JOURNAL OF VIROLOGY, May 2009, p. 5181–5191 Vol. 83, No. 10
0022-538X/09/$08.00+0 doi:10.1128/JVI.00090-09

Phosphorylation of the U_L31 Protein of Herpes Simplex Virus 1 by the U_S3-Encoded Kinase Regulates Localization of the Nuclear Envelopment Complex and Egress of Nucleocapsids[▽]

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Received 14 January 2009/Accepted 26 February 2009

Herpes simplex virus 1 nucleocapsids bud through the inner nuclear membrane (INM) into the perinuclear space to obtain a primary viral envelope. This process requires a protein complex at the INM composed of the U_L31 and U_L34 gene products. While it is clear that the viral kinase encoded by the U_S3 gene regulates the localization of pU_L31/pU_L34 within the INM, the molecular mechanism by which this is accomplished remains enigmatic. Here, we have determined the following. (i) The N terminus of pU_L31 is indispensable for the protein's normal function and contains up to six serines that are phosphorylated by the U_S3 kinase during infection. (ii) Phosphorylation at these six serines was not essential for a productive infection but was required for optimal viral growth kinetics. (iii) In the presence of active U_S3 kinase, changing the serines to alanine caused the pU_L31/pU_L34 complex to aggregate at the nuclear rim and caused some virions to accumulate aberrantly in herniations of the nuclear membrane, much as in cells infected with a U_S3 kinase-dead mutant. (iv) The replacement of the six serines of pU_L31 with glutamic acid largely restored the smooth distribution of pU_L34/pU_L31 at the nuclear membrane and precluded the accumulation of virions in herniations whether or not U_S3 kinase was active but also precluded the optimal primary envelopment of nucleocapsids. These observations indicate that the phosphorylation of pU_L31 by pU_S3 represents an important regulatory event in the virion egress pathway that can account for much of pU_S3's role in nuclear egress. The data also suggest that the dynamics of pU_L31 phosphorylation modulate both the primary envelopment and the subsequent fusion of the nascent virion envelope with the outer nuclear membrane.

The U_L31 and U_L34 proteins of herpes simplex virus 1 (HSV-1) form a complex that accumulates at the inner nuclear membrane (INM) of infected cells (26, 27). This complex is essential for the budding of nucleocapsids through the INM into the perinuclear space (26, 28). pU_L34 is a type 2 integral membrane protein with a 247-amino-acid nucleoplasmic domain that binds pU_L31 and holds the latter in close approximation to the INM (16, 19, 26, 31, 36, 37). Both proteins become incorporated into nascent virions, indicating that they directly or indirectly interact with nucleocapsids during the budding event (27). Interestingly, the coexpression of the pseudorabies virus homologs of HSV pU_L31 and pU_L34 are sufficient to induce budding from the INM in the absence of other viral proteins (13).

The most prominent model of nuclear egress proposes that the step following primary envelopment involves the fusion of the perinuclear virion envelope with the outer nuclear membrane (ONM), allowing subsequent steps in which the deenveloped capsid engages budding sites in the Golgi or trans-Golgi network (20, 32). The U_S3 protein is a promiscuous kinase that phosphorylates pU_L31, pU_L34, and several other viral and cellular components (1, 2, 5, 11, 15, 21–23, 25). In the absence of pU_S3 kinase activity, (i) virions accumulate within distensions of the perinuclear space that herniate into the nucleoplasm (14, 27, 29), (ii) the pU_L31/pU_L34 complex is mislocalized at the nuclear rim from a smooth pattern to discrete foci that accumulate adjacent to nuclear membrane herniations (12, 14, 27, 29), and (iii) the onset of infectious virus production is delayed (21, 29).

Aberrant accumulations of perinuclear virions similar to those observed in cells infected with U_S3 kinase-dead viruses have been observed in cells infected with viruses lacking the capacity to produce glycoproteins H and B (gH and gB, respectively) (8). Because these proteins are required for fusion with the plasma membrane or endocytic vesicles during HSV entry (3, 4, 9, 10, 18, 30, 33), it has been proposed that the accumulation of perinuclear virions in the absence of gH and gB reflects a failure in the apparatus that normally mediates the fusion between the nascent virion envelope and the ONM (8). By extension of this hypothesis, pU_S3 might act to trigger or otherwise regulate this perinuclear fusion event.

The substrate(s) of the pU_S3 kinase responsible for the altered localization of the pU_L31/pU_L34 complex and the aberrant accumulation of perinuclear virions were heretofore unknown. In one study to identify such a substrate, it was determined that precluding the phosphorylation of pU_L34 was not responsible for the nuclear egress defects induced by the absence of pU_S3 or its kinase activity (29). The current study was therefore undertaken to investigate the hypothesis that the pU_S3-mediated phosphorylation of pU_L31 is critical to regulate nuclear egress. The presented evidence indicates that aspects of the U_S3 kinase-dead phenotype, including the reten-

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▽ Published ahead of print on 11 March 2009.

TABLE 1. Vectors and primers used to generate the U_L31 subclones

Plasmid	U_L31 codon (aa)[a]	Vector(s)	Primers[b]
U_L31(dN)-pCDNA3	45–306	pcDNA3	5′-ATACTCGAGATGCCGCCTCACG-3′, 5′-TATACTCGAGCTACGGCGGAGGAAAC-3′
U_L31-Flag	1–306	pBudCE 4.1	5′-ATACTCGAGACCTATGTATGACACCGACCC-3′, 5′-ATAAGATCTTAC**TTATCGTCGTCATCC**TTGTAATCCGGCGGAGGAAACTC-3′
U_L31(dN)-Flag	45–306	pBudCE 4.1	5′-ATACTCGAGACCTATGCCGCCTCACG-3′, 5′-ATAAGATCTTAC**TTATCGTCGTCATCC**TTGTAATCCGGCGGAGGAAACTC-3′
GST-U_L31 (1–50 aa)	1–50	pCR2.1, pGEX4T-2	5′-ATGTATGACACCGACCCCCATC-3′, 5′-TTTGCGGGCGTGAGGC-3′
GST-U_L31 (52–165 aa)	52–165	pCR2.1, pGEX4T-1	5′-TCTCGAGATGGAGGAGCTGTGTTTAC-3′, 5′-TCTAGGCCAACACGACCAA-3′
GST-U_L31 (159–265 aa)	159–265	pCR2.1, pGEX4T-1	5′-GCTCGAGATGGTGGCCTCCTTGGTC-3′, 5′-CCTAGTCCGTGGGTTTCTCTGCGTT-3′
GST-U_L31 (255–306 aa)	255–306	pCR2.1, pGEX4T-1	5′-CCTCGAGATGGGCAACGCAGAGAAACC-3′, 5′-CCTACGGCGGAGGAAACTC-3′
GST-U_L31 (SA3)	1–306	pGEX4T-1	5′-TAGAATTCATGTATGACACCGACCCCCATC-3′, 5′-TATACTCGAGCTACGGCGGAGGAAAC-3′
GST-U_L31 (SA6)	1–306	pGEX4T-1	5′-TAGAATTCATGTATGACACCGACCCCCATC-3′, 5′-TATACTCGAGCTACGGCGGAGGAAAC-3′

[a] aa, amino acids.
[b] Restriction sites used for cloning are underlined, and Flag sequences are in bold.

tion of virions in the perinuclear space, the mislocalization of the pU_L31/pU_L34 complex, and the delayed onset of virus replication, can be replicated by precluding pU_L31 phosphorylation in the presence or absence of pU_S3 kinase activity. The data also suggest that the dynamic phosphorylation of pU_L31 is important during the primary envelopment of nucleocapsids.

MATERIALS AND METHODS

Cells and viruses. Wild-type virus HSV-1(F), U_L31 null virus, and mutant virus containing a lysine-to-alanine mutation in U_S3 codon 220 (K220A) have been described and were obtained from B. Roizman and R. J. Roller, respectively (6, 7, 29). The HSV-1(F) and U_S3(K220A) viruses were propagated and titers determined on Vero cells, whereas the U_L31 null virus was grown and titers determined on U_L31-expressing rabbit skin cells described previously (17).

Plasmids. For transient expression, U_L31 fused to DNA encoding a FLAG epitopic tag at the 3′ end was cloned into the pcDNA3 vector (Invitrogen) or into the pBudCE4.1 vector (Invitrogen) as indicated in Table 1. Full-length U_L31 in pcDNA3 was previously described (26). This plasmid served as a template for other pcDNA3 or pBudCE4.1 constructs using the PCR primers listed in Table 1.

A plasmid bearing glutathione *S*-transferase (GST) fused to full-length U_L31 was also reported previously (26). In the current study, a series of U_L31 fragments were cloned into pGEX4T vectors (Amersham) for the expression of the encoded GST fusion proteins in *Escherichia coli*. Briefly, U_L31 sequences were amplified from HSV-1(F) genomic DNA using the primers listed in Table 1. The amplicons were first ligated into the pCR2.1 vector (Invitrogen) using TA cloning, such that they were flanked by EcoRI restriction sites. The EcoRI fragments containing U_L31 sequences were subcloned into the EcoRI site of the pGEX4T vector (Amersham) in frame with the GST gene.

To generate plasmids bearing U_L31 with point mutations, U_L31 was amplified from the corresponding mutant viral BAC DNAs (described below), and the amplicons were cloned into the pGEX4T vector at the EcoRI and XhoI sites.

Recombinant viruses and BAC mutagenesis. A series of recombinant viruses were constructed using en passant mutagenesis of a bacterial artificial chromosome (BAC) containing the entire HSV-1(F) genome as previously described (34, 35). Briefly, a PCR amplicon bearing fragments containing a kanamycin resistance (Kan^r) cassette, an Sce-I restriction endonuclease site, and desired mutations flanked by homologous regions to the target gene was generated with the pEPkan-S plasmid as a template and the primer pairs listed in Table 2. The resulting amplicons were electroporated into recombination competent GS1783 *E. coli* (a kind gift of Greg Smith, Northwestern University), which harbors the target BAC as an episome and an Sce1 endonuclease gene in the chromosome. Following confirmation by Kan^r screening and restriction length polymorphism, the SceI cleavage was initiated by arabinose-induced SceI expression in GS1783, causing the Kan^r sequence to be removed efficiently by Red recombination between internal homologous sequences. Positive clones were verified through Kan selection and further by restriction length polymorphism and DNA sequencing (not shown). Rabbit skin cells were transfected with the BACs and a plasmid encoding FLP recombination target recombinase to remove BAC sequences as described previously (17). Viral plaques were propagated into viral stocks using Vero cells.

All the BACs were designated according to their genotypes. Before obtaining the U_L31(SA6) BAC, U_L31 nucleotides 3 to 132 were first deleted from wild-type HSV-1(F) BAC, producing an intermediate BAC designated U_L31(dN). The deleted region was restored with homologous sequences bearing designed mutations, thus changing codons 11, 24, and 40 from serine to alanine. The resulting BAC was named U_L31(SA3). This BAC then served as the parental BAC to generate U_L31(SA6) BAC by changing serine codons 26, 27, and 43 to alanine. The repair U_L31(SAR) BAC was created by repeating the deletion step starting with the U_L31(SA6) BAC, thus regenerating the intermediate U_L31(dN) genotype, followed by the insertion of the wild-type sequences. In a separate experiment, this U_L31(dN) BAC served as the recipient of mutations changing codons 11, 24, 26, 27, 40, and 43 to those encoding glutamic acid. This BAC was designated U_L31(SE6). The U_L31(SAE) BAC was derived from the U_L31(SA6) BAC by mutating alanine codons 26, 27, and 43 into glutamic acid codons. The incorporation of mutations substituting U_S3 lysine 220 with alanine in the U_L31(SE6) BAC led to the U_L31(SE6)/U_S3(K220A) BAC double mutant.

In vitro U_S3 kinase assays. A kinase assay was performed using purified GST-pU_S3 essentially as described previously (21). Briefly, 0.1 μg GST-pU_S3 was incubated separately with approximately 1 μg of pU_L31-GST fusion proteins partially purified from *E. coli* and bound to glutathione Sepharose beads. The kinase reaction was performed for 30 min at 30°C in 50 μl pU_S3 specific kinase buffer (50 mM Tris [pH 9.0], 20 mM $MgCl_2$, 0.1% NP-40, 1 mM dithiothreitol) containing 10 μM ATP and 10 μCi [γ-^{32}P]ATP (Amersham). The Sepharose beads bearing the fusion proteins were then washed three times with TNE buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA). The bound proteins were eluted in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer (10 mM Tris-HCl [pH 8.0], 10 mM β-mercaptoethanol, 20% glycerol, 5% SDS, trace amounts of bromophenol blue) and subjected to electrophoresis in a 12% polyacrylamide gel in the presence of 0.1% SDS. The gels were then stained with Coomassie brilliant blue, dried, and autoradiographed using X-ray film (Pierce).

Transient complementation assay. Hep2 cells were seeded at 90% confluence in 12-well plates and were transfected with 1.6 μg plasmid DNA using Lipofectamine (Invitrogen). After 20 h of incubation at 37°C, the cells were infected with 5.0 PFU per cell of the U_L31 null virus. At 24 h postinfection (hpi), whole-cell cultures were lysed for viral titering on U_L31-expressing rabbit skin cells. The experiments were performed in duplicate. The means and standard deviations were calculated and plotted.

TABLE 2. Primers for BAC mutagenesis

BAC	Primers
U_L31(dN)	5′-ATC TCG CTC CTG TCC CTG GAG CAC ACC CTG TGT ACC TAT GCC GCC TCA CGC CCG CAA ACA TAG GGA TAA CAG GGT-3′, 5′-GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG CGT GAG GCG GCA TAG GTA CAC AGG GTG TGC GCC AGT GTT ACA ACC-3′
U_L31(SA3)	5′-TCG ATC TCG CTC CTG TCC CTG GAG CAC ACC CTG TGT ACC TAT GTA TGA CAC CGA CCC CAT CGC CGC GGC GCC CGG CCC GGG CCC TAT CAC GGC AAG GAG CGC CGG CGG GCG CGC TCC TCT GCG GCC GGC GTA GGG ATA ACA GGG T-3′, 5′-GGC CCC GAT AGC GCT GGC GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG CGT GAG GCG GCA GGC TCT TCC GGG CGG CCC GAC GCA CCA CGC CCA GAG TCC CGC CGG CCG CAG AGG AGC GCG CCC GCC GGC GCT CCT TGC CAG TGT TAC AAC C-3′
U_L31(SA6)	5′-GCC CGG GCC CTA TCA CGG CAA GGA GCG CCG GCG G GCG CGC GCT GCT GCG GCC GGC GGG ACT CTG GGC GTG GTG CGT CGG GCC GCC CGG AAG TAG GGA TAA CAG GGT-3′, 5′-CGT GTA AAC ACA GCT CCT GTT TGC GGG CGT GAG GCG GCA GTG CCT TCC GGG CGG CCC GAC GCA CCA CGC CCA GAG TCC CGC CGC CAG TGT TAC AAC C-3′
U_L31(SAR)	5′-GGG CCT CCC GGA AGA GCC TGC CGC CTC ACG CCC GCA AAC AGG AGC TGT GTT TAC ACG AGC TAG GGA TAA CAG GGT-3′, 5′-GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG CGT GAG GCG GCA TAG GTA CAC AGG GTG TGC GCC AGT GTT ACA ACC-3′[b]
U_L31(SAE)	5′-CCC GGG CCC TAT CAC GGC AAG GAG CGC CGG CGG GCG CGC GAA GAA GCG GCC GGC GGG ACT CTG GGC GTG GTG CGT CGG GCC GCC CTA GGG ATA ACA GGG T-3′, 5′-GTG TAA ACA CAG CTC CTG TTT GCG GGC GTG AGG CGG CAG TTC CTT CCG GGC GGC CCG ACG CAC CAC GCC CAG AGT CCC GCC GGC CGC CAG TGT TAC AAC C-3′
U_L31(SE6)	5′-TCG ATC TCG CTC CTG TCC CTG GAG CAC ACC CTG TGT ACC TAT GTA TGA CAC CGA CCC CAT CGC CGC GGC GAA CGG CCC GGG CCC TAT CAC GGC AAG GAG CGC CGG CGG GAA CGC GAA GAA GCG GCC GGC GTA GGG ATA ACA GGG T-3′, 5′-GGC CCC GAT AGC GCT GGC GCT CGT GTA AAC ACA GCT CCT GTT TGC GGG CGT GAG GCG GCA GTT CCT TCC GTT CGG CCC GAC GCA CCA CGC CCA GAG TCC CGC CGG CCG CTT CTT CGC GTT CCC GCC GGC GCT CCT TGC CAG TGT TAC AAC C-3′
U_L31(SE6)/U_S3(K220A)	5′-TGA CAG CAG CCA TCC AGA TTA CCC CCA ACG GGT AAT CGT GGC GGC GGG GTG GTA CAC GAG CAT AGG GAT AAC AGG GTA ATC GAT TT-3′, 5′-CAG TCG CGC CTC GTG GCT CGT GCT CGT GTA CCA CCC CGC CGC CAC GAT TAC CCG TTG GGG GTG CCA GTG TTA CAA CCA ATT AAC C-3′

[a] Introduced mutations are underlined.
[b] Amplicons generated using these primers were PCR fused with a second amplicon generated from wild-type HSV-1(F) genomic DNA with primer pair 5′-TGCATAGCGTTGCGTGCC-3′ and 5′-TTTGCGGGCGTGAGGC-3′ to obtain the 5′ extension required for homologous recombination.

One-step viral growth assay. The 100% confluent Vero cells grown in 12-well plates were infected with 5.0 PFU per cell of the viruses shown in Fig. 4 and 8. After the cells were incubated for 1 h at 37°C, residual surface infectivity was inactivated with a low-pH wash (40 mM citric acid [pH 3.0], 10 mM KCl, 135 mM NaCl). At various time points, the cultures were frozen and thawed to lyse the cells, and infectious virus was titrated by plaque assay on Vero cells. The mean values of the results from two independent experiments and the corresponding standard deviations were calculated and plotted.

Antibodies and immunofluorescence assay. The polyclonal chicken antibody against pU_L34 was a kind gift from R. J. Roller (27). Rabbit anti-pU_L31 antisera were prepared in our laboratory and were described previously (26). M2 mouse monoclonal antibody against the Flag epitopic tag was purchased from Sigma.

Hep2 cells growing on glass coverslips were mock infected or were infected with the viruses shown in Fig. 5 and 7 at a multiplicity of infection (MOI) of 5 PFU/cell for 16 h. The cells were fixed with 3% paraformaldehyde for 15 min. For the experiments for which the preadsorbed pU_L31-specific rabbit polyclonal antibody was to be used, the cells were treated for another 15 min at −20°C in methanol. The cells were then permeabilized with 0.1% Triton X-100 and reacted with 10% human serum in phosphate-buffered saline to block nonspecific immunoreactivity, and they were subsequently probed with the preadsorbed pU_L31 antiserum diluted 1:50 in phosphate-buffered saline supplemented with 1% bovine serum albumin. Thereafter, 10% BlockHen II (Aves Lab) was used for a second round of blocking before probing with a 1:400 dilution of chicken anti-pU_L34 polyclonal antibody. Bound primary antibodies were recognized by the corresponding secondary antibodies conjugated with fluorescein isothiocyanate or Texas Red, respectively (Jackson ImmunoResearch). Digital images were recorded using an Olympus confocal microscope and were processed with Adobe Photoshop.

Electron microscopy. The cells were first fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate (pH 7.4) (Electron Microscopy Sciences) for 30 min at room temperature and then 90 min at 4°C. After three 5-min washes with the same buffer, the cells were fixed with 2% OsO_4 in the same buffer and rinsed again. The cells were then dehydrated at 4°C using a graduated series of ethanol concentrations (20%, 50%, 70%, and 100%), a subsequent acetone-ethanol mixture (1:1), and, finally, 100% acetone. This was followed by stepwise infiltration with Epon-Araldyte resin (EM Sciences) over the course of 64 h. The samples were dispensed into Beem capsules, and the resin was polymerized at 80°C overnight. Thin sections (60- to 90-nm thick) were collected on 300-mesh copper grids and viewed with a Philips 201 transmission electron microscope. Conventionally rendered negatives of electron microscopic images were scanned by using Microtek Scanmaker 5 and Scanwizard Pro PPC 1.02 software. Positive images were rendered from digitized negatives with Adobe Photoshop software.

RESULTS

The N-terminal domain of pU_L31 is functionally important to viral propagation. The HSV-1 U_L31 protein is highly conserved among members of the *Herpesviridae*. Interestingly, the first 50 amino acids of the N terminus of the U_L31 protein share little homology at the primary sequence level with other herpesvirus homologs. The clustered charged residues and the hydrophilic nature of this region distinguishes it from the remainder of HSV-1 pU_L31, which is mostly hydrophobic (not shown). To investigate the functional relevance of this charged region of pU_L31, the truncated U_L31(dN) gene lacking the region from codon 2 to 44 was transfected into Hep2 cells, and its ability to support the replication of the U_L31 null HSV-1 mutant was determined by a plaque assay. Plasmids bearing the full-length U_L31 open reading frame and vector DNA (pcDNA3) served as positive and negative controls, respec-

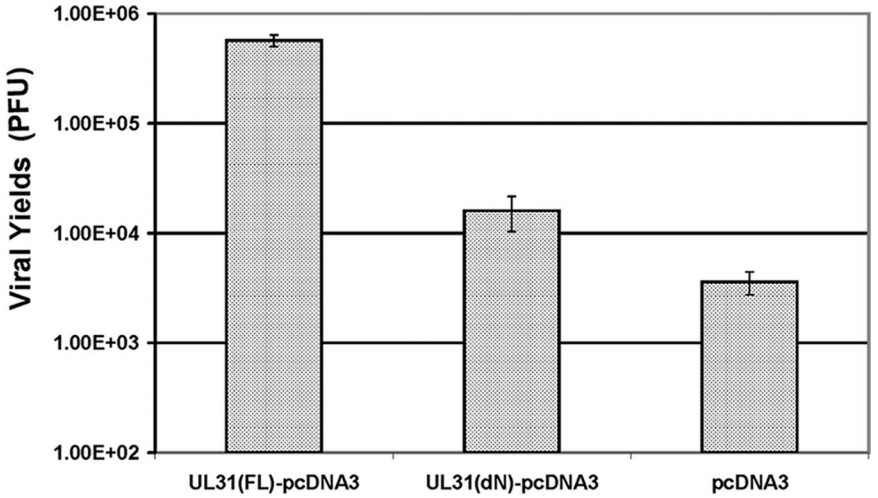

FIG. 1. Functional analysis of the N terminus of pU_L31. Hep2 cells were transfected with a pcDNA3 vector plasmid or plasmids bearing full-length U_L31(FL) or the N-terminally deleted U_L31(dN). The cells were then infected with the U_L31 null virus at 5.0 PFU/cell. Infectivity at 24 hpi was determined by freeze-thawing the infected cells, followed by titration on a U_L31-complementing cell line. Experiments were performed in duplicate. Each histogram represents the mean value, and the standard deviations are indicated by error bars.

tively. Viral infectivity at 24 hpi was titrated on U_L31-complementing cells.

As shown in Fig. 1, the expression of full-length pU_L31 increased the yield of the infectious virus more than 100-fold above the background levels produced by the transfection of the vector DNA. In contrast to this result, the plasmid bearing N-terminally truncated pU_L31 produced only a fivefold enhancement of the viral yield over the background. This result indicated that the N-terminal domain is important for the full function of the HSV-1 U_L31 protein.

The pU_L31 N-terminal domain harbors multiple phosphorylation sites of the viral U_S3 kinase. The N-terminal sequence of pU_L31 is arginine and serine rich, which is reminiscent of U_S3 serine/threonine kinase phosphorylation motifs (24). Because it was already known that pU_L31 was a substrate of pU_S3, we hypothesized that the U_S3 kinase phosphorylated at least some of the putative U_S3 phosphorylation motifs located at the pU_L31 N terminus. The pU_S3 phosphorylation sites of pU_L31 were therefore mapped as follows. The U_L31 codons 1 to 50, 51 to 165, 155 to 165, and 255 to 306 (Fig. 2A) were fused to the gene encoding GST, and the corresponding fusion proteins were purified from *E. coli* by GST affinity chromatography. The purified proteins were then incubated with partially purified recombinant baculovirus-expressed U_S3 kinase in buffer containing [γ-^{32}P]ATP. The reaction components were then separated on an SDS polyacrylamide gel, and the gel was stained with Coomassie brilliant blue, dried, and exposed to X-ray film. As shown in Fig. 2B, equal amounts of all four fragments were present in the reactions (top panel), but only the N-terminal 50 amino acids of pU_L31 were labeled with ^{32}P (bottom panel).

An analysis of the primary sequence of the first 50 amino acids of pU_L31 indicated that six serine residues were in a context potentially consistent with pU_S3 phosphorylation sites (Fig. 2A). Of the six residues, serines 11, 24, and 40 completely matched the predicted pU_S3 consensus motif (RnXS/T [$n \geq 2$]) (24), while the other three serines (S26, S27, and S43) could not be excluded since the kinase was found to be more promiscuous than originally predicted (21). We therefore systematically substituted serines encoded by this region with alanines and tested the corresponding mutants for their capacity to be phosphorylated by pU_S3 in vitro. The constructs were designated pU_L31(SA3) bearing S11, S24, and S40 changed to alanine codons and pU_L31(SA6) bearing all six serines, S11, S24, S26, S27, S40, and S43, changed to alanines. Mixing GST fusion proteins bearing these mutations with active U_S3 kinase and [^{32}P]ATP revealed that wild-type pU_L31 was heavily phosphorylated by U_S3 kinase, whereas mutating all six serines completely abolished phosphorylation. In contrast, the SA3 mutations decreased but did not eliminate U_S3-dependent phosphorylation (Fig. 2C). The results reveal that the pU_L31 N terminus contains multiple sites that the U_S3 kinase can phosphorylate in vitro and suggest that the protein is phosphorylated by the U_S3 kinase during infection as indicated previously (11).

Precluding the phosphorylation of the pU_L31 N terminus impairs viral propagation. We next proceeded to determine whether the N terminus of pU_L31 was phosphorylated by U_S3 during viral infection. Recombinant viruses bearing the six serine-to-alanine mutations in U_L31 and its corresponding genetically repaired virus U_L31(SAR) were generated through BAC mutagenesis. Cells were infected with 5.0 PFU/cell of the wild-type virus HSV-1(F), the kinase-dead virus U_S3(K220A), the U_L31 mutant virus U_L31(SA6), or the repaired virus U_L31(SAR). At 16 hpi, the cells were harvested, and electrophoretically separated proteins were subjected to immunoblotting with pU_L31-specific antiserum.

The results, shown in Fig. 3, indicated the following. (i) Consistent with previous reports (11), bacteriophage lambda phosphatase (λ-PPase) treatment caused an increase in the electrophoretic mobility of the pU_L31-specific band of HSV-1(F) (lanes 1 and 2). The migration of this band was similar to that of nontreated pU_L31 from cells infected with U_S3(K220A) (lane 3), indicating that pU_S3 played an important role in the

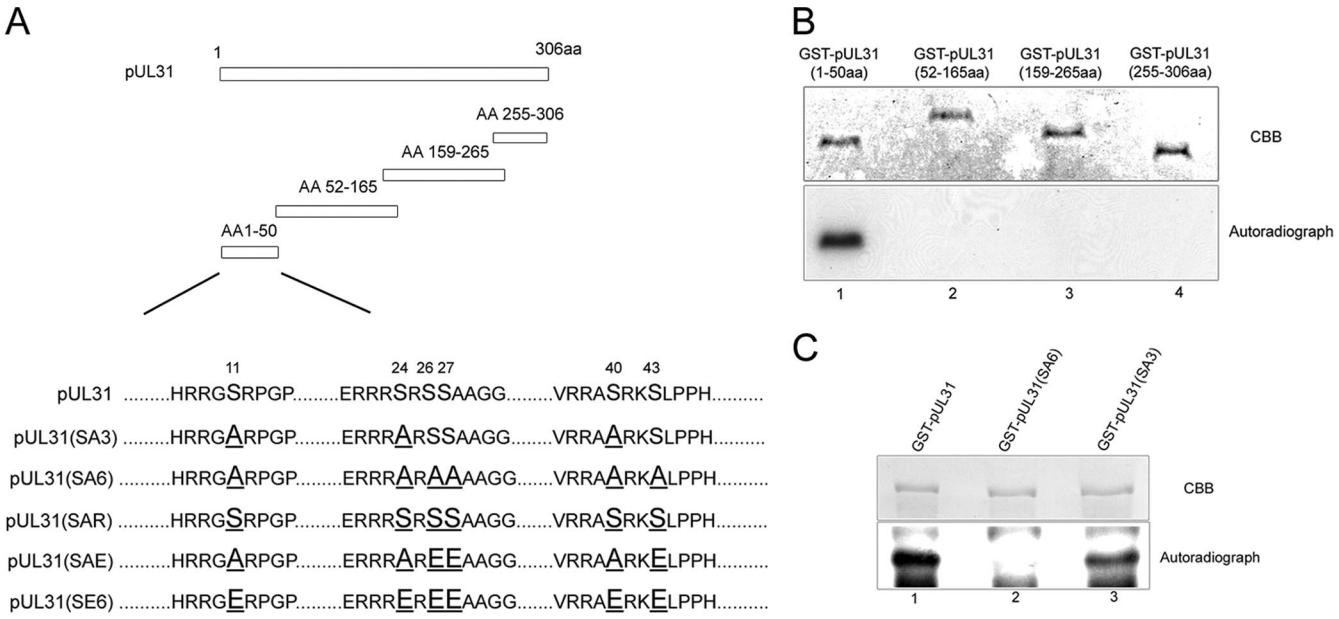

FIG. 2. Mapping the pU_L31 phosphorylation sites of the pU_S3 kinase. (A) Schematic illustration of the full-length U_L31 protein and its subfragments as shown in panel B and the primary sequence showing potential phosphorylation sites (underlined). A series of constructs with point mutations used in subsequent experiments is listed. (B) In vitro kinase assay with subfragments of pU_L31. GST fusion proteins purified from *E. coli* were incubated with purified U_S3 kinase and [γ-P^{32}]ATP. The reaction products were electrophoretically separated on a denaturing gel and were stained by Coomassie brilliant blue (CBB), dried, and exposed to X-ray film. (C) Wild-type and mutant pU_L31 reacted with purified U_S3 kinase in vitro. Reactions were performed as described for panel B. Reactants were separated on a denaturing gel, visualized by CBB staining, and dried. The presence or absence of phosphorylation was determined by autoradiography of the same gel.

phosphorylation of pU_L31 during infection directly or indirectly. (ii) The substitution of all six serines by alanines increased the electrophoretic migration of the U_L31 protein. Phosphatase treatment did not cause a mobility change of this mutant protein (lanes 5 and 6), suggesting that these were the only sites in pU_L31 that were phosphorylated, at least as detected by one-dimensional electrophoresis. In contrast, the mobility of pU_L31 expressed by the genetically repaired virus U_L31(SAR) (lanes 7 and 8) was greatly increased by λ-PPase treatment, confirming that pU_L31 phosphorylation was successfully blocked by the SA6 mutations. (iii) All of the nonmutated pU_L31 proteins (lanes 1, 3, and 7) produced wider bands than their λ-PPase-treated counterparts (lanes 2, 4, and 8), implying the existence of heterogeneously phosphorylated pU_L31 species during infection.

We then conducted one-step viral growth assays to gauge the infectivity of the U_L31(SA6) virus. Cells were infected with 5 PFU/cell of U_L31(SA6), the restored virus U_L31(SAR), the wild-type HSV-1(F), or U_S3(K220A) at an MOI of 5, and viral yields at the indicated time points (Fig. 4) were determined on Vero cells. As shown in Fig. 4, the U_L31(SA6) and U_S3(K220A) viruses displayed a similar pattern of viral growth over time. Of note was a nearly 10-fold decrease in their titers at 12 hpi compared to that of the wild-type virus. On the other hand, both recombinant viruses produced titers similar to that of HSV-1(F) at 24 hpi. Impaired viral growth was eliminated by repairing the SA6 mutation inasmuch as the virus U_L31(SAR) replicated as efficiently as HSV-1(F). We conclude from these data that precluding the phosphorylation of pU_L31 mimics the growth defect of the U_S3 kinase-dead virus.

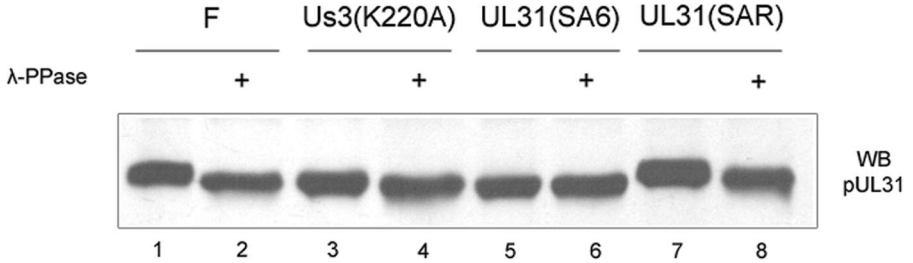

FIG. 3. Immunoblot to determine the phosphorylation state of pU_L31 during infection with wild-type and mutant viruses. Hep2 cells were infected with wild-type HSV-1(F) (lanes 1 and 2), U_S3(K220A) (lanes 3 and 4), U_L31(SA6) (lanes 5 and 6), and U_L31(SAR) (lanes 7 and 8) viruses at an MOI of 5.0 PFU/cell for 16 h. Prepared lysates were treated or untreated with λ-PPase as indicated. Cell lysates were electrophoretically resolved on denaturing polyacrylamide gels, electrically transferred to nitrocellulose, and probed with antibody to pU_L31.

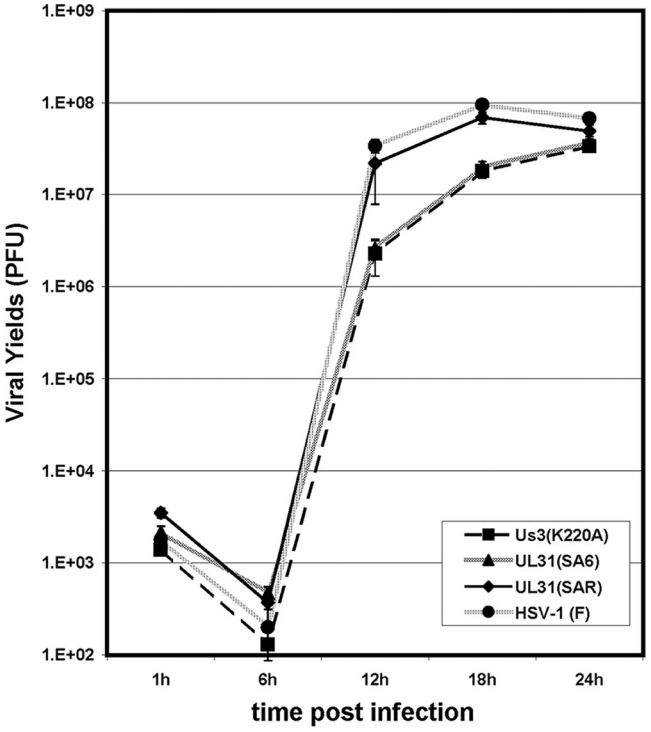

FIG. 4. One-step growth curves of the wild-type and mutant viruses. Hep-2 cells were infected with the wild-type HSV-1(F), U_S3(K220A), U_L31SA6, or U_L31SAR virus at an MOI of 5. After adsorption for 1 h, residual infectivity was inactivated by a low-pH wash. At the indicated time points, pooled intracellular and extracellular viral infectivity was titrated on Vero cells. Experiments were done in duplicate. Mean values are plotted, and deviations are represented by error bars.

Precluding the phosphorylation of the pU_L31 N terminus mimics the effects of the U_S3 kinase deficiency in pU_L31/pU_L34 complex localization and perinuclear virion accumulation. Although the absence of U_S3 kinase activity causes a defect in capsid nuclear egress, including the mislocalization of pU_L34 at the nuclear rim and the accumulation of primary enveloped virions in the perinuclear space, the substrate responsible for these changes had not been identified. Because the growth kinetics of the U_L31(SA6) and Us3(K220A) viruses were very similar, we compared the localization of pU_L34 and virions in cells infected with the U_L31(SA6) and U_S3 kinase-dead viruses.

Hep2 cells were infected with the wild-type HSV-1(F), U_S3(K220A), U_L31(SA6), or U_L31(SAR) virus for 16 h, at which time the cells were fixed, permeabilized, immunostained with antibodies against pU_L31 and pU_L34, and visualized by confocal microscopy (Fig. 5). Consistent with previous reports, the pU_L31 and U_L34 proteins colocalized smoothly along the nuclear envelope in cells infected with HSV-1(F). The deactivation of the U_S3 kinase in cells infected with the U_S3(K220A) virus induced discrete foci of pU_L31 and pU_L34 at the nuclear rim. In cells infected with the U_L31(SA6) virus, the focal distribution of pU_L31/pU_L34 appeared very similar to that of cells infected with U_S3(K220A). The aberrant distribution of pU_L31/pU_L34 was restored to the wild-type distribution in cells infected with the genetically repaired virus U_L31(SAR).

To examine the effects of pU_L31 phosphorylation on the distribution of virions, cells were infected with the wild-type HSV-1(F), U_S3(K220A), U_L31(SA6), or U_L31(SAR) virus and embedded, and thin sections were stained and examined by electron microscopy. As shown in Fig. 6, U_L31(SA6) viral infection produced nuclear membrane invaginations containing multiple primary enveloped virions. Enumerating these invaginations indicated that in 25 sections of cells infected with each virus, U_L31(SA6)-infected cell sections contained an average of 1.6 invaginations/section (range, 0 to 5), whereas the U_S3(K220A)-infected cells yielded 3.75 invaginations per section (range, 0 to 14). The presence of the virions in these invaginations implied an egress defect for both viruses. The appearance of these infected cells was dissimilar to that of cells infected with HSV-1(F) or the restored virus U_L31(SAR) where such invaginations were not observed (not shown).

We conclude that the phosphorylation of pU_L31 is necessary for the proper localization of the pU_L31/pU_L34 complex in the nuclear rim and for the optimal egress of virions from the perinuclear space.

Proper pU_L31/pU_L34 distribution can be restored by increasing the acidity of the pU_L31 N terminus. In preliminary studies, the localization of pU_L31/pU_L34 in cells infected with a virus [designated U_L31(SA3)] expressing pU_L31 with point mutations S11A, S24A, and S40A was undistinguishable from that in cells infected with the wild-type virus (not shown). This observation suggested that phosphorylation at sites S26, S27, and/or S43 were sufficient to enable pU_L31 to function properly. With this in mind, two pseudophosphorylated pU_L31-expressing viruses were generated, one termed U_L31(SAE), which encoded residues 11, 24, and 40 as alanines while residues 26, 27, and 43 were changed to glutamic acid, and one termed U_L31(SE6), which had all six residues changed to glutamic acid (diagrammed in Fig. 2A).

An immunofluorescence analysis of pU_L31 and pU_L34 distribution in cells infected with these viruses revealed phenotypic differences (Fig. 7). Specifically, U_L31(SAE) still produced punctate pU_L31/pU_L34-specific staining like the U_L31(SA6) or U_S3(K220A) viruses, whereas these proteins were distributed smoothly throughout the nuclear rim in cells infected with U_L31(SE6). Taken together with the distribution patterns of pU_L31/pU_L34 in cells infected with the U_L31(SA3) and U_L31(SA6) viruses, the data indicated that the acidification of the pU_L31 N terminus, either through phosphorylation or the experimental introduction of glutamic acid, was critical for proper pU_L31/pU_L34 localization at the nuclear rim.

In order to confirm the rescuing effect of the acidity of pU_L31 in pU_L31/pU_L34 complex localization and to see if effecters of U_S3 other than U_L31 played a role, a novel virus was generated in which the mutation abolishing U_S3 kinase activity was introduced into the U_L31(SE6) virus, resulting in a U_L31(SE6)/U_S3(K220A) double mutant virus. An analysis of the distribution of the pU_L31/pU_L34 complex in cells infected with this mutant indicated that the complex localized in a smooth distribution at the nuclear rim in contrast to what would be expected in cells infected with U_S3(K220A). Thus, the rescuing phenotype of pseudophosphorylated pU_L31 was dominant over the inactive U_S3 kinase phenotype. These observations suggested that pU_L31 is the major substrate of U_S3

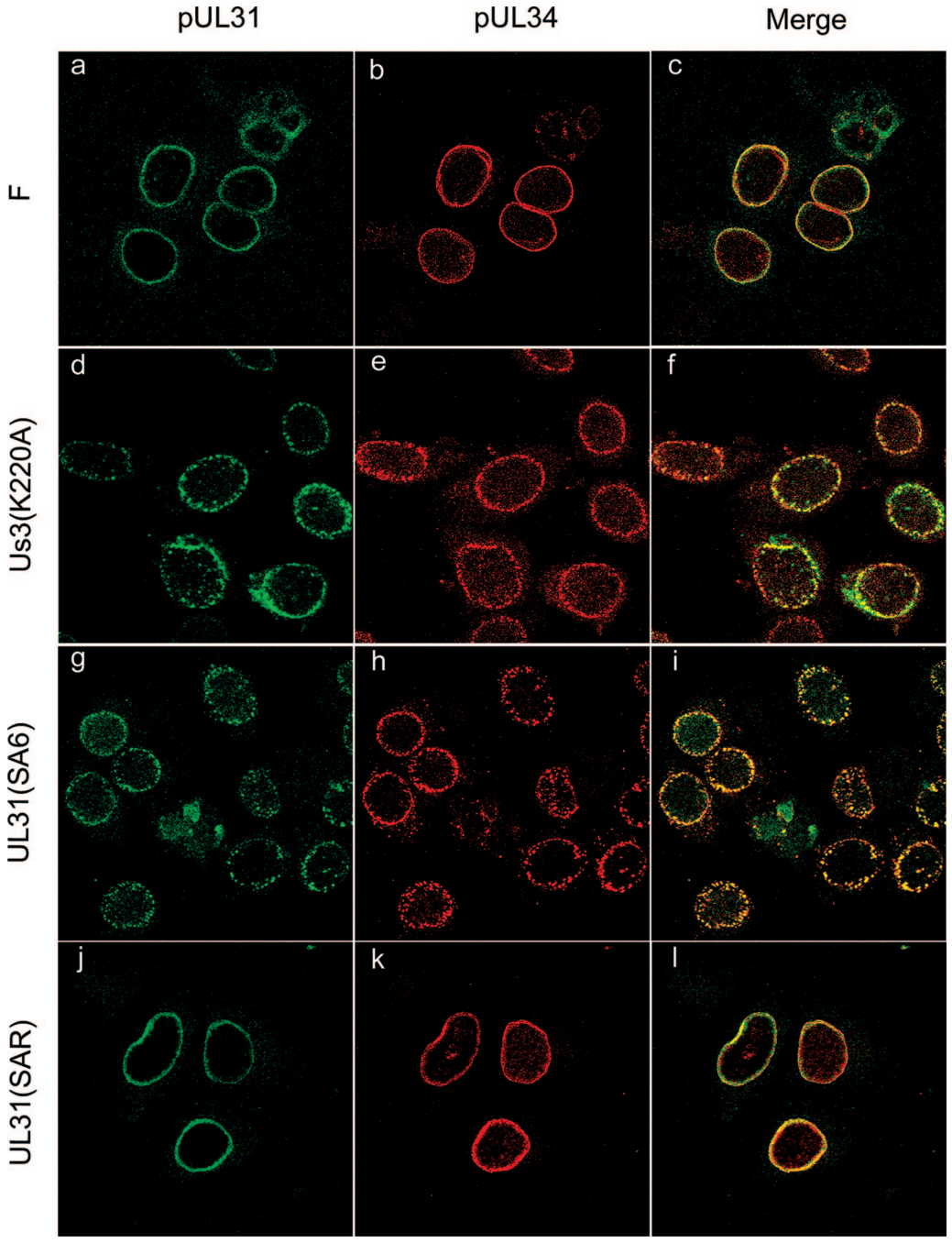

FIG. 5. Confocal immunofluorescence staining of pU_L31 and pU_L34 in HSV-1-infected Hep2 cells. Cells were infected with the wild-type HSV-1(F), U_S3(K220A), U_L31(SA6), or U_L31(SAR) virus at 5.0 PFU/cell. After 16 h, the cells were fixed in paraformaldehyde and methanol, permeabilized, immunostained for pU_L31 (green) and pU_L34 (red), and visualized by confocal microscopy. Optical sections were taken through the middle of the cells.

kinase responsible for regulating the localization of pU_L31/pU_L34 at the nuclear rim.

The pseudophosphorylation of pU_L31 inhibits the primary envelopment of nucleocapsids. Based on the immunofluorescence data above, we presumed that unlike the U_S3 kinase-dead virus, the U_L31(SE6) virus and U_L31(SE6)/U_S3(K220A) viruses (i) would not induce herniations of the nuclear membrane containing perinuclear virions and (ii) would replicate as efficiently as wild-type HSV-1. To test the first prediction, cells were infected with these viruses and examined by electron microscopy 16 h later. Consistent with the hypothesis, no excessive perinuclear virion accumulation was observed in cells infected with either virus over that seen in cells infected with wild-type HSV-1(F) (data not shown). Inconsistent with the

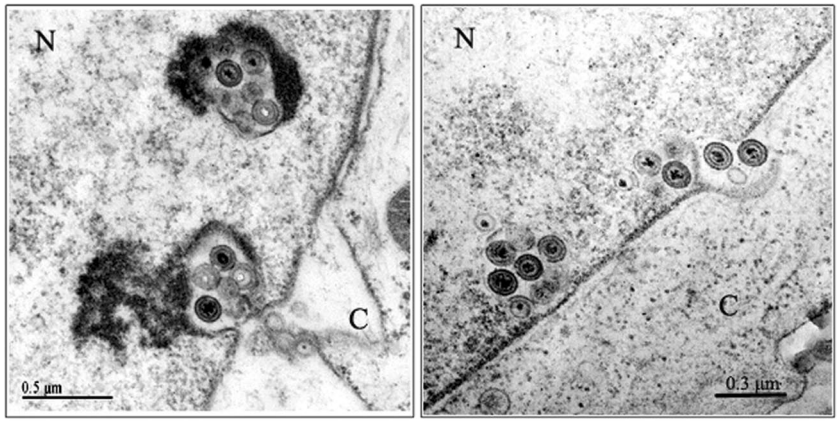

FIG. 6. Electron microscopy of Hep2 cells infected with HSV mutants. Cells were infected for 16 h at an MOI of 5.0 PFU/cell with the indicated viruses, fixed, embedded, sectioned, stained, and examined in a transmission electron microscope. Images show primary enveloped virion accumulation in Hep2 cells infected by U_S3(K220A) viruses or U_L31(SA6) viruses. N, nucleus; C, cytoplasm. The scale in μm is indicated.

second hypothesis above, however, very few viral particles were detected in the cytoplasm or on the surface of cells infected with the U_L31(SE6) virus or the U_L31(SE6)/U_S3(K220A) viruses (not shown). This was the case despite ample numbers of capsids within the nuclei of these cells. Counting a representative nine cell sections indicated that whereas the ratio of cytoplasmic to intranuclear capsids was approximately 1.55 in cells infected with HSV-1(F), this ratio was only 0.11 in cells infected with the U_L31(SE6) mutant virus and 0.02 for the U_L31(SE6)/U_S3(K220A) double mutant (Table 3). These data suggest that the mutant virions were enveloped at the INM less efficiently than their wild-type counterparts.

To further investigate the phenotypes of the U_L31(SE6) and U_L31(SE6)/U_S3(K220A) viruses, cells were infected with these viruses at 5.0 PFU/cell and the amount of infectious virus was determined at various times after infection. As shown in Fig. 8, the U_L31(SE6) and U_L31(SE6)/U_S3(K220A) viruses produced less infectious virus throughout the infection than HSV-1(F). The most marked discrepancy was observed at 12 hpi, when the amount of infectious U_L31(SE6) virus was reduced approximately 10-fold compared to that of HSV-1(F), whereas the U_L31(SE6)/U_S(K220A) virus titer was reduced more than 20-fold. As shown previously, the U_S3(K220A) virus also displayed a 10-fold lower titer at 12 hpi compared to HSV-1(F) but eventually produced infectious virus titers approaching those of the wild-type virus. In contrast to this result, the two U_L31 mutants reached their peak titers at 18 hpi and never reached infectious titers approaching those of wild-type HSV-1(F). These data indicate that the nuclear egress defects of the U_L31 mutants are more persistent than that of the U_S3(K220A) virus, suggesting a defect in primary envelopment that does not diminish as infection proceeds. We also noted that the double mutant U_L31(SE6)/U_S3(K220A) replicated slightly less efficiently than either of the viruses with single mutations, indicating that the pseudophosphorylated pU_L31 is insufficient to complement fully the contribution of U_S3 kinase activity to viral infectivity.

DISCUSSION

Nucleocapsid budding through the INM and fusion of the nascent virion envelope with the ONM is believed to be highly efficient. This conclusion comes from the observation that during wild-type virus infection, there is rarely extensive virion accumulation in the perinuclear space. In cells infected with U_S3 kinase-dead viruses, however, the accumulation of perinuclear virions in herniations of the perinuclear space is commonplace. This aberrant virion accumulation likely reflects an imbalance between the rate of delivery into localized regions of the perinuclear space and the rate of exit from these regions. With this paradigm in mind, the U_S3 kinase might serve to decrease the numbers of virions in particular perinuclear regions because its activity disperses the nuclear envelopment complex widely throughout the nuclear membrane. Thus, the rate of exit from the nucleoplasm is similar to that of the wild-type virus, but the number of envelopment sites is increased. As a result, perinuclear virions are not observed in particular regions. U_S3 may also promote the fusion of the perinuclear virion envelope with the ONM, thereby increasing the rate of exit from the perinuclear space (14).

Perhaps the most important finding of the current work is that most aspects of the U_S3 nuclear egress phenotype, including the mislocalization of the pU_L31/pU_L34 complex and virion accumulation in herniations of the perinuclear space, can be mimicked by precluding the phosphorylation of pU_L31 at its N terminus. Thus, U_S3 mediates much of its effects in nuclear egress through the phosphorylation of pU_L31. Although we have no evidence indicating that the rate of budding into the perinuclear space is affected by the absence of the phosphorylation of pU_L31, the aggregation of pU_L31/pU_L34 may restrict the number of budding sites, causing nucleocapsids to continue budding into herniations of the perinuclear space that already contain multiple perinuclear virions. The machinery that mediates the fusion of the perinuclear virion and the ONM therefore may become overwhelmed, leading to the observed phenotype.

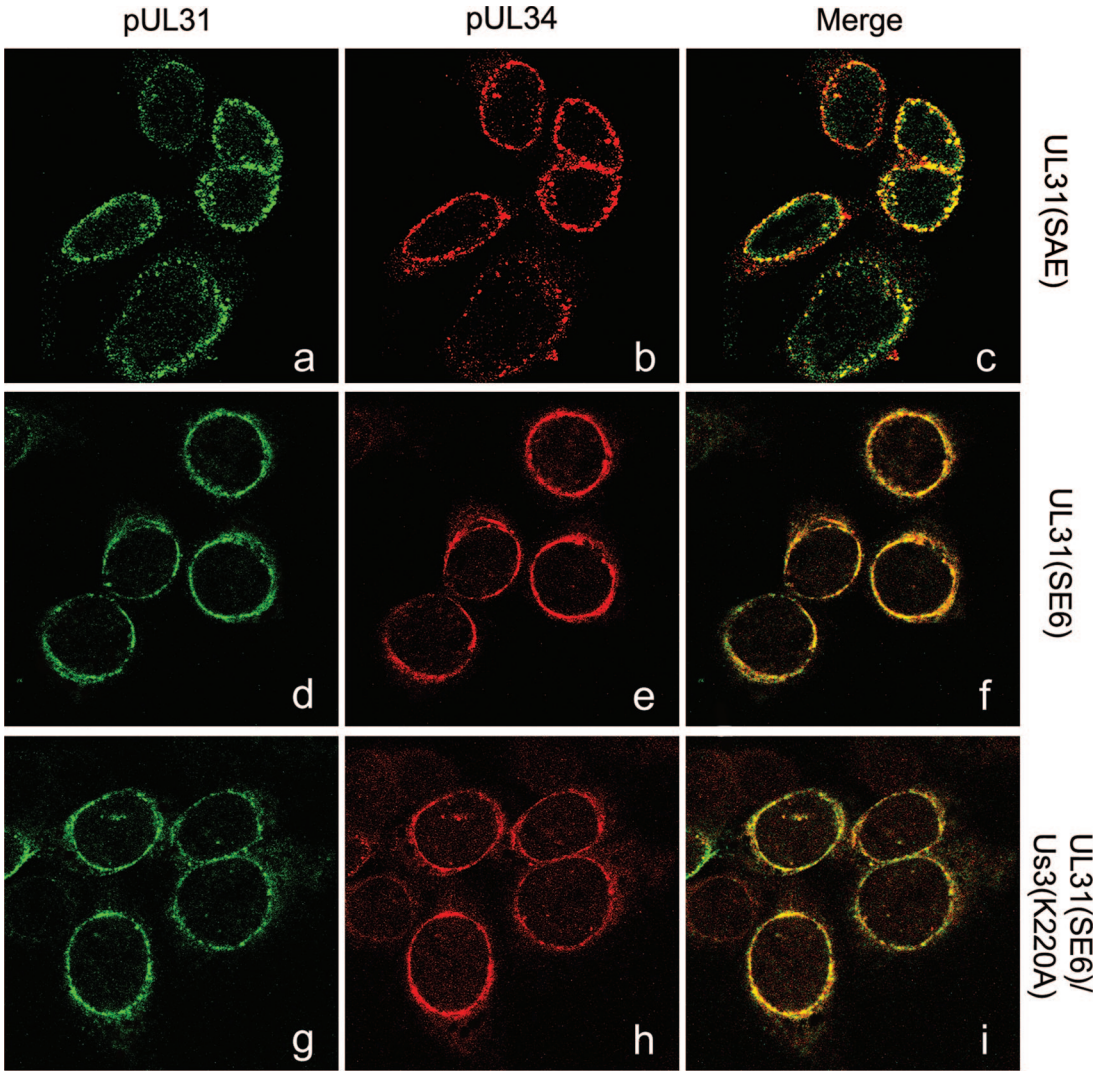

FIG. 7. pU_L31 and pU_L34 localization in Hep2 cells infected with pseudophosphorylated U_L31 viruses. Hep2 cells were infected with the indicated viruses and were fixed at 16 h after infection in paraformaldehyde and methanol. The cells were then immunostained for pU_L31 (green) and pU_L34 (red) and visualized by confocal microscopy. Optical sections were taken through the middle of the cells.

Our data also indicate that pseudophosphorylated pU_L31(SE6) is defective in the primary envelopment of nucleocapsids at the INM and is insufficient to complement the full contribution of U_S3 kinase to replication. We cannot exclude the possibility that the serine-to-glutamic-acid mutations cause some misfolding of pU_L31, thereby impairing its ability to mediate nuclear egress. Alternatively, the data suggest that substrates of U_S3 kinase other than pU_L31 are important to nuclear egress. We speculate that these other substrates include an impaired or mistriggered apparatus responsible for fusing the nascent virion envelope with the ONM (possibly through effects on gB and gH). This is difficult to assess because the paucity of perinuclear virions in cells infected with pU_L31(SE6) precludes an evaluation of whether the mutation also affects egress at later steps.

TABLE 3. Subcellular distribution of viral particles in Hep2 cells infected for 16 h and examined by electron microscopy

Virus	Total no. of intracellular viral particles			No. of cells	Average ratio (no. of cytoplasmic particles/no. of nuclear particles)
	Nuclear	Cytoplasmic	Perinuclear		
HSV-1(F)	73	113	10	9	1.55
U_L31(SE6)	186	21	0	9	0.11
U_L31(SE6)/U_S3 (K220A)	129	3	2	9	0.02

The data presented herein suggest that the function of pU_L31 is tightly and dynamically controlled by phosphorylation as diagrammed in Fig. 9. We hypothesize that there is a mixed population of both unphosphorylated and phosphorylated pU_L31 and that most pU_L31 at the INM is phosphorylated, thereby preventing pU_L31/pU_L34 aggregation and premature budding. This conclusion is consistent with the observations that (i) pU_L31/pU_L34 aggregates aberrantly to

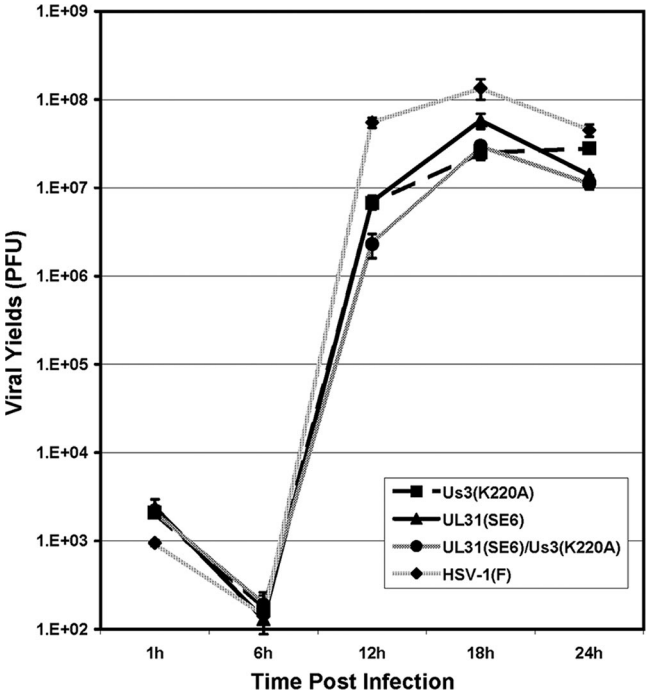

FIG. 8. One-step growth curves of pseudophosphorylated U_L31 viruses. Hep-2 cells were infected with wild-type HSV-1(F), U_S3(K220A), U_L31(SE6), or U_L31(SE6)/U_S3(K220A) at an MOI of 5 PFU/cell. Residual viruses were inactivated by a low-pH wash at 1 hpi. At the indicated time points, virus in the whole culture was collected and titrated on Vero cells. Experiments were done in duplicate. Mean values are plotted, and simple standard deviations are represented by error bars.

form foci when pU_L31 phosphorylation by pU_S3 is precluded, (ii) pU_L31/pU_L34 foci do not form upon the pseudophosphorylation of pU_L31, and (iii) the foci comprise pU_L31/pU_L34 associated with perinuclear virions and pU_L31/pU_L34 located at the INM but free from virions (27). We further propose that at primary envelopment sites in the INM, the unphosphorylated form is involved in the initiation of the budding reaction, possibly by direct or indirect interaction with the nucleocapsid or other egress components. Once in the perinuclear space, newly phosphorylated pU_L31 is needed to promote the fusion of the perinuclear virion with the ONM, probably by activating the fusion properties of gH and gB. This phosphorylation of pU_L31 may also decrease the affinity of pU_L31/pU_L34 for nucleocapsids, facilitating nucleocapsid release into the cytosol after membrane fusion.

Finally, we note that in our previous study of cellular lamin A/C, we reasoned that the punctate distribution of pU_L31/pU_L34 in cells infected with U_S3 kinase-dead viruses might be consequential to physical constraints induced by a hypophosphorylated, and therefore less flexible, nuclear lamina (21). The current observation that the U_L31/U_L34 proteins are of punctate distribution in cells infected with the U_L31(SA6) mutant argue against this possibility because these cells contain active pU_S3, which would be expected to phosphorylate lamin A/C. Thus, the phosphorylation of lamin A/C by pU_S3 is separable from functions required for the proper localization of pU_L31/pU_L34. Instead, the latter is dependent on the pU_S3-mediated phosphorylation of pU_L31.

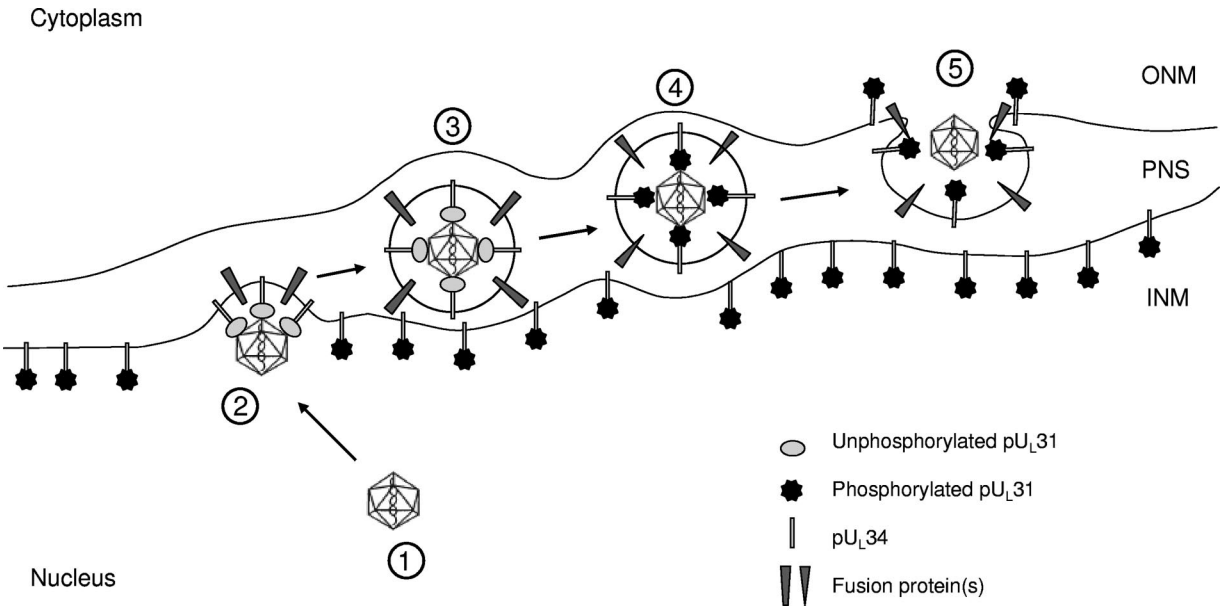

FIG. 9. Model of virion egress consistent with presented data. (1) Nucleocapsids are produced in the nucleoplasm. (2) The nucleocapsid engages unphosphorylated pU_L31 associated with pU_L34 at the INM directly or indirectly. pU_L31 in the U_L31/U_L34 complex is mostly phosphorylated to preclude promiscuous budding and pU_L31/pU_L34 aggregation. (3) The completion of budding produces perinuclear virions bearing fusion proteins, the U_S3 kinase (not shown), and pU_L34 bound to unphosphorylated pU_L31. (4) pU_L31 becomes phosphorylated by pU_S3 in the virion conferring a signal to initiate fusion between the virion envelope and the inner surface of the ONM. (5) Fusion protein conformation is altered and fusion occurs. Phosphorylated pU_L31, pU_L34, and fusion proteins remain at the ONM while the nucleocapsid is released into the cytosol. PNS, perinuclear space.

ACKNOWLEDGMENTS

We thank Greg Smith for the *E. coli* strain GS1783, Richard Roller for viruses and anti-pU_L34 IgY, and Yasushi Kawaguchi for interesting discussions.

These studies were supported by R01 grant AI52341 from the National Institutes of Health.

REFERENCES

1. **Benetti, L., J. Munger, and B. Roizman.** 2003. The herpes simplex virus 1 U_S3 protein kinase blocks caspase-dependent double cleavage and activation of the proapoptotic protein BAD. J. Virol. **77:**6567–6573.
2. **Benetti, L., and B. Roizman.** 2004. Herpes simplex virus protein kinase US3 activates and functionally overlaps protein kinase A to block apoptosis. Proc. Natl. Acad. Sci. USA **101:**9411–9416.
3. **Browne, H., B. Bruun, and T. Minson.** 2001. Plasma membrane requirements for cell fusion induced by herpes simplex virus type 1 glycoproteins gB, gD, gH and gL. J. Gen. Virol. **82:**1419–1422.
4. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. **62:**2596–2604.
5. **Cartier, A., T. Komai, and M. G. Masucci.** 2003. The Us3 protein kinase of herpes simplex virus 1 blocks apoptosis and induces phosphorylation of the Bcl-2 family member Bad. Exp. Cell Res. **291:**242–250.
6. **Chang, Y. E., C. Van Sant, P. W. Krug, A. E. Sears, and B. Roizman.** 1997. The null mutant of the U_L31 gene of herpes simplex virus 1: construction and phenotype of infected cells. J. Virol. **71:**8307–8315.
7. **Ejercito, P. M., E. D. Kieff, and B. Roizman.** 1968. Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. J. Gen. Virol. **2:**357–364.
8. **Farnsworth, A., T. W. Wisner, M. Webb, R. Roller, G. Cohen, R. Eisenberg, and D. C. Johnson.** 2007. Herpes simplex virus glycoproteins gB and gH function in fusion between the virion envelope and the outer nuclear membrane. Proc. Natl. Acad. Sci. USA **104:**10187–10192.
9. **Heldwein, E. E., H. Lou, F. C. Bender, G. H. Cohen, R. J. Eisenberg, and S. C. Harrison.** 2006. Crystal structure of glycoprotein B from herpes simplex virus 1. Science **313:**217–220.
10. **Hutchinson, L., K. Goldsmith, D. Snoddy, H. Ghosh, F. L. Graham, and D. C. Johnson.** 1992. Identification and characterization of a novel herpes simplex virus glycoprotein, gK, involved in cell fusion. J. Virol. **66:**5603–5609.
11. **Kato, A., M. Yamamoto, T. Ohno, H. Kodaira, Y. Nishiyama, and Y. Kawaguchi.** 2005. Identification of proteins phosphorylated directly by the Us3 protein kinase encoded by herpes simplex virus 1. J. Virol. **79:**9325–9331.
12. **Kato, A., M. Yamamoto, T. Ohno, M. Tanaka, T. Sata, Y. Nishiyama, and Y. Kawaguchi.** 2006. Herpes simplex virus 1-encoded protein kinase UL13 phosphorylates viral Us3 protein kinase and regulates nuclear localization of viral envelopment factors UL34 and UL31. J. Virol. **80:**1476–1486.
13. **Klupp, B. G., H. Granzow, W. Fuchs, G. M. Keil, S. Finke, and T. C. Mettenleiter.** 2007. Vesicle formation from the nuclear membrane is induced by coexpression of two conserved herpesvirus proteins. Proc. Natl. Acad. Sci. USA **104:**7241–7246.
14. **Klupp, B. G., H. Granzow, and T. C. Mettenleiter.** 2001. Effect of the pseudorabies virus US3 protein on nuclear membrane localization of the UL34 protein and virus egress from the nucleus. J. Gen. Virol. **82:**2363–2371.
15. **Leach, N., S. L. Bjerke, D. K. Christensen, J. M. Bouchard, F. Mou, R. Park, J. Baines, T. Haraguchi, and R. J. Roller.** 2007. Emerin is hyperphosphorylated and redistributed in herpes simplex virus type 1-infected cells in a manner dependent on both UL34 and US3. J. Virol. **81:**10792–10803.
16. **Liang, L., and J. D. Baines.** 2005. Identification of an essential domain in the herpes simplex virus 1 U_L34 protein that is necessary and sufficient to interact with U_L31 protein. J. Virol. **79:**3797–3806.
17. **Liang, L., M. Tanaka, Y. Kawaguchi, and J. D. Baines.** 2004. Cell lines that support replication of a novel herpes simplex 1 UL31 deletion mutant can properly target UL34 protein to the nuclear rim in the absence of UL31. Virology **329:**68–76.
18. **Manservigi, R., and P. G. Spear.** 1977. Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA **74:**3913–3917.
19. **McGeoch, D. J., M. A. Dalrymple, A. J. Davison, A. Dolan, M. C. Frame, D. McNab, L. J. Perry, J. E. Scott, and P. Taylor.** 1988. The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. **69:**1531–1574.
20. **Mettenleiter, T. C., B. G. Klupp, and H. Granzow.** 2006. Herpesvirus assembly: a tale of two membranes. Curr. Opin. Microbiol. **9:**423–429.
21. **Mou, F., T. Forest, and J. D. Baines.** 2007. U_S3 of herpes simplex virus type 1 encodes a promiscuous protein kinase that phosphorylates and alters localization of lamin A/C in infected cells. J. Virol. **81:**6459–6470.
22. **Munger, J., and B. Roizman.** 2001. The US3 protein kinase of herpes simplex virus 1 mediates the posttranslational modification of BAD and prevents BAD-induced programmed cell death in the absence of other viral proteins. Proc. Natl. Acad. Sci. USA **98:**10410–10415.
23. **Poon, A. P., and B. Roizman.** 2005. Herpes simplex virus 1 ICP22 regulates the accumulation of a shorter mRNA and of a truncated U_S3 protein kinase that exhibits altered functions. J. Virol. **79:**8470–8479.
24. **Purves, F. C., A. D. Deana, F. Marchiori, D. P. Leader, and L. A. Pinna.** 1986. The substrate specificity of the protein kinase induced in cells infected with herpesviruses: studies with synthetic substrates indicate structural requirements distinct from other protein kinases. Biochim. Biophys. Acta **889:**208–215.
25. **Purves, F. C., D. Spector, and B. Roizman.** 1991. The herpes simplex virus protein kinase encoded by the U_S3 gene mediates posttranslational modification of the phosphoprotein encoded by the U_L34 gene. J. Virol. **65:**5757–5764.
26. **Reynolds, A. E., B. Ryckman, J. D. Baines, Y. Zhou, L. Liang, and R. J. Roller.** 2001. U_L31 and U_L34 proteins of herpes simplex virus type 1 form a complex that accumulates at the nuclear rim and is required for envelopment of nucleocapsids. J. Virol. **75:**8803–8817.
27. **Reynolds, A. E., E. G. Wills, R. J. Roller, B. J. Ryckman, and J. D. Baines.** 2002. Ultrastructural localization of the HSV-1 U_L31, U_L34, and U_S3 proteins suggests specific roles in primary envelopment and egress of nucleocapsids. J. Virol. **76:**8939–8952.
28. **Roller, R., Y. Zhou, R. Schnetzer, J. Ferguson, and D. Desalvo.** 2000. Herpes simplex virus type 1 U_L34 gene product is required for viral envelopment. J. Virol. **74:**117–129.
29. **Ryckman, B. J., and R. J. Roller.** 2004. Herpes simplex virus type 1 primary envelopment: UL34 protein modification and the US3-UL34 catalytic relationship. J. Virol. **78:**399–412.
30. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex virus type 1. III. Role of glycoprotein VP7(B2) in virion infectivity. J. Virol. **29:**1149–1158.
31. **Shiba, C., T. Daikoku, F. Goshima, H. Takakuwa, Y. Yamauchi, O. Koiwai, and Y. Nishiyama.** 2000. The UL34 gene product of herpes simplex virus type 2 is a tail-anchored type II membrane protein that is significant for virus envelopment. J. Gen. Virol. **81:**2397–2405.
32. **Stackpole, C. W.** 1969. Herpes-type virus of the frog renal adenocarcinoma. I. Virus development in tumor transplants maintained at low temperature. J. Virol. **4:**75–93.
33. **Subramanian, R. P., and R. J. Geraghty.** 2007. Herpes simplex virus type 1 mediates fusion through a hemifusion intermediate by sequential activity of glycoproteins D, H, L, and B. Proc. Natl. Acad. Sci. USA **104:**2903–2908.
34. **Tanaka, M., H. Kagawa, Y. Yamanashi, T. Sata, and Y. Kawaguchi.** 2003. Construction of an excisable bacterial artificial chromosome containing a full-length infectious clone of herpes simplex virus type 1: viruses reconstituted from the clone exhibit wild-type properties in vitro and in vivo. J. Virol. **77:**1382–1391.
35. **Tischer, B. K., J. von Einem, B. Kaufer, and N. Osterrieder.** 2006. Two-step RED-mediated recombination for versatile high-efficiency markerless DNA manipulation in *Escherichia coli*. BioTechniques **40:**191–197.
36. **Yamauchi, Y., C. Shiba, F. Goshima, A. Nawa, T. Murata, and Y. Nishiyama.** 2001. Herpes simplex virus type 2 UL34 protein requires UL31 protein for its relocation to the internal nuclear membrane in transfected cells. J. Gen. Virol. **82:**1423–1428.
37. **Ye, G. J., and B. Roizman.** 2000. The essential protein encoded by the UL31 gene of herpes simplex virus 1 depends for its stability on the presence of the UL34 protein. Proc. Natl. Acad. Sci. USA **97:**11002–11007.