSV-1(F) (MOI, 10) or were mock infected in the presence or absence of 200 μg/ml PAA. At 12 h postinfection total cell lysates were collected and proteins separated by SDS-PAGE. The immunoblot was reacted with antibody to pU_{31} or lamin A/C as a loading control. The results are presented in Fig. 6.

The presence of PAA significantly diminished pU_{31} expression in HSV-1-infected cells. These data are consistent with U_{31} expression as a late gene but are not inconsistent with an important role(s) of pU_{31} early in infection.

**DISCUSSION**

Besides its well-documented role in nuclear egress, the current data argue that pU_{31} plays a role in optimizing viral protein production and activation of JNK and NF-κB, especially in Hep2 and HeLa cells. These functions are separable kinetically. Specifically, U_{31} acts to increase early gene expression at times before the activation of JNK and NF-κB, which normally occurs at 6 h postinfection with HSV-1. It is therefore unclear whether pU_{31} itself, or another viral protein whose optimal expression depends on pU_{31}, is responsible for activation of NF-κB and JNK. After 6 h, however, the failure to activate signaling molecules in cells infected with the U_{31}-null virus can no longer be kinetically separated from defects in viral gene expression.

The degree to which pU_{31} mediates its effects on viral protein accumulation varies in different cell lines. For example, the block in NF-κB and JNK activation is relieved with time in RSC and Vero cells, but less so in Hep2 and HeLa cells. The previously noted restriction of U_{31} deletion mutant replication in Vero cells, and relative permissivity of RSC (21), is therefore experimentally separable from U_{31}-mediated effects on NF-κB and JNK activation. We have also shown that the defects of the U_{31} deletion virus cannot be rescued by expression of ICP27 in trans, suggesting that pU_{31} performs different functions than this potent viral regulator. Perhaps most importantly, the effect of pU_{31} on viral protein accumulation can be distinguished from its interaction with pU_{34}, because protein expression defects were not observed in cells infected with a U_{34} deletion virus, as noted previously (31). It follows that because the nuclear lamina is not perforated and particles do not bud from the nuclear membrane in cells in-
fect with the U_34 deletion virus (27, 28, 31), the function of pU_31 on protein expression is independent of its roles in nuclear egress or effects on the nuclear lamina. While we do not know the mechanism by which pU_31 augments viral protein expression and cell signaling, its localization in the nucleoplasm must be sufficient to mediate these functions, because pU_31 is located solely in the nucleoplasm of cells with the U_34 deletion virus (28). Such localization suggests possible roles in transcription, stability, or export of mRNAs, and studies are under way to distinguish between these possibilities. It is possible that the presence of pU_31 early in infection is important for blocking nascent expression of the NF-κB repressor IκBα during the second wave of NF-κB activation (2). How and which of these putative activities are consequential to (or dependent on) U_31-dependent JNK and NF-κB activation will require further studies. It is logical that incomplete blocks in these processes could be relieved by prolonged incubation, just as we observed in cells infected with the U_31 deletion virus.

ACKNOWLEDGMENTS

We thank Bill Ruyechan for the ICP8 antibody, Bernard Roizman for the ΔU_31 and ΔU_31-R viruses, Richard Roller for the ΔU_34 virus, and Stephen Rice for the V27 cell line and the d27-1 virus. These studies were supported by grants R01 AI52341 and T32 AI007618 from the National Institutes of Health.

REFERENCES