VP22 of Herpes Simplex Virus 1 Promotes Protein Synthesis at Late Times in Infection and Accumulation of a Subset of Viral mRNAs at Early Times in Infection

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VP22, encoded by the U1,49 gene, is one of the most abundant proteins of the herpes simplex virus 1 (HSV-1) tegument. In the present study we show VP22 is required for optimal protein synthesis at late times in infection. Specifically, in the absence of VP22, viral proteins accumulated to wild-type levels until ~6 h postinfection. At that time, ongoing synthesis of most viral proteins dramatically decreased in the absence of VP22, whereas protein stability was not affected. Of the individual proteins we assayed, VP22 was required for optimal synthesis of the late viral proteins gE and gD and the immediate-early protein ICP0 but did not have discernible effects on accumulation of the immediate-early proteins ICP4 or ICP27. In addition, we found VP22 is required for the accumulation of a subset of mRNAs to wild-type levels at early, but not late, times in infection. Specifically, the presence of VP22 enhanced the accumulation of gE and gD mRNAs until ~9 h postinfection, but it had no discernible effect at later times in infection. Also, VP22 did not significantly affect ICP0 mRNA at any time in infection. Thus, the protein synthesis and mRNA phenotypes observed with the U1,49-null virus are separable with regard to both timing during infection and the genes affected and suggest separate roles for VP22 in enhancing the accumulation of viral proteins and mRNAs. Finally, we show that VP22’s effects on protein synthesis and mRNA accumulation occur independently of mutations in genes encoding the VP22-interacting partners VP16 and vhs.

Herpes simplex virus 1 (HSV-1) virions are composed of a nucleocapsid enclosing the double-stranded linear DNA genome, a proteinaceous layer termed the tegument that surrounds the nucleocapsid, and a host-derived lipid membrane envelope that contains viral glycoproteins. The tegument is unique to herpesviruses and is composed of at least 20 different viral proteins of varied stoichiometries. Tegument proteins have been shown to play a variety of roles in infection, including the regulation of viral and host gene expression and the promotion of virus assembly and egress (1, 16, 34, 38). Tegument proteins enter the cell upon fusion of the viral envelope with the host cell membrane during infection initiation. Thus, these proteins provide a potential means to modulate the host cell and advance viral infection at both very early times in infection upon tegument delivery and at late times in infection, when the tegument proteins are produced in high amounts.

VP22, encoded by the U1,49 gene, is one of the most abundant HSV-1 tegument proteins, with an average stoichiometry of 2,000 copies per virion. A number of functions have been attributed to VP22, including association with and reorganization of microtubules in infected and uninfected cells (10, 22) and incorporation of RNA into the virion (42). In previous studies, U1,49 truncation or deletion mutants propagated on VP22-expressing cell lines were shown to be significantly debilitated in production of infectious virus on noncomplementing cells at low multiplicities of infection and produced plaques of significantly smaller size (6, 35). Further characterization revealed significant defects in release of infectious virus into the medium in the absence of VP22, thereby decreasing viral spread (6). Virions produced by this U1,49− virus upon infection of noncomplementing cells contained decreased levels of ICP0, gD, and gE. These observations are potentially consistent with reports that VP22 interacts with the cytoplasmic tails of gD and gE (4, 14, 33) and is necessary for localization of ICP0 to putative sites of viral assembly within the cytoplasm (8). In contrast to the small plaque phenotype displayed by the U1,49− virus on noncomplementing cells after propagation on complementing cells, passage of the U1,49− virus on noncomplementing cells rapidly and consistently caused the normally defective virus to produce plaques of wild-type size (5). These data suggest a strong selection for a secondary compensatory mutation(s) that rescues the U1,49− small plaque phenotype.

Also pertinent to this report is the observation that VP22 interacts with VP16 (9). VP16 was one of the first transcription factors described and binds host octamer transcription factor 1 and other host factors to mediate transcription from viral immediate-early promoters expressed in the absence of viral protein production (23–26, 40). Thus, VP16 within the tegument is delivered to the nucleus upon initiation of infection to mediate preferential transcription of viral mRNAs over those of the host.

Previous studies indicated that VP16 and the VP16/VP22 complex can interact with the virion host shutoff protein (vhs) (46). vhs, encoded by the U1,41 gene, is a riboendonuclease that specifically cleaves host and viral mRNAs (7, 13, 27, 28, 38, 45, 48). The RNase activity of vhs is believed to promote
expression of viral proteins early in infection by degrading host mRNAs. Likewise, vhs is believed to promote the transition from the early phase of viral transcription to the late phase by degrading early viral mRNAs at the appropriate time in infection. In contrast, the production of VP16 late in infection suppresses the RNase activity of vhs (29, 41, 44), suggesting a mechanism to ensure appropriate accumulation of late transcripts. Interestingly, a previously described U1-49-null mutant derived from an HSV-1 bacterial artificial chromosome accumulated deletion and frameshift mutations in U1-41 when propagated on noncomplementing cells (43), suggesting the presence of vhs was detrimental in the absence of VP22.

The present studies were initiated to further characterize the phenotype of the U1-49− virus we described previously (6). The data presented herein show VP22 is required for optimal protein synthesis at late times in infection and the accumulation of at least gE, gD, and vhs mRNAs at early times in infection. Interestingly, the protein and mRNA phenotypes are separable with regard to both timing during infection and the genes affected, suggesting separate roles for VP22 in both protein and mRNA accumulation. Moreover, these effects were mediated in the absence of mutations in VP16 or vhs.

MATERIALS AND METHODS

Viruses and cells. Viral stocks of wild-type HSV-1(1F) (wild type [WT]), the U1-49 deletion virus (U1-49−), and the U1-49 repair viruses (U1-49R) described previously (6) were propagated exclusively on V49 cells, a Vero-derived cell line that constitutively expresses VP22 (35). Vero and V49 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4.0 mM l-glutamine, 4.5 g/liter glucose, 125 units/ml penicillin, 0.125 mg/ml streptomycin, and either 10% newborn calf serum or 10% fetal bovine serum, respectively.

Time course of protein synthesis. Six-well plates of Vero cells were infected with WT, U1-49−, or U1-49R viruses in medium 199V at a multiplicity of infection (MOI) of 10 at 4°C for 1 hour to allow viral attachment. Virus-containing medium was then replaced with fresh medium 199 supplemented with 1% newborn calf serum (medium 199V), and the plates were shifted to 37°C to allow synchronization of infection. At various times after the 37°C shift, the medium overlying each well was replaced with DMEM (high glucose, with pyridoxine hydrochloride, no l-glutamine, no L-methionine, no L-cysteine) supplemented with 20 μCi/ml Trans35S-label ([35S]methionine/cysteine; MP Biomedicals, Solona, CA). Cells were labeled for 15 min, the medium was then replaced with fresh medium 199V, and the plates were shifted to 37°C to allow synchronization of infection. At various times after the 37°C shift, the medium overlying each well was replaced with DMEM (high glucose, with pyridoxine hydrochloride, no l-glutamine, no l-methionine, no l-cysteine) supplemented with 20 μCi/ml Trans35S-label ([35S]methionine/cysteine; MP Biomedicals, So- lon, OH). Cells were labeled for the time periods shown in the figures and figure legends. At the end of each labeling period the media were removed and the cells were collected, washed with phosphate-buffered saline (PBS), resuspended in 150 μl sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (50 mM Tris-Cl [pH 6.8], 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 10 min, and sonicated briefly. Radiolabeled lysates were separated by SDS–12% PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Labeled proteins were visualized by autoradiography on Pierce Cl-X Posure film (Pierce Biotechnology, Rockford, IL).

Pulse-chase analyses. Six-well plates of Vero cells were infected with WT, U1-49−, or U1-49R viruses in medium 199V at an MOI of 10 at 4°C for 1 hour to allow viral attachment. Virus-containing medium was then replaced with fresh medium 199V and the plates were shifted to 37°C to allow synchronization of infection. Total RNA was extracted from the infected cells at 3, 6, 9, and 15 h postinfection using the RNeasy RNA isolation kit (Ambion, Inc., Austin, TX). Residual DNA was removed by DNase I treatment and DNase I was then removed using DNase inactivation reagent (Ambion, Inc.). Before proceeding to the reverse transcription reaction (RT) without termination, the samples were heat denatured by heating through trial quantitative PCRs (qPCRs) using primers to both 18S and gE and Maxima Sybr green qPCR master mix (Fermentas Inc., Glen Burnie, MD). All RNA samples were found to be free of amplifiable DNA, as PCR product did not accumulate above the level detected in no-template control reaction mixtures. Samples (0.5 μg) of DNase I-treated total RNA were used as the templates for single-stranded cDNA synthesis reactions primed with random decamers using the RETRO-script kit (Ambion, Inc.). cDNAs were tested for template quality by constructing standard curves using serial dilutions of each cDNA template as starting material using the SYBR green qPCR master mix and PCR conditions described above and cDNA concentrations approaching the level of interest in, and in separate reactions, primers to 18S rRNA as a control to normalize template input. All qRT-PCRs were performed in triplicate using Maxima SYBR green qPCR master mix (Fermentas Inc., Glen Burnie, MD) in a MyiQ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA). Primer pair sequences were as follows: gD, 5′-AGCTTCGCGGTTGTTGGAGATGG-3′ and 5′-GATGCGACAGGGAGAAGCG-3′; gE, 5′-GGTCTGGTGTTCTGTTGTGTTGTCG-3′ and 5′-CTCCTCATGATCTTCTGCCATT-3′; IC0, 5′-CTCCTGGCGCATCACACAGAAGCC-3′ and 5′-CAGTCTGGTCTGCGAGAAGAC-3′; vhs, 5′-ATCCAAACAAATATACGAAAAAGCTCTACAGCAG-3′ and 5′-CGGACAAACCTACCTACAAAAACACG-3′; 18S rRNA, 5′-CCGTAATGCGGCTGTACAGACG-3′ and 5′-GCTCTCATAAACACCTACTCCTGGTG-3′ (Integrated DNA Technologies, Coralville, IA). SYBR green fluorescence was measured over the course of 40 amplification cycles. For each template quantified, the mean cycle number at which product accumulation entered the linear range (Ct) was calculated. Replicate Ct values were within 0.4 cycles of each other. The 18S rRNA Ct value for a given template was subtracted from the Ct obtained for each mRNA of interest (gD, gE, gIC0, and vhs) to obtain the normalized Ct values (ΔCt) for each template. The change was then calculated using the ΔΔCt method relative to wild type.

Viral DNA purification and U1-41 and U1-48 sequencing. Viral DNA was purified from plaques produced on noncomplementing Vero cells by the WT, U1-49−, or U1-49R viruses as well as from two independent large plaques derived from the U1-49− virus after two passages on noncomplementing Vero cells. Plaques were picked by Pasteur pipette aspiration through an agarose overlay into 1 ml of medium 199V. Viral DNA was isolated by boiling the plaques for 10 min followed by two phenol-chloroform-
RESULTS

Steady-state levels of ICP0, gE, and gD are decreased at late, but not early, times in U49-infected cells. Previous studies showed that virions produced by various HSV-1 U49 viruses contained decreased levels of ICP0, ICP4, gE, and gD compared to WT and U49R virions but contained WT levels of ICP4 and gD but WT levels of ICP0 and gD. Another possibility is that these proteins are not efficiently incorporated into virions. To test the latter possibility, lysates of equal numbers of Vero cells infected with the WT, U49-, and U49R infections. Vero cells were mock infected or mock infected with the WT, U49R-infected cells were incubated in medium containing [35S]methionine/cysteine for 3 hours. Following each 35S-labeling period, the medium was removed, cells were washed and lysed, and the labeled proteins were separated by SDS-PAGE followed by electrophoretic transfer and autoradiography to visualize individual proteins. Aliquots of labeled cell lysates were also analyzed by scintillation counting (B). Example cellular proteins that undergo shutoff in WT-, U49-, and U49R-infected cells are indicated by †, and example proteins whose synthesis does not decrease at late times during U49-infected are indicated by ∗.

FIG. 1. Immunoblot analysis of steady-state ICP0, ICP4, gE, gD, and β-actin protein levels throughout WT, U49-, and U49R infections. Lysates of Vero cells infected with the WT, U49-, or U49R virus for 2, 4, 6, 8, or 10 h were subjected to immunoblotting using antibodies against ICP0, gE, gD, and β-actin. As shown in Fig. 1, steady-state levels of ICP0, ICP4, gE, gD, and β-actin were very similar in WT-, U49-, and U49R-infected cells at 2, 4, and 6 h postinfection, but at 8 and 10 h postinfection, U49-infected cells contained decreased levels of ICP0, gE, and gD but WT levels of ICP4 and β-actin. Thus, VP22 is required for accumulation of ICP0, gE, and gD to WT levels at late, but not early, times in infection.

FIG. 2. Analysis of global protein synthesis and accumulation throughout WT, U49-, and U49R infections. Vero cells synchronously infected with the WT, U49-, and U49R viruses were incubated in the presence of [35S]methionine/cysteine from 0 to 3, 3 to 6, 6 to 9, 9 to 12, 12 to 15, or 15 to 18 h postinfection (hpi). Following cell washing and lysis, labeled proteins were detected by either autoradiography following SDS-PAGE separation (A) or scintillation counting (B). Example cellular proteins that undergo shutoff in WT-, U49-, and U49R-infected cells are indicated by †, and example proteins whose synthesis does not decrease at late times during U49-infected are indicated by ∗.

isoamyl alcohol (25:24:1) extractions, one chloroform-isoamyl alcohol (24:1) extraction, and ethanol precipitation. Isolated DNAs from individual plaques were used as templates to amplify the upstream, coding, and downstream regions of the U41 and U48 genes by PCR. The primers used were 5′-GCACAAAGCGATACCCAGG-3′ and 5′-GGAATCCGTCATCCCAACGCGGG-3′, which amplified HSV-1 bp 93123 to bp 93140 (the UL41 gene was in reverse orientation from HSV-1 bp 92637 to bp 91170). The amplified UL41 genes were sequenced. Lysates of Vero cells infected with the WT, U49-, and U49R infections. Vero cells were mock infected or mock infected with the WT, U49-, or U49R-infected cells were incubated in medium containing [35S]methionine/cysteine for 3 hours. Following each 35S-labeling period, the medium was removed, cells were washed and lysed, and the labeled proteins were separated by SDS-PAGE followed by electrophoretic transfer and autoradiography to visualize individual proteins. Aliquots of labeled cell lysates were also analyzed by scintillation counting to determine total protein production and accumulation during each labeling period.

As shown in Fig. 2, protein production in WT-, U49-, and U49R-infected cells was very similar from 0 to 3 and 3 to 6 h postinfection with regard to both the individual proteins pro-
duced (Fig. 2A) and total protein accumulation (Fig. 2B). By comparing the electrophoretic profiles of labeled polypeptides from WT-, UL49R-, and U149R-infected cells to those of mock-infected cells, it was also clear that all three viruses were able to mediate shut off of host protein synthesis (Fig. 2A). These data indicate that the absence of VP22 has virtually no effect on viral protein synthesis at early times in infection or on the onset of host shut off.

In contrast to observations at early times, protein production dramatically decreased in U149R-infected cells starting at ~6 h post infection. Total 35S incorporation in U149R-infected cells was 110% and 94% of WT incorporation from 0 to 3 and 3 to 6 h post infection, respectively, but dropped to 46% from 6 to 9 h post infection, 37% from 9 to 12 h post infection, 32% from 12 to 15 h post infection, and 38% from 15 to 18 h post infection. Figure 2A shows the majority of individual proteins in U149R-infected cells were produced in decreased amounts starting at ~6 h post infection, corresponding with the drop in total 35S incorporation measured by scintillation counting. Interestingly, production of a few proteins was unaffected by the absence of VP22 (Fig. 2A). These data show VP22 is necessary for accumulation of most proteins to WT levels at late times in infection.

To determine whether the decreased accumulation of proteins at late times during U149R infection was a consequence of decreased protein synthesis, decreased protein stability, or both, we performed pulse-chase assays. Vero cells infected with the WT, U149R, and UL49R viruses for 8 h (a time in infection corresponding to the observed decrease in protein accumulation) were pulse labeled with [35S]methionine/cysteine for 15 min at 37°C. Following the pulse, the 35S-containing medium was replaced with medium 199V and the infected, labeled cells were incubated at 37°C. At 0, 15, 30, 60, or 120 min after pulse-labeling the cells were washed and lysed. Labeled polypeptides were separated by SDS-PAGE, electrophoretically transferred, and visualized by autoradiography (Fig. 3A). Aliquots of pulse-labeled cell lysates were also analyzed by scintillation counting to determine total protein degradation in cells infected by each virus over time (Fig. 3B and C).

Figure 3B shows there was less total 35S incorporation in U149R-infected cells collected immediately after the short 15-min labeling (0 min post pulse) compared to WT- and UL49R-infected cells, indicating protein synthesis is decreased in U149R-infected cells at 8 h post infection. In Fig. 3C, the amount of 35S incorporation measured following the chases at 15, 30, 60, and 120 min was normalized to each virus's amount of incorporation following the initial labeling (0 min post pulse) and expressed as the percent degradation. These data show there is not a global increase in protein degradation in U149R-infected cells compared to WT- and UL49R-infected cells at 8 h post infection. Figure 3A shows the degradation of individual proteins over time. Overall, the proteins that appeared most stable in WT- and UL49R-infected cells were also most stable in U149R-infected cells. Likewise, the most unstable proteins in WT- and UL49R-infected cells were relatively unstable in U149R-infected cells. We conclude from the above data that VP22 is required for WT levels of protein synthesis, but not protein stability, at late times in infection.

VP22 enhances mRNA accumulation in a manner separate from its effects on protein synthesis. To determine whether VP22's role in protein synthesis reflected a role in accumulation of mRNAs, we performed quantitative real-time RT-PCR to quantify ICP0, gE, gD, and β-actin mRNA levels over time. Total RNA was collected from cells infected with the WT,
wise, gD mRNA levels were reduced 36-fold at 3 h postinfection and were increased 3-fold at 15 h postinfection in U1.49−-infected cells compared to WT-infected cells. As an additional control, Northern blot analysis was performed and indicated that, once detectable, the size of gE mRNA did not change significantly during the course of infection (data not shown). Thus, the quantitative PCR results were a reliable indicator of mRNA accumulation rather than spurious transcription. Interestingly, gE and gD U1.49− mRNA levels did not correlate with gE and gD protein levels in U1.49−-infected cells; while gE and gD protein levels were unaffected at early times in infection and dropped off starting at ~8 h postinfection (Fig. 1), levels of gE and gD mRNAs were reduced in U1.49−-infected cells at early times in infection and present at wild-type levels later in infection.

The above data show (i) a subset of mRNAs (e.g., gD and gE, but not ICP0) are present in reduced levels early in U1.49− infections, and (ii) the protein synthesis shutdown observed at late times in U1.49− infections does not appear to occur at an mRNA regulatory level. Together, the above data indicate VP22 plays roles in both protein synthesis and mRNA accumulation. Interestingly, these roles are distinct and separable with regard to both timing during infection and the genes affected.

Effects of VP22 on protein synthesis and mRNA accumulation are not mediated by antiapoptotic activities. HSV-1 has been shown to induce apoptosis at early times in infection and later block the apoptotic pathway through the production of antiapoptotic viral proteins (31). To determine whether VP22 promotes protein synthesis and mRNA accumulation through an antiapoptotic role during HSV-1 infection, we used immunoblot assays to study PARP accumulation and cleavage over time in cells infected with the WT, U1.49−, and U1.49R viruses. PARP is a substrate for caspase-3, a protease that is activated during apoptosis, and PARP cleavage is an indicator of unblocked apoptosis. In Vero cells infected with HSV-1 and treated with cycloheximide, PARP cleavage is seen at 24 h postinfection (32). Figure 5A shows that U1.49−-infected cells did not show either decreased PARP accumulation or increased PARP cleavage compared to WT- or U1.49R-infected cells over the course of infection. We therefore conclude that VP22 does not promote protein synthesis and mRNA accumulation through antiapoptotic activities.

Effects of VP22 on protein synthesis occur independently of ICP27, VP16, vhs, or phosphorylated eIF2α protein levels. ICP27 is an immediate-early protein that contributes to HSV-1 infection through roles in transcriptional regulation of HSV-1 early and late genes (19, 47), translation initiation (12, 15), and mRNA export (2, 3, 21). VP22 could contribute to global protein synthesis at late times and/or the accumulation of viral mRNAs throughout the viral life cycle by increasing the mRNA regulatory level. Together, the above data indicate VP22 affects ICP27 accumulation, and (i) the protein synthesis shutdown observed at late block the apoptotic pathway through the production of antiapoptotic viral proteins (31). To determine whether VP22 promotes protein synthesis and mRNA accumulation through an antiapoptotic role during HSV-1 infection, we used immunoblot assays to study PARP accumulation and cleavage over time in cells infected with the WT, U1.49−, and U1.49R viruses. PARP is a substrate for caspase-3, a protease that is activated during apoptosis, and PARP cleavage is an indicator of unblocked apoptosis. In Vero cells infected with HSV-1 and treated with cycloheximide, PARP cleavage is seen at 24 h postinfection (32). Figure 5A shows that U1.49−-infected cells did not show either decreased PARP accumulation or increased PARP cleavage compared to WT- or U1.49R-infected cells over the course of infection. We therefore conclude that VP22 does not promote protein synthesis and mRNA accumulation through antiapoptotic activities.

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We next asked whether VP22 promotes mRNA accumulation through an effect on VP16 accumulation. We previously showed VP16 is incorporated into virions at near-WT levels (6). Therefore, VP16's role in immediate-early mRNA synthesis should not be affected by a lack of VP22, consistent with the WT levels of ICP0 mRNA (Fig. 4) and ICP4 and ICP27 proteins (Fig. 1 and 5B) we observed throughout U149R infection. At late times in infection, VP16 suppresses the RNA degradative activity of vhs, suggesting a mechanism that ensures appropriate accumulation of late transcripts (29). Given its potential relevance to the VP22 phenotypes shown herein, we examined the accumulation of VP16 in WT-, U149R-, and U149R-infected cells over time. Like the majority of other proteins studied herein, we found VP16 accumulated to WT levels in U149R-infected cells at early times in infection (Fig. 5B, 3 and 6 h postinfection), but relative VP16 levels decreased in U149R-infected cells at ~9 h postinfection and beyond.

Because vhs both interacts with and is modulated by VP16 (29, 41, 44), the decreased levels of VP16 in U149R-infected cells could affect mRNA accumulation and/or protein synthesis if vhs were present at WT levels in U149R cells. Therefore, we next examined vhs protein levels in WT-, U149R-, and U149R-infected cells over the course of infection. We found very little vhs protein present in U149R-infected cells (Fig. 5B). As vhs was first detected in WT- and U149R-infected cells at 9 h postinfection, a time at which shutdown of protein synthesis has already begun in U149R-infected cells, it is reasonable that the lack of vhs observed in U149R-infected cells is due to this U149R phenotype. To ensure the decreased vhs protein levels found in U149R-infected cells were not due to a disruption in vhs transcription, we performed qRT-PCR using primers against vhs and 18S (Fig. 6). As with gE and gD, we found vhs mRNA levels were greatly reduced at early, but not late, times in U149R infection. Specifically, vhs mRNA levels were reduced 50-fold at 3 h postinfection, 70-fold at 6 h, and 3.6-fold at 9 h and were increased 1.5-fold at 15 h postinfection in U149R-infected cells compared to WT-infected cells. Together, the above data indicate that the decreased protein synthesis and mRNA accumulation observed in U149R-infected cells is not due to an increase in the ratio of vhs versus VP16 protein levels and that the absence of VP22 affects vhs, gD, and gE mRNA and protein levels in a very similar manner.

Finally, we asked whether VP22 promotes protein synthesis late in infection by modulating, directly or indirectly, the phosphorylation status of the translation initiation factor eIF-2α. During the normal course of infection, double-stranded RNA produced through complementary transcript annealing leads to the activation of protein kinase R, the subsequent phosphorylation of eIF-2α at serine-51, and a consequent shutdown in protein synthesis (17, 39). The HSV-1 ICP34.5 protein acts to preclude the shutoff of protein synthesis by directing the dephosphorylation of eIF-2α by the host protein phosphatase 1 (5, 18). It is possible that VP22 increases protein synthesis late in infection through an ICP34.5-dependent or -independent role in eIF-2α dephosphorylation. To determine whether U149R-infected cells contained increased levels of phosphorylated eIF-2α, we performed immunoblot assays with lysates from cells infected for 3, 6, 9, 12, 15, and 18 h with the WT, U149R, and U149R viruses using an antibody specific to the phosphorylated form of eIF-2α's serine-51. We found approximately equal and barely detectable levels of phosphorylated eIF-2α in cells infected with all three viruses (data not shown). Therefore, we conclude VP22 does not promote protein synthesis at late times in infection by modulating eIF-2α's phosphorylation status.

Small and large plaque variants of the U149R virus do not possess secondary mutations in U141 or U146. In WT- and U149R-infected cells, vhs is produced starting at ~9 h postinfection (Fig. 5B), the time corresponding to the global dropoff in protein synthesis observed in U149R-infected cells. Therefore, the lack of vhs observed in U149R-infected cells (Fig. 5B) was not surprising. However, a recent study by Sciortino et al. showed that three independently derived U149 deletion viruses contained secondary mutations that led to either the truncation or deletion of vhs (43). To ensure the lack of vhs
expression observed during infection with our $U_{49}^{\textnormal{r}}$ virus was due to the lack of VP22 and not to secondary mutations in $U_{41}$ encoding vhs, and to ensure the vhs protein encoded by our $U_{49}^{\textnormal{r}}$ virus did not contain mutations that could preclude interaction with or modulation by VP16, we sequenced the $U_{41}$ coding region from WT, $U_{49}^{\textnormal{r}}$, and $U_{49R}$ viral DNA obtained from individual plaques and from the virus stocks used in the above studies. The sequenced region started 307 bp upstream of the $U_{41}$ start codon and extended through the $U_{41}$ promoter, the $U_{41}$ open reading frame, and down-stream of the stop codon through the $U_{41}$ poly(A) tract. There were no DNA sequence differences between WT, $U_{49}^{\textnormal{r}}$, and $U_{49R}$ viral DNAs for the entire $U_{41}$ coding region of the genome (data not shown).

We previously reported that transfection of $U_{49}^{\textnormal{r}}$ BAC DNA into or passage of the $U_{49}^{\textnormal{r}}$ virus on noncomplementing cells reproducibly led to the production of $U_{49}^{\textnormal{r}}$ large plaque variants, presumably due to the acquisition of secondary compensatory mutations. The $U_{49}$ deletion viruses carrying secondary mutations in the $U_{41}$ gene constructed by Sciortino et al. were generated through transfection of noncomplementing cells with $U_{49}^{\textnormal{r}}$ BAC DNA (43). To determine whether large plaque variants produced by our $U_{49}^{\textnormal{r}}$ virus after two passages on noncomplementing cells had acquired secondary mutations in the $U_{41}$ gene, we sequenced the above $U_{41}$ coding regions of viral DNA obtained from two independent $U_{49}^{\textnormal{r}}$ large plaque variants. We found no sequence changes within the entire $U_{41}$ coding region for either of the large plaque variants. We therefore conclude that passage of the $U_{49}^{\textnormal{r}}$ virus on noncomplementing cells can lead to the production of $U_{49}^{\textnormal{r}}$ large plaque variants by means other than the acquisition of secondary mutations in the $U_{41}$ region of the genome.

Secondary mutations in the $U_{48}$ gene encoding VP16 could affect the ability of VP16 to modulate vhs, rendering the trace amounts of vhs present in $U_{49}^{\textnormal{r}}$-infected cells very active in mRNA degradation. Therefore, we also sequenced the $U_{48}$ coding region, starting 416 bp upstream of the $U_{48}$ start codon and extending through the $U_{48}$ promoter, the $U_{48}$ open reading frame, and downstream of the stop codon through the $U_{48}$ poly(A) tract, from the above DNAs obtained from WT, $U_{49}^{\textnormal{r}}$, and $U_{49R}$ plaques. There were no DNA sequence differences between WT, $U_{49}^{\textnormal{r}}$, and $U_{49R}$ viral DNA for the entire $U_{48}$ coding region of the genome (data not shown).

**DISCUSSION**

In previous studies of a $U_{49}$ deletion mutant we showed VP22 greatly enhanced plaque size and viral replication at low multiplicities of infection (6). We also showed $U_{49}^{\textnormal{r}}$ virions contained decreased amounts of ICP0, gE, and gD. In the current study we aimed to determine whether the latter phenomenon was due to decreased virion incorporation or decreased synthesis of ICP0, gE, and gD in the absence of VP22. We found that at late times in infection, VP22 is required for WT levels of protein synthesis on a near-global scale. We also found VP22 contributes to the accumulation of some viral mRNAs at early, but not late, times in infection. Interestingly, VP22’s effects on protein and mRNA levels are distinct and separable with regard to both timing during infection and the genes affected. Specifically, (i) gE and gD mRNA levels were reduced in $U_{49}^{\textnormal{r}}$-infected cells at early times in infection whereas gE and gD protein levels were reduced at late times in infection, and (ii) gE, gD, and ICP0 protein levels were all reduced late in $U_{49}^{\textnormal{r}}$ infections whereas only gE and gD mRNA levels were substantially reduced in $U_{49}^{\textnormal{r}}$-infected cells relative to WT- and $U_{49R}$-infected cells.

**Role of VP22 in protein synthesis at late times in infection.**

Immunoblot analyses of steady-state ICP0, ICP4, ICP27, gE, gD, and $\beta$-actin levels in WT-, $U_{49}^{\textnormal{r}}$, and $U_{49R}$-infected cells at various times in infection showed these proteins were present in similar amounts regardless of the infecting virus at early times in infection. However, beginning at ~6 to 8 h postinfection ICP0, gE, and gD levels dropped dramatically in $U_{49}^{\textnormal{r}}$-infected cells, whereas ICP4, ICP27, and $\beta$-actin levels were not affected. Using $[^35]S$-methionine/cysteine labeling, we found the drop-off in protein synthesis during $U_{49}^{\textnormal{r}}$ infection took place on a near-global scale and decreased total steady-state protein levels by up to 68%. Using pulse-chase analyses, we determined the defect was in protein synthesis, rather than protein stability. We also determined that this drop-off in protein synthesis during $U_{49}^{\textnormal{r}}$ infections did not reflect an aberrant induction of apoptosis or an increase in cIIF-2a phosphorylation, either of which might account for a general failure of protein synthesis.

VP22’s function in protein synthesis at late times in infection appears to involve direct and/or indirect roles in mRNA export and/or mRNA translation. VP22 has been shown to interact with mRNA (42) and is found in both the nucleus and cytoplasm (11, 36), pointing to a possible direct role for VP22 in mRNA export and/or translation. Alternatively, VP22 may aid another viral protein in these activities. Although we found no difference in ICP27 protein levels in $U_{49}^{\textnormal{r}}$-infected cells compared to cells infected with wild-type virus, it is possible that VP22 serves to activate ICP27 at late times in infection, boosting ICP27’s mRNA export and translation initiation activities.

**Role of VP22 in mRNA accumulation at early times in infection.**

We have shown that whereas ICP0 mRNAs were present at WT levels throughout the course of $U_{49}^{\textnormal{r}}$ infection, gE and gD mRNA levels were significantly reduced early in infection and rose to WT levels by late times in infection. VP22 may act to increase steady-state mRNA levels early in infection via a direct or indirect route. For example, VP22’s mRNA binding activity may serve to directly protect viral mRNAs from degradation. Alternatively, VP22 may contribute to mRNA accumulation by modulating the RNase activity of vhs. VP22, VP16, and vhs are known to form a complex (46) and VP16 is known to modulate the vhs RNase activity (29). Although the amount of vhs present in $U_{49}^{\textnormal{r}}$-infected cells is severely decreased relative to WT-infected cells, we cannot exclude the hypothesis that VP22 may contribute to VP16’s modulation of vhs, and thus, in the absence of VP22 the small amount of vhs present may be extremely active.

In summary, we have further characterized an HSV-1 $U_{49}$-null virus and have discovered roles for VP22 in both protein synthesis at late times in infection and mRNA accumulation of some viral mRNAs at early times in infection. Future studies will focus on gaining a more comprehensive view of the genes affected by the absence of VP22 and determining VP22’s pre-
cise role(s) in enhancing protein synthesis and mRNA accumulation.

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