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Deletion of U_L21 Causes a Delay in the Early Stages of the Herpes Simplex Virus 1 Replication Cycle

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The herpes simplex virus 1 (HSV-1) U_L21 gene encodes a 62-kDa tegument protein with homologs in the alpha-, beta-, and gammaherpesvirus subfamilies. In the present study, we characterized a novel U_L21-null virus and its genetic repair to determine whether this protein plays a role in early stages of the HSV-1 replication cycle. Single-step growth analyses, protein synthesis time courses, and mRNA quantifications indicated that the absence of U_L21 results in a delay early in the HSV-1 replication cycle.

Herpes simplex virus 1 (HSV-1) virions, like those of all herpesviruses, contain a proteinaceous layer, termed the tegument, located between the nucleocapsid and viral envelope. The HSV-1 tegument is composed of ~20 different viral proteins of varying 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 (2, 5, 12, 13). Tegument proteins are delivered to the host cell upon infection and, thus, can play roles at early times in infection as well as at late times when they are produced.

The U_L21 protein is a 535-amino-acid component of the HSV-1 tegument (1, 17). The majority of work on U_L21 has been performed with HSV-1 and pseudorabies virus (PRV), though homologs of this protein have been identified in the alpha-, beta-, and gammaherpesvirus subfamilies. Although the role of U_L21 late in infection has been studied in both HSV-1 and PRV, the role this protein plays at early times in infection is unknown. In the present study, we sought to characterize the role(s) of the U_L21 protein at early times in the HSV-1 replication cycle.

To identify the function(s) of U_L21 at early times in HSV-1 infection, we generated both a U_L21-null virus (U_L21^-), which lacks the entire U_L21 ORF, and a repair virus (U_L21R), in which the U_L21 ORF was restored using the HSV-1(F) BAC pYEbac102 (18) with the "en passant" recombination system (19) and the PCR primers 5′-CCGTAGGGGGCCTCTGGGCCGTGTT-3′, ACGTCGCCGCCCGCGAAGACCCCAATAAACGTATATAGG GATAACAGGGTAATCGATTT-3′, and 5′-ACACAAGGGTGT AGTAGCGATATACGTTTATTGGGGTCTTCGCGGGCGGCG ACGTAACACGCCAGTGTTACAACCAATTAACC-3′. The deletion in the U_L21-null virus spanned HSV-1 bp 42074 to 43678, a region beginning with the start codon and ending with the stop codon of the U_L21 open reading frame (ORF). This entire sequence was restored to its original location in the U_L21R virus. Following transfection of BAC DNAs into Vero cells and subsequent virus stock production, restriction fragment length polymorphism analysis of purified viral DNAs was performed and showed the genotype was as expected (data not shown). Viral DNAs were also used for PCR amplification of the manipulated areas. Sequencing of the PCR products showed that the genetic manipulations were made as planned (data not shown). Southern blot analysis showed that the deletion in the U_L21-null genome and the repair in the U_L21R genome were of the correct sizes (data not shown). Immunoblot analysis showed that the U_L21 protein was present in lysates of Vero cells infected with the wild-type and U_L21R viruses but not the U_L21-null virus (data not shown).

To determine whether the newly generated U_L21-null virus possessed a defect in virus replication, single-step growth analyses were performed. Vero cells infected at a multiplicity of infection (MOI) of 5 PFU/cell with the wild-type (WT), U_L21^-, and U_L21R viruses were collected at 0, 6, 12, 18, and 24 h postinfection and lysed, and intracellular virus was quantified by plaque assay (Fig. 1A). Medium overlying the infected cell monolayers was clarified and assayed separately (Fig. 1B). The U_L21-null virus showed a 99% (two-log-unit) reduction in both intracellular and extracellular virus yields as early as 6 h postinfection compared to the wild-type and U_L21 repair viruses. This reduction was less pronounced late in infection, with intracellular and extracellular virus yields being reduced by approximately 5- and 10-fold, respectively, at 18 and 24 h postinfection (hpi). These data indicate that the absence of U_L21 causes a delay in the production of infectious virus.

A delay in virus production could result from a delay in the initiation of virus infection. To measure the kinetics and efficiency of U_L21-null virus attachment, 25-cm^2 flasks of Vero cells were infected at room temperature with 100 PFU of WT, U_L21^-, or U_L21R viruses for 5, 10, 20, 30, or 45 min. At each time point, infected cells were washed to remove unattached virus and overlaid with medium containing human gamma globulins to neutralize any remaining unattached virus. Infected cells were then incubated at 37°C for ~2 days, at which time viral plaques were counted to determine the percentage of the original infecting dose that had initiated viral infection by each time point. We found no difference in the number of viral plaques produced by the wild-type, U_L21-null, and U_L21 repair viruses (data not shown).

The reduced intra- and extracellular virus yields observed early in U_L21-null virus infections could be caused by a defect or delay

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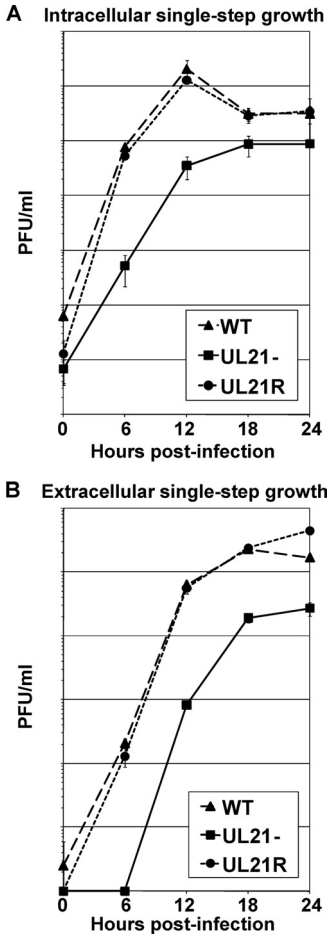

FIG 1 Single-step growth analyses of the WT, U_L21^-, and U_L21R viruses. Vero cell monolayers were infected with the WT, U_L21^-, and U_L21R viruses at an MOI of 5 PFU/cell for 1 h to allow virus adsorption. The cells were then washed extensively with citrate buffer to neutralize and remove unbound virus. The cells were overlaid with medium and held at 37°C. At the indicated times postinfection, the infected cells (A) and the overlying medium (B) were analyzed separately by plaque assay to determine intracellular and extracellular virus yields, respectively. Data points are the arithmetic means from three independent experiments; error bars represent one standard deviation.

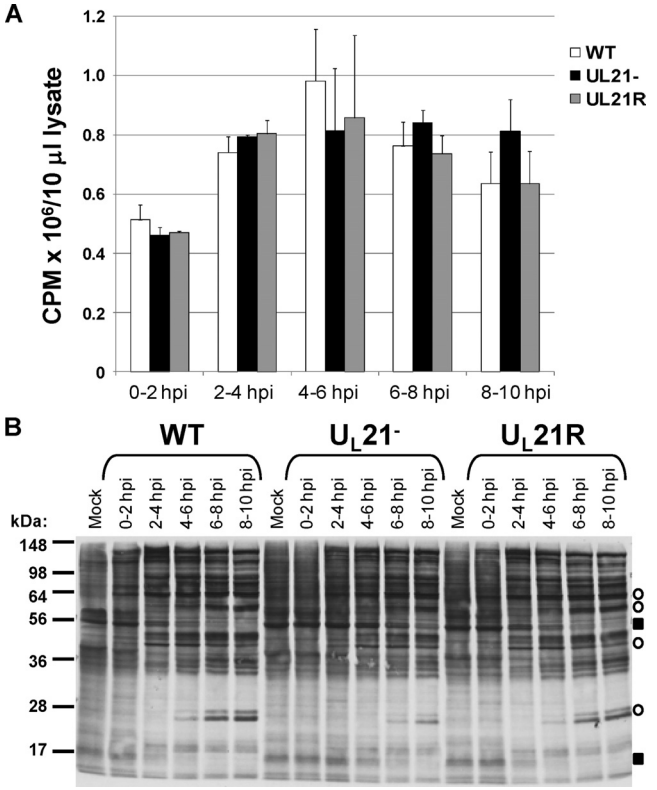

FIG 2 Analysis of global protein synthesis and accumulation at early times in WT, U_L21^-, and U_L21R infections. Vero cells synchronously infected with the WT, U_L21^-, and U_L21R viruses were incubated in the presence of [^{35}S]methionine and [^{35}S]cysteine from 0 to 2, 2 to 4, 4 to 6, 6 to 8, or 8 to 10 h postinfection. (A) Following cell washing and lysis, labeled proteins were detected by scintillation counting. The values are arithmetic means from three independent experiments; error bars represent one standard deviation. (B) Labeled proteins were also detected by autoradiography following SDS-PAGE separation. The experiment was performed three times with similar results. Examples of viral proteins that undergo delayed induction in U_L21^--infected cells are indicated by circles, and examples of cellular proteins that undergo delayed shutoff in U_L21^--infected cells are indicated by squares.

in viral protein synthesis. We examined whether total protein synthesis was decreased at various times early in U_L21^- virus infections by performing ^{35}S-labeling time courses. Vero cells were either mock infected or infected with WT, U_L21^-, or U_L21R viruses at an MOI of 10 PFU/cell by slow rocking at 4°C for 1 h, followed by removal of unattached virus and incubation at 37°C to synchronize virus entry. At 0, 2, 4, 6, or 8 h postinfection, medium overlying the infected cells was replaced with medium containing 200 μCi [^{35}S]methionine and [^{35}S]cysteine, and cells were returned to 37°C for 2 h. Following each 2-h labeling period, the infected, labeled cells were washed, collected, and boiled in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Ten-microliter samples were assayed for incorporated radiolabel via scintillation counting (Fig. 2A). We found no difference in total protein synthesis between cells infected with the wild-type, U_L21-null, or U_L21-repair viruses for any of the time periods assayed. To examine the relative abundance of individual proteins synthesized between 0 and 2, 2 and 4, 4 and 6, 6 and 8, and 8 and 10 h postinfection, we performed autoradiography following SDS-PAGE separation of the radiolabeled cell lysates (Fig. 2B). The absence of U_L21 caused a 2- to 4-h delay in the induction of viral protein synthesis, inasmuch as some proteins synthesized in infected cells, but not in mock-infected cells, began accumulating 2 to 4 h later in U_L21^- infections than in WT and U_L21R infections. The absence of U_L21 also corresponded to a 2- to 4-h delay in the shutoff host protein synthesis, inasmuch as proteins present in mock-infected cells were synthesized in U_L21^--infected cells for 2 to 4 h longer than in either WT- or U_L21R-infected cells.

We next examined how the absence of U_L21 affects the accumulation of specific immediate-early, early, and late viral proteins. We analyzed the accumulation, over time, of ICP0, ICP4, ICP27, ICP8, and VP16 by performing immunoblotting of lysates from Vero cells infected with WT, U_L21^-, or U_L21R viruses at an MOI of 10 PFU/cell for 2, 4, 6, 8, or 10 h (Fig. 3A). For each experiment, immunoblots were quantified, and the signal from each sample was normalized to the signal obtained from the WT 10-hpi sample. Normalized values from three experiments were used to calculate relative means and standard deviations (Fig. 3B). We found

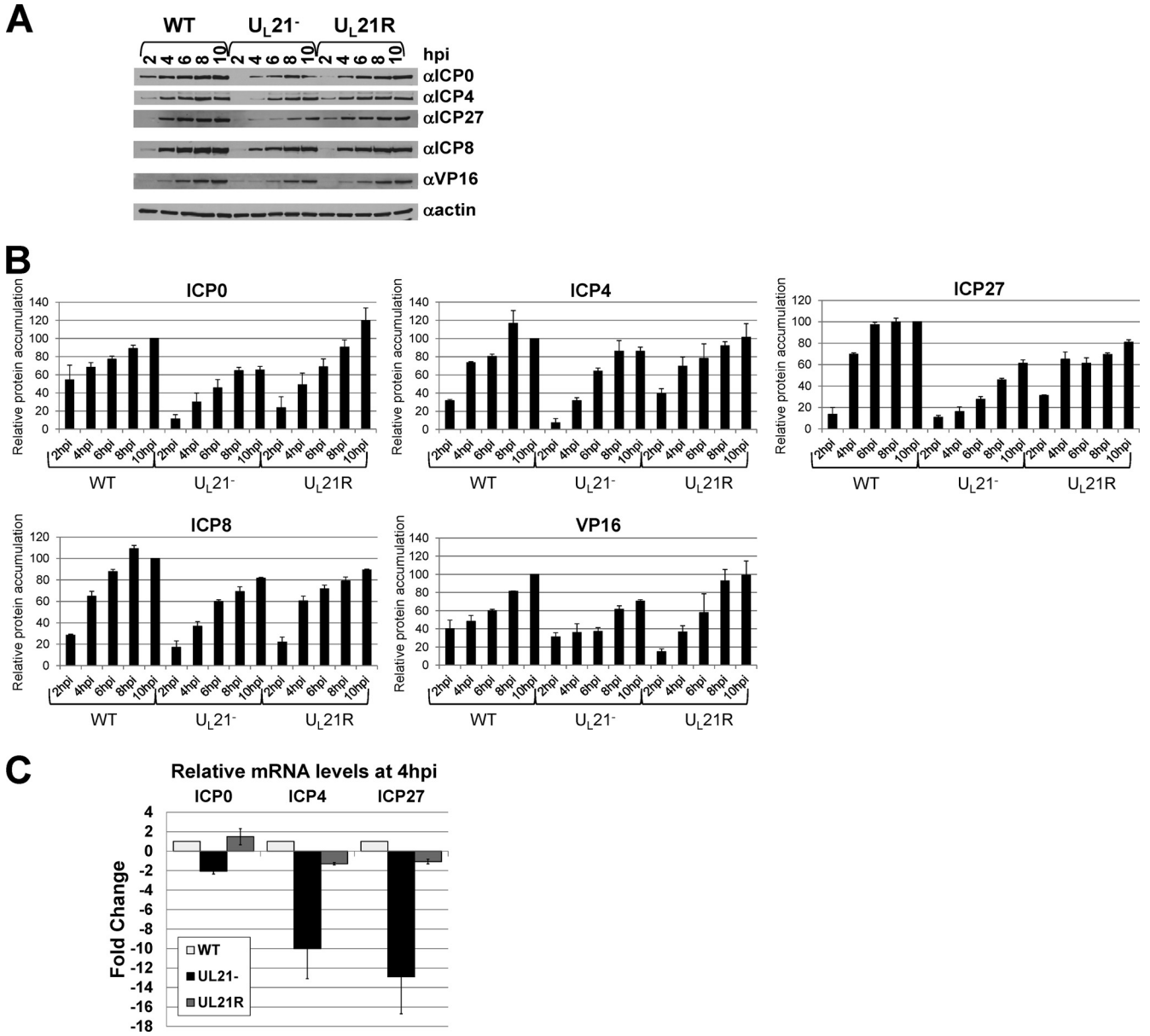

FIG 3 Analysis of individual protein and mRNA levels. (A and B) Immunoblot analysis of steady-state ICP0, ICP4, ICP27, ICP8, VP16, and β-actin protein levels at early times in WT, U_L21^-, and U_L21R infections. Lysates of Vero cells infected with the WT, U_L21^- and U_L21R viruses for 2, 4, 6, 8, or 10 h were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies specific to ICP0 (Abcam), ICP4 (Rumbaugh-Goodwin Institute for Cancer Research), ICP27 (Virusys), ICP8 (16), VP16 (Santa Cruz Biotechnology), and β-actin (Santa Cruz Biotechnology). The experiment was performed three times. (A) Autoradiographic visualization of immunoblots. Similar results were obtained in three experiments. (B) Quantification of immunoblots. For each experiment, bands were normalized to the signal obtained from the WT 10-hpi band. Normalized values from each experiment were used to calculate relative means. Error bars represent one standard deviation. (C) Relative quantitative real-time RT-PCR analysis of ICP0, ICP4, and ICP27 mRNA levels in Vero cells infected with the WT, U_L21^-, and U_L21R viruses for 4 h. Primer pair sequences were as follows: ICP0, 5′-CCTCTCCGCATCACCACAGAAGCC-3′ and 5′-CAGGTCTCGGTCGCAGGGAAACAC-3′; ICP4, 5′-CCGCCGTCGCAGCCGTATC-3′ and 5′-CCCGCCCTCCTCCGTCTCC-3′; ICP27, 5′-GGTGTCGGGGTCGGAGAGAAGATG-3′ and 5′-GGCGGTGCGTGTCTAGGATTTCG-3′; 18S rRNA, 5′-CCAGTAAGTGCGGGTCATAAGC-3′ and 5′-GCCTCACTAAACCATCCAATCGG-3′. The values are arithmetic means from two experiments, each performed in triplicate. Error bars represent one standard deviation.

that the delay in protein synthesis observed in the absence of U_L21 was more pronounced for the immediate-early proteins ICP0, ICP4, and ICP27 than for either the early protein ICP8 or the late protein VP16. We therefore focused on ICP0, ICP4, and ICP27 for the experiments examining mRNA levels. The quantifications also revealed that the largest difference between wild-type and U_L21^- ICP0, ICP4, and ICP27 levels occurred at 4 h postinfection. This time point was therefore chosen to examine relative mRNA levels.

To determine whether the delay in accumulation of ICP0, ICP4, and ICP27 proteins in U_L21^- infections reflected a delay in accumulation of their respective mRNAs, we performed quantitative real-time reverse transcription-PCR (qRT-PCR). Total RNA was collected from cells infected at an MOI of 10 PFU/cell with the WT, U_L21^-, and U_L21R viruses for 4 h. The RNAs were treated with DNase to remove contaminating DNA and then used as templates to synthesize cDNAs using random primers. Equal

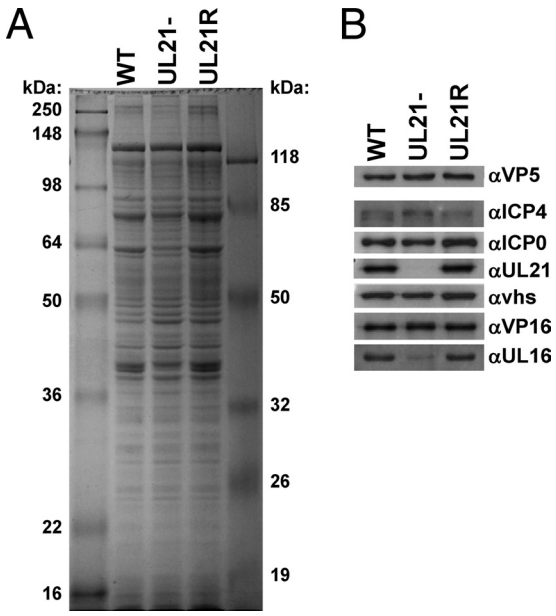

FIG 4 Immunoblot analysis of purified WT, U_L21^-, and U_L21R virions. Purified virions were separated by SDS-PAGE and either stained with Coomassie brilliant blue (A) or transferred to a nitrocellulose membrane for immunoblot analysis (B) using antibodies against VP5 (3), ICP4 (Rumbaugh-Goodwin Institute for Cancer Research), ICP0 (Abcam), U_L21 (1), vhs (8), VP16 (Santa Cruz Biotechnology), and U_L16 (10). The experiment was performed twice, with similar results.

amounts of cDNA were used in qRT-PCR mixtures containing primers specific to the gene of interest and SYBR green for product quantification. Parallel qRT-PCR mixtures contained primers specific to 18S rRNA as a control to normalize template input. The 18S rRNA cycle threshold (C_T) value for a given template was subtracted from the C_T obtained for each mRNA of interest (ICP0, ICP4, and ICP27) from the same template to obtain the normalized C_T value (ΔC_T) for each template. The change (fold, relative to the wild type) was then calculated using the $\Delta\Delta C_T$ method. At 4 h postinfection, steady-state ICP4 and ICP27 mRNA levels were decreased ~10-fold and ~13-fold, respectively, in U_L21^--infected cells compared to WT- and U_L21R-infected cells (Fig. 3C). ICP0 mRNA levels were also affected by the absence of U_L21, although to a lesser extent, showing a 2-fold decrease in U_L21^--infected cells compared to WT- and U_L21R-infected cells.

These findings show that the absence of U_L21 leads to a delay in the accumulation of ICP0, ICP4, and ICP27 mRNAs, their corresponding proteins, and infectious intra- and extracellular virus. U_L21 could promote timely progression through the HSV-1 replication cycle by promoting efficient packaging of tegument proteins into virions. For example, if the absence of U_L21 leads to decreased levels in virions of tegument proteins with known regulatory roles, such as VP16, a delay in IE transcription could result during subsequent infection with these virions. To test this hypothesis, we purified virions from WT-, U_L21^--, and U_L21R-infected Vero cells as described previously (4) and examined their relative protein compositions. Figure 4A shows WT, U_L21^-, and U_L21R purified virions separated by SDS-PAGE and stained with Coomassie brilliant blue. Figure 4B shows a panel of immunoblots of the purified virions. VP5 was used as a loading control. As expected, the U_L21 protein (65 kDa) was absent in U_L21^- virions. Of note, VP16, vhs, ICP0, and ICP4 were present in approximately equimolar amounts in WT, U_L21^-, and U_L21R virions. As shown previously (9), levels of the U_L16 protein (41 kDa) were reduced in U_L21^- virions. These data show the delay in the HSV-1 replication cycle observed in the absence of U_L21 is not caused by reduced virion packaging of the regulatory proteins VP16, vhs, ICP0, or ICP4.

By specifically studying the role of U_L21 in early stages of HSV-1 replication, we made the novel finding that deletion of U_L21 causes a delay early in the virus replication cycle. Single-step growth analyses showed that the deletion of U_L21 results in a delay in the production of infectious virions. This was previously demonstrated in a study by Baines et al. in which an HSV-1 mutant virus missing the first 484 codons of U_L21 was generated (1). Compared to the wild-type and U_L21 repair viruses, the U_L21 mutant produced virus yields that were reduced by 1.5 log units on Vero cells at 8 h postinfection. However, by 20 and 28 h postinfection, the difference between wild-type and U_L21-null virus yields decreased to approximately 0.5 log units. In the present study, the delay in the virus replication cycle was also observed in ^{35}S-labeling protein synthesis time courses (Fig. 2B). By monitoring the accumulation of individual proteins over time via immunoblots (Fig. 3A and B), we found that the U_L21^--associated delay in viral protein synthesis was more pronounced for the immediate-early proteins ICP0, ICP4, and ICP27 than for either the early protein ICP8 or the late protein VP16. Similarly, steady-state ICP4, ICP27, and, to a lesser extent, ICP0 mRNA levels were decreased at 4 h postinfection in U_L21^--infected cells compared to WT- and U_L21R-infected cells (Fig. 3C).

Interestingly, virus-induced shutoff of several host proteins was also delayed in U_L21^--infected cells compared to WT- and U_L21R-infected cells (Fig. 2B). The effect of U_L21 on host protein shutoff is likely an indirect consequence of its role in promoting the synthesis of ICP27, since several studies have shown that ICP27 inhibits host cell pre-mRNA splicing, resulting in the shutoff of host protein synthesis (6, 7, 14, 15).

There are a number of possible roles for U_L21 in promoting timely progression through early stages of the HSV-1 replication cycle. For example, U_L21 may play a role in ensuring timely transport of the DNA-filled capsid to the nucleus and/or in nuclear genome delivery. Alternatively, U_L21 delivered to infected cells may act directly or indirectly to aid in guiding the cellular transcription machinery toward the synthesis of viral mRNAs and away from the synthesis of cellular mRNAs. Finally, the delay in HSV-1 replication we observed in the absence of U_L21 may be an indirect consequence of reduced U_L16 quantities present in U_L21^- virions (Fig. 4B) (9). Sequence analysis of U_L16 homologs from several herpesviruses identified a possible zinc-binding domain in the C terminus (20), and studies with the U_L16 protein from HSV-2 have shown that this protein may possess DNA binding activity (11). It is not known whether U_L16 is involved in transcriptional regulation, but if so, U_L21 could act indirectly to promote timely viral protein synthesis by promoting the incorporation of U_L16 into virions. In any case, future studies that further define the role of U_L21 in promoting timely progression through early stages of the HSV-1 replication cycle will be of interest.

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