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U_L34, the Target of the Herpes Simplex Virus U_S3 Protein Kinase, Is a Membrane Protein Which in Its Unphosphorylated State Associates with Novel Phosphoproteins

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Previous studies (F. C. Purves, D. Spector, and B. Roizman, J. Virol. 65:5757–5764, 1991) have shown that the protein kinase encoded by the U_S3 gene mediates posttranslational modification of a viral phosphoprotein with an apparent M_r of 30,000 encoded by the U_L34 gene. Here we report the following. (i) U_L34 protein is not phosphorylated in cells infected with recombinant viruses deleted in the U_S3 gene. (ii) Several new phosphoproteins (apparent M_rs, 25,000 to 35,000) are present in cells infected with recombinant viruses deleted in the U_S3 gene or with viruses carrying a mutation in the U_L34 gene that precluded phosphorylation of the U_L34 gene product by the U_S3 protein kinase, but not in cells infected under conditions which permit phosphorylation of the U_L34 protein. These proteins are genetically unrelated to the product of the U_L34 gene. (iii) Polyclonal rabbit anti-U_L34 protein serum precipitated not only the U_L34 protein but also the other (25,000- to 35,000-M_r) phosphoproteins from lysates of cells infected with U_S3^- virus. (iv) The U_L34 gene product is a membrane protein inasmuch as the polyclonal anti-U_L34 serum reacted with surfaces of intact, unfixed, infected cells and the antigen-antibody complex formed in this reaction contained the U_L34 protein. (v) Small amounts of the U_L34 protein were present in virions of infected cells. We conclude that the U_L34 gene product is a membrane protein exclusively phosphorylated by the U_S3 protein kinase which can either directly or indirectly form complexes with several other phosphoproteins. Experiments done thus far suggest that these phosphoproteins are present only under conditions in which the U_L34 protein is not phosphorylated.

Earlier studies have shown that herpes simplex virus 1 (HSV-1) open reading frame U_S3 encodes a novel protein kinase (U_S3-PK) (4, 16, 21). The amino acid sequence that encodes this enzyme is conserved at least among the *Alphaherpesvirinae* subfamily of the family *Herpesviridae* (1, 2, 15). The key finding of an earlier report from this laboratory was that one of the targets of U_S3-PK was a phosphoprotein encoded by the U_L34 gene (22). Specifically, we demonstrated that a wild-type phosphoprotein with an apparent M_r of 30,000 was no longer labeled with ^{32}P in cells infected with R7041, a U_S3-PK^- recombinant virus. The 30,000-M_r phosphoprotein was mapped to open reading frame U_L34. The observation that the protein specified by U_L34 was predicted to contain an amino acid sequence which corresponded to the idealized substrate of U_S3-PK as previously defined by using synthetic peptides (10, 20) reinforced the hypothesis that it was the target of U_S3-PK. Definitive evidence that this was the case emerged from experiments involving site-specific mutagenesis designed to destroy this putative kinase target site of the U_L34 protein. Thus, two recombinant viruses containing an intact U_S3 gene but mutated in the U_L34 gene target site (R7310 and R7311) failed to specify a ^{32}P-labeled 30,000-M_r protein in infected cells (22). The key question was whether the U_L34 protein was also phosphorylated by another infected-cell kinase, since we also observed that whenever the ^{32}P-labeled band corresponding to the 30,000-M_r gene product of U_L34 was absent, either owing to the absence of U_S3-PK or because of specific mutations introduced within the U_L34 gene U_S3-PK target site, a new ^{32}P-labeled band with an apparent M_r of 33,000 appeared. The regularity with which the ^{32}P-labeled 30,000-M_r band was replaced by the ^{32}P-labeled 33,000-M_r band suggested that they were the products of the same gene, i.e., that phosphorylation of the U_L34 gene product by U_S3-PK yielded the ^{32}P-labeled 30,000-M_r band and that in the absence of this kinase, the product of the U_L34 gene was phosphorylated by another kinase to yield the ^{32}P-labeled 33,000-M_r band.

Subsequent studies, reported here, utilized a polyclonal antibody made against a fusion product of the U_L34 gene. We report that the U_L34 gene product is a membrane phosphoprotein exclusively phosphorylated by U_S3-PK and that the 33,000-M_r phosphoprotein is one of a group of at least four other phosphoproteins (M_rs, 25,000 to 35,000) present in infected cells under conditions in which the U_L34 gene product is not phosphorylated. These other phosphoproteins are genetically unrelated to the U_L34 gene product. Immunoprecipitation studies indicate that the U_L34 protein can complex either directly or indirectly with these other phosphoproteins under conditions in which the U_L34 protein is not phosphorylated. Whether the phosphorylated form of the U_L34 protein can complex either directly or indirectly with the various unphosphorylated members of this other group of proteins is not known.

MATERIALS AND METHODS

Cells and viruses. The isolation and properties of HSV-1(F) and HSV-2(G), the prototype HSV-1 and HSV-2 strains used in this laboratory, have been described elsewhere (3). The construction and characterization of HSV-1 recombi-

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nant viruses R7037, R7039, R7041, R7306, and R7314 have also been previously reported (11, 17, 22). R7037 is deleted in the U_S3 and U_S4 open reading frames; the deletion extends from the *Pst*I restriction enzyme site located 69 amino acids in from the 5′ end of the U_S3 gene to the *Nsi*I restriction enzyme site located 3 nucleotides downstream from the 3′ coterminus of the mRNAs that specify the proteins encoded by U_S3 and U_S4. R7039 is deleted in the U_S2 and U_S3 open reading frames; the deleted sequences include the promoter-regulatory domains, the first 47 amino acids of U_S2, and the first 357 amino acids of U_S3 and extend from the *Kpn*I restriction site at amino acid 47 of U_S2 to the *Bam*HI restriction site at amino acid 357 of U_S3. R7041 harbors an 860-bp deletion within the U_S3 open reading frame defined by the *Pst*I-*Bam*HI restriction endonuclease fragment, deleting predicted amino acids 69 to 357 of U_S3-PK. In R7306, the deleted US3 sequences of R7041 were restored by marker rescue with the U_S3 gene. In R7314, the native U_L34 gene was tagged at its amino terminus with a 17-amino-acid epitope from the HSV-1 α4 protein (6). The titers of all virus stocks were determined on Vero cells.

Preparation of polyclonal antiserum to the U_L34 fusion protein. A female New Zealand White rabbit (Charles River Laboratories, Rome, Italy) was injected subcutaneously with affinity-purified U_L34 fusion protein. Each injection consisted of 20 μg of protein. In the first injection, the protein was emulsified with complete Freund adjuvant; in the following injections, the protein was emulsified with incomplete Freund adjuvant. The rabbit was bled 1 month after the last of five injections given at 15-day intervals, and the serum was tested for reactivity with the U_L34 protein by immunoblotting as described below.

Preparation of radioactively labeled infected-cell lysates. Flasks (25 cm^2) of BHK-C13 cells were infected with 10 PFU of the indicated viruses per cell. The cells were infected and maintained in medium 199V consisting of mixture 199 supplemented with 1% calf serum. Cells were labeled with either $^{32}P_i$ or [^{35}S]methionine. At 13 h postinfection, the cells were preincubated for 1 h in either phosphate-free medium or methionine-free 199V. Cells were then labeled with either 100 to 200 μCi of $^{32}P_i$ (carrier free; The Radiochemical Center, Amersham, England) or 100 μCi of [^{35}S]methionine (1,300 Ci/mmol; The Radiochemical Center) in a final volume of 3 ml for 4 h. Cells were washed and scraped into 1 ml of ice-cold phosphate-buffered saline lacking Ca^{2+} and Mg^{2+} (PBS)-A, centrifuged for 5 min in a microcentrifuge at 4°C, and suspended in 350 μl of PBS-A containing 0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK), 0.1 mM tosyl-L-phenylalanine chloromethyl ketone (TLCK), 1.0% (vol/vol) Nonidet P-40, 40 mM β-glycerophosphate, and 1.0% (wt/vol) sodium deoxycholate (PBS-A*). Lysates were sonicated and frozen in aliquots at −70°C.

Polyacrylamide gel electrophoresis and immunoblotting. Infected-cell lysates containing approximately 50 μg of protein were electrophoretically separated in denaturing gels consisting of 0.1% sodium dodecyl sulfate (SDS) and 10% polyacrylamide and electrically transferred onto a nitrocellulose sheet. Reagents for probing of immunoblots were obtained from Promega Laboratories (Protoblot AP kits for mouse and rabbit antibodies). The nitrocellulose membrane was blocked in PBS containing 5% skim milk (Carnation). The membrane was washed in PBS containing bovine serum albumin (1 mg/ml) and incubated in the same buffer by using a 1:200 dilution of the primary U_L34 polyclonal antiserum. The membrane was washed with 5% skim milk in PBS and treated with a 1:4,000 dilution of either goat anti-rabbit or goat anti-mouse immunoglobulin G coupled to alkaline phosphatase in PBS containing skim milk for 1 h. The membrane was washed once with skim milk in PBS and several times with PBS and then developed by using the reagents and protocols supplied with the Protoblot AP kit.

Immunoprecipitation of the U_L34 gene product from infected-cell lysates. Approximately 50 μg of an infected-cell lysate radiolabeled with either $^{32}P_i$ or [^{35}S]methionine was incubated with a 1:200 dilution of polyclonal anti-U_L34 serum for at least 1 h at 4°C. The immune complexes were then precipitated by using 5 mg of protein A-conjugated Sepharose beads (Sigma). After extensive washing of the precipitate, the immune complexes were solubilized in SDS disruption buffer and boiled for 2 min and the beads were removed by centrifugation in a microcentrifuge for 2 min at room temperature. The immunoprecipitated proteins were electrophoretically separated in denaturing polyacrylamide gels.

Immunoprecipitation of the U_L34 gene product from the infected-cell surface. To immunoprecipitate U_L34 protein from the infected-cell surface, radiolabeled cells were washed with phosphate- or methionine-free medium and then blocked for 1 h in medium containing 1% horse serum. After removal of the blocking medium, a mixture of a 1:200 dilution of the primary anti-U_L34 polyclonal antiserum and anti-U_S11 monoclonal antibody was added to the monolayer and incubated for 2 h at room temperature. The U_S11 protein is not present on the surface of HSV-1(F)-infected cells; therefore, inclusion of the U_S11 monoclonal antibody served as a control for cell integrity. The cell monolayers were washed extensively with 199V and then PBS and then gently scraped into PBS and solubilized in PBS-A*. Immune complexes were precipitated from the infected-cell lysate by using protein A-conjugated Sepharose beads. The precipitated immune complexes were washed three times with PBS-A* and once with a buffer containing 10 mM Tris–100 mM NaCl, suspended in SDS sample buffer, boiled for 2 min, and centrifuged to remove the beads, and the immunoprecipitated proteins were electrophoretically separated in denaturing gels.

Black-plaque assay. Surface labeling of unfixed Vero Cell monolayer cultures exhibiting HSV-1 plaques with a 1:200 dilution of the polyclonal anti-U_L34 serum was done as previously described (9).

Preparation of HSV-1(F) virions. Six 150-cm^2 flasks of HEp-2 cells were infected with 10 PFU of HSV-1(F) per cell. At 8 h postinfection, one of the six flasks was overlaid with phosphate-free medium supplemented with 1 mCi of $^{32}P_i$. The infected cells were maintained for 2 days at 34°C. Virions were extracted from infected cells and purified by centrifugation in dextran T10 gradients as described by Spear and Roizman (24).

Flotation of HSV-1(F)-infected cell membranes in sucrose density gradients. Four 150-cm^2 flasks were infected with 10 PFU of HSV-1(F) per cell and maintained for 2 days at 34°C. The cells were harvested, disrupted by 20 strokes in a Dounce homogenizer, and subjected to low-speed centrifugation to remove the nuclei as previously described (24). The cytoplasmic extract was adjusted to 50% sucrose by weight and placed in a 40-ml ultracentrifuge tube. A continuous 10 to 45% (wt/wt) sucrose gradient in PBS lacking Ca^{2+} and Mg^{2+} was layered over the cytoplasmic extract, and the gradients were spun at 20,000 rpm in an SW28 rotor for 20 h. Fractions (0.2 ml) were collected from the top of the gradient, diluted at least 10-fold with PBS, and then pelleted at 30,000 rpm for 90 min in a Beckman Vti30 rotor. The pellet from each fraction was suspended in 200 μl of SDS disrup-

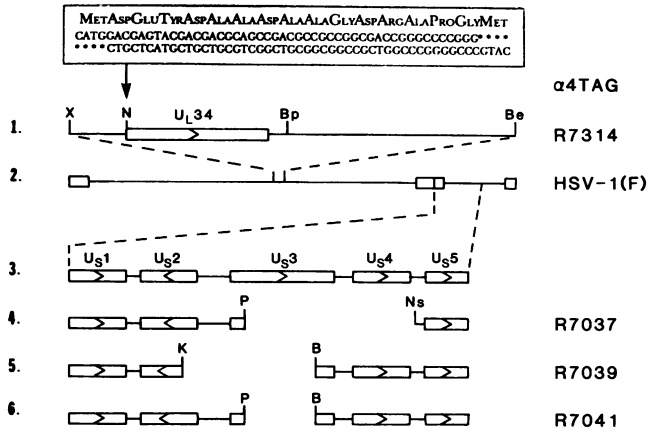

FIG. 1. Schematic representation of the DNA sequence arrangements in the genomes of HSV-1(F) and the various recombinant viruses. Line 1, DNA sequence arrangement of the U_L34 gene present in virus R7314, in which a 17-amino-acid epitope from the HSV-1 α4 protein was inserted at the amino terminus of the U_L34 gene. Line 2, sequence arrangement of the HSV-1 genome. The rectangles represent the inverted repeats flanking the unique sequences (represented by thin lines) of the long and short components. Line 3, arrangement of the first five open reading frames (U_S1 to U_S5) in the U_S sequence. The arrow indicates the direction of the transcript. Line 4, DNA arrangement of R7037, a recombinant virus deleted in both the U_S3 and the U_S4 open reading frames. The deletion extends from the *Pst*I site located at amino acid 69 of the U_S3 gene to the *Nsi*I site 3 nucleotides from the 3′ terminus of the U_S4 mRNA. Line 5, DNA sequence arrangement of recombinant virus R7039, which harbors a deletion in both the U_S2 and U_S3 open reading frames. The deleted sequences include the promoter-regulatory domains, the first 47 amino acids of U_S2, and the first 357 amino acids of U_S3 and extends from the *Kpn*I site at amino acid 47 of the U_S2 open reading frame to the *Bam*HI site at amino acid 357 of the U_S3 open reading frame. Line 6, DNA sequence arrangement of R7041, a recombinant virus with an 860-bp deletion in the U_S3 open reading frame. The deletion extends from the *Pst*I site at amino acid 69 to the *Bam*HI site at amino acid 357 of the U_S3 open reading frame. Abbreviations: B, *Bam*HI; K, *Kpn*I; Ns, *Nsi*I; P, *Pst*I; Be, *Bst*EII; Bp, BspEI; N, *Nco*I; X, *Xba*I.

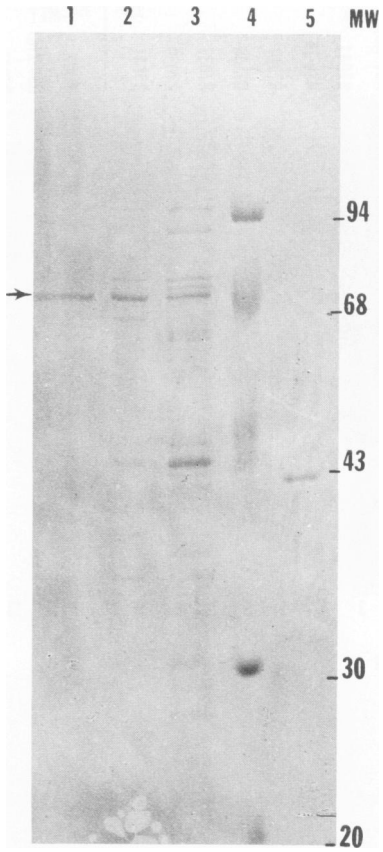

FIG. 2. Photograph of electrophoretically separated MBP-U_L34 fusion protein in denaturing gels stained with Coomassie brilliant blue R. Lanes: 1, 2 μg of affinity-purified MBP-U_L34; 2, insoluble material associated with *E. coli* membranes after initial lysis of the bacteria; 3, approximately 20 μg of *E. coli* lysate which was applied to the amylose affinity column; 4, molecular weight markers; 5, 2 μg of affinity-purified MBP (M_r, 42,000). The arrow indicates the MBP-U_L34 fusion protein, with an apparent M_r of 74,000, which was used as an antigen to generate the polyclonal rabbit antiserum. The molecular weight markers (MW) are in thousands.

tion buffer and subjected to electrophoresis in denaturing gels.

RESULTS

Experimental design. The objective of the studies described in this report was to characterize the U_L34 gene product and its relationship to a 33,000-M_r phosphoprotein observed only under conditions in which either U_S3-PK was missing or the target site of U_S3-PK on the U_L34 protein was destroyed by mutagenesis. To attain this objective, we produced a polyclonal antiserum to the U_L34 protein. The relevant sequence arrangements of the HSV-1 genome and of the recombinant viruses described in this report are shown in Fig. 1.

Production of the MBP–U_L34 protein fusion product. To make antibody reactive with the U_L34 gene product, we fused in frame the coding sequences of the *malE* gene which encodes the maltose-binding-protein (MBP) with those of the U_L34 gene. Specifically, the entire U_L34 gene and an additional 17 codons upstream of the natural ATG were inserted in frame as an *Nru*I-*Bsp*EI fragment from pRB4247 (described earlier [22]) into the *Stu*I site of plasmid pMALTM-c (New England BioLabs). The resulting plasmid, designated pRB4334, was used to transform *Escherichia coli* XL-1 blue (Stratagene). The fusion protein was predicted to have an M_r of approximately 74,000. After induction with isopropyl-β-D-thiogalactopyranoside, the cell lysate was passed through an amylose column as described in the manufacturer's instructions. A 74,000-M_r MBP-U_L34 fusion protein was eluted from the amylose resin with buffer containing 10 mM maltose and 50 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Maltose was removed from the eluate by dialysis, and the purified fusion protein was concentrated to 0.5 mg/ml by using Aquacide II (Boehringer Mannheim). To demonstrate the purity of the affinity-purified product, a sample of the concentrated protein was subjected to electrophoresis in denaturing gels. The 74,000-apparent-molecular-weight fusion protein is shown in Fig. 2, lane 1.

The U_L34 protein is phosphorylated exclusively by U_S3-PK. Lysates of cells infected as described in Materials and Methods and labeled with either $^{32}P_i$ or [^{35}S]methionine were electrophoretically separated in denaturing gels, electrically transferred to nitrocellulose, and reacted with the polyclonal anti-U_L34 serum prepared against the MBP-U_L34 fusion

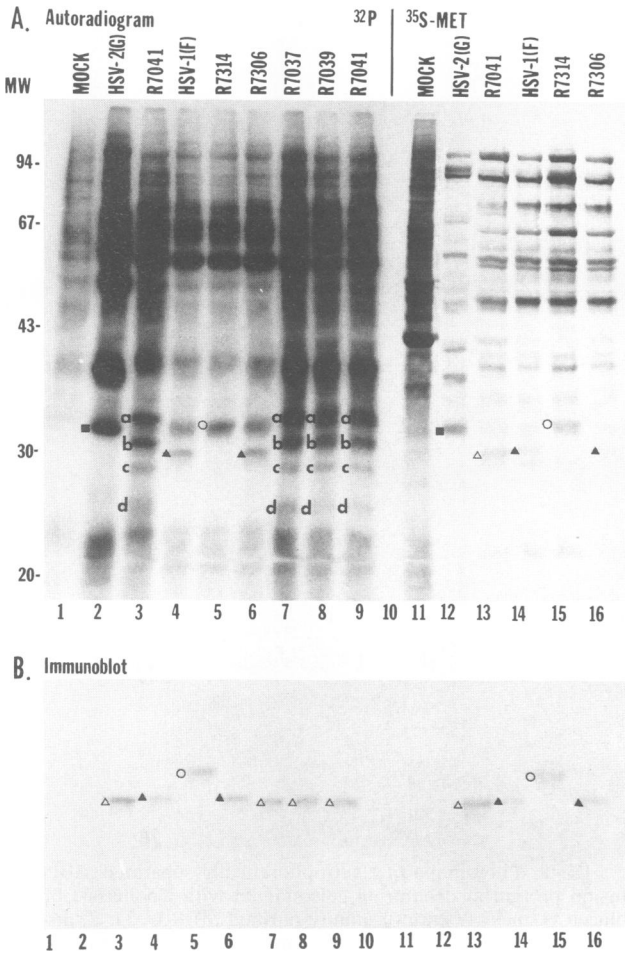

FIG. 3. Autoradiographic and photographic images of electrophoretically separated, ^{32}P- and [^{35}S]methionine (^{35}S-MET)-labeled lysates of cells infected with wild-type and recombinant HSV-1. (A) Autoradiogram of ^{32}P- and [^{35}S]methionine-labeled proteins separated in denaturing gels. (B) Photograph of corresponding electrophoretically separated proteins transferred to nitrocellulose and reacted with polyclonal anti-U_L34 serum. Symbols: ▲, wild-type HSV-1 U_L34 protein; ■, HSV-2 U_L34 protein; ○, more slowly migrating HSV-1 U_L34 protein containing the additional 17 amino acids of the α4 protein in lysates of cells infected with recombinant virus R7314; △, U_S3-PK$^-$ form of the U_L34 protein present as an unphosphorylated species in R7037-, R7039-, and R7041-infected cell lysates. The letters A, B, C, and D indicate four new phosphoprotein species which are present in cells infected with viruses whose U_L34 protein is not phosphorylated. The phosphoprotein identified with the letter B is the 33,000-M_r phosphoprotein first noted in the previous studies (22). The molecular weights (MW) of marker proteins are in thousands.

protein as described in Materials and Methods. The salient features of the results shown in Fig. 3 are as follows. The U_L34 phosphoprotein in the lysates of cells infected with either HSV-1(F) (lanes 4 and 14) or R7306, a recombinant virus in which the deleted U_S3 sequences of R7041 have been restored (lanes 6 and 16), reacted strongly with the anti-U_L34 serum. As expected, addition of the 17-amino-acid α4 epitope to the U_L34 protein in R7314 resulted in a lower-mobility species of the U_L34 phosphoprotein which also reacted strongly with the anti-U_L34 serum (lanes 5 and 15). In the lysates of cells infected with the U_S3^- R7041 virus (lanes 3 and 13), the anti-U_L34 serum reacted with a band which migrated slightly faster than the U_L34 protein made in HSV-1(F)-infected cells. This band (Fig. 3, △) was not labeled with ^{32}P and did not correspond in electrophoretic mobility to any ^{32}P-labeled band in cells infected with HSV-1(F) or R7306. Moreover, the polyclonal anti-U_L34 serum did not react with the ^{32}P-labeled 33,000-M_r phosphoprotein band (B in Fig. 3A, lanes 3, 7, 8, and 9) present in electrophoretically separated lysates of U_S3^- virus-infected cells. We conclude that the product of the U_L34 gene is the substrate of U_S3-PK and that in cells infected with the U_S3^- virus, the U_L34 protein is not phosphorylated.

The 33,000-M_r phosphoprotein is one of a group of at least four phosphoproteins present in cells infected with U_S3-PK$^-$ virus which are not genetically related to either the U_L34 or the U_S3 protein. The results presented in Fig. 3 indicate that the 33,000-M_r phosphoprotein (protein B) observed previously was one of at least four phosphoproteins with M_rs of 25,000 to 35,000 (proteins A, B, C, and D) labeled with $^{32}P_i$ in cells infected with U_S3-PK$^-$ virus. Whereas the 33,000-M_r phosphoprotein (protein B) was invariably present, phosphoproteins C and D occasionally labeled less intensely with ^{32}P and were sometimes difficult to visualize. As none of these phosphoproteins reacted with polyclonal antiserum to the U_L34 gene product, we conclude that the amino acid sequences of these phosphoproteins differ from that of the U_L34 gene product.

To ensure that these phosphoproteins were not the result of an unexpected fusion protein created by serendipitous fusion of open reading frames during deletion of the U_S3 gene in R7041, we compared the phosphoprotein profiles of two other U_S3^- recombinant viruses that exhibit different deletions within the HSV-1 genome. The deletion in the genome of R7037 spans both the U_S3 and U_S4 open reading frames, whereas the deletion in R7039 spans both the U_S2 and U_S3 open reading frames. As can be seen in Fig. 3A, lanes 7 and 8, the phosphoprotein profiles of both of these U_S3 deletion mutants are identical to that of R7041, whose phosphoprotein profile is shown in Fig. 3A, lanes 3 and 9. Thus, we conclude that the presence of phosphoproteins A, B, C, and D in infected-cell lysates correlates with the absence of U_S3-PK. The hypothesis that these phosphoproteins are labeled with $^{32}P_i$ only in the absence of phosphorylation of the U_L34 protein by U_S3-PK is supported by the observation, reported earlier, that the 33,000-M_r (B) phosphoprotein was also present in cells infected with recombinant viruses in which U_S3-PK was intact, but the target site of the kinase on the U_L34 gene product was destroyed by site-specific mutagenesis (22). We should stress that in this instance only the B phosphoprotein was prominent and was invariably detected.

The U_L34 gene product is a virion component. To determine whether the U_L34 gene product is a virion component, ^{32}P-labeled virions from cells infected with HSV-1(F) were prepared and purified by the method of Spear and Roizman (24). Virion proteins were separated by electrophoresis in denaturing gels, transferred to nitrocellulose, and reacted with anti-U_L34 serum. The results are shown in Fig. 4. Lane 1 displays the electrophoretically separated polypeptides stained with Coomassie blue, lane 2 shows the autoradiographic image of a profile of phosphoproteins identical to that in lane 1, and lane 3 shows a photograph of lane 2 electrically transferred to a nitrocellulose sheet and stained with the polyclonal anti-U_L34 serum. The results show that the U_L34 gene product is present in purified virions, but in amounts too small to be detected by Coomassie blue.

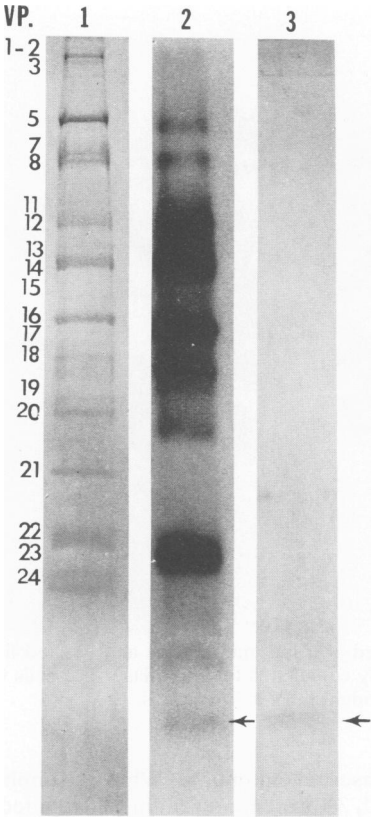

FIG. 4. Photographic and autoradiographic images of ^{32}P-labeled HSV-1(F) virion polypeptides (VP.) (5) electrophoretically separated in denaturing gels and either stained with Coomassie blue (lane 1) or transferred to nitrocellulose (lane 2) and subjected to autoradiography. The two dots in lane 3 located between VP21 and VP22 do not pertain to this lane. The electrophoretically separated proteins transferred to nitrocellulose were reacted with the anti-U_L34 serum (lane 3). The arrows indicate the small quantity of U_L34 protein detected as a phosphoprotein in the virion.

The U_L34 gene product is associated with cellular membranes and is present on the infected-cell surface. Analysis of the predicted amino acid sequence of the U_L34 gene product, reported elsewhere (22), indicated that the amino acid sequence of the N-terminal domain of the U_L34 polypeptide has properties similar to those of signal sequences. In addition, hydrophobicity analysis revealed that the U_L34 protein is predicted to have a hydrophobic C terminus, highly conserved among herpesviruses, which could conceivably anchor the protein in membranes. Consequently, several experiments were done to localize the U_L34 gene product within infected cells.

In the first experiment, cell membranes of HSV-1(F)-infected cells were separated from dense cytoplasmic constituents by flotation through a continuous sucrose gradient. Individual fractions of the gradient were subjected to electrophoresis on denaturing gels, transferred to nitrocellulose, and then reacted first with anti-U_L34 serum and subsequently with an anti-glycoprotein D monoclonal antibody (H170-1). Glycoprotein D, a known membrane constituent, served as a control. As shown in Fig. 5, the peak concentrations of U_L34 protein and glycoprotein D coincided in fraction 18 of the flotation gradient.

In the second experiment, monolayers of confluent Vero cells were infected with HSV-1(F) at low multiplicity to obtain individual plaques. At 48 h postinfection, the cell monolayer was overlaid with the anti-U_L34 serum and then reacted with goat anti-rabbit serum conjugated with peroxidase by the black-plaque procedure, described elsewhere (9). Since the cells are not fixed, the antibody reacts only with antigens reactive on the cell surface. As shown in Fig. 6, the anti-U_L34 serum reacted with the unfixed infected cells but not with the uninfected cells. It is noteworthy that cells infected with the R7041 virus also stained darkly in the black-plaque assay, indicating that both the phosphorylated and the unphosphorylated forms of the U_L34 gene product were present on the infected-cell surface (data not shown). The results of the black-plaque assays were consistent with those of immunofluorescence studies performed on HEp-2 cells or BHK-C13 cells infected with either HSV-1(F) or a

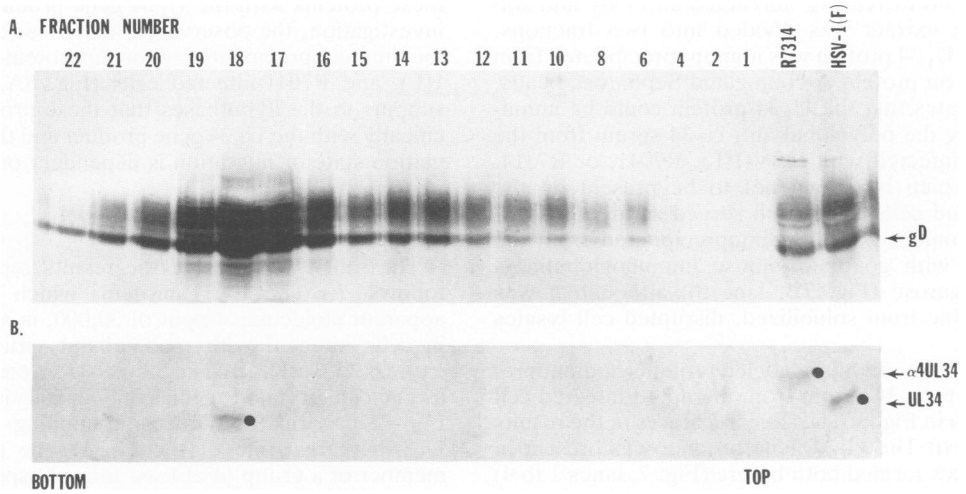

FIG. 5. Immunoblot of electrophoretically separated proteins contained in membranes of HSV-1(F)-infected cells. The infected-cell membranes were separated from dense cytoplasmic constituents by flotation through a continuous sucrose gradient. Individual fractions were subjected to electrophoresis in denaturing gels, transferred to nitrocellulose, and then reacted first with anti-U_L34 serum as shown in panel B and then with anti-gD (H170-1) monoclonal antibody as shown in panel A. Lysates of cells infected with HSV-1(F) and R7314 were included on the gel as markers for the position of the U_L34 protein. The U_L34 protein is identified by the closed circles in panel B.

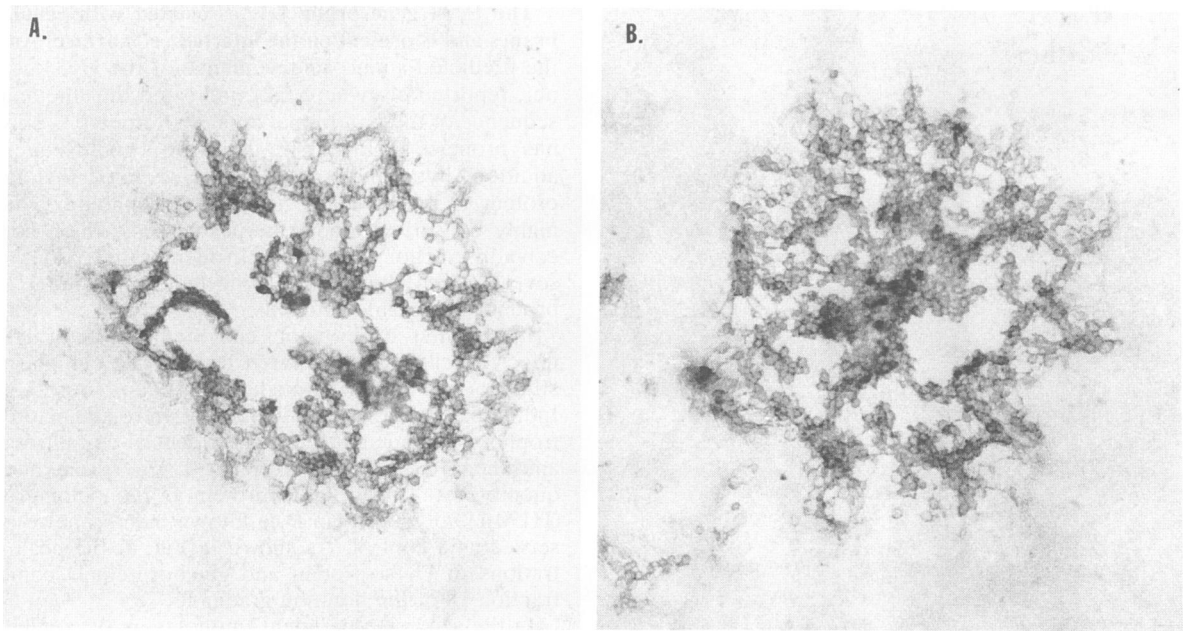

FIG. 6. Photograph of two HSV-1(F) plaques on monolayers of Vero cells infected at a low multiplicity and reacted first with rabbit polyclonal anti-U_L34 serum and then with a goat anti-rabbit immunoglobulin G antibody conjugated to peroxidase. The cells were not fixed. The procedure has been described previously (9). Panels A and B represent two individual HSV-1(F) plaques.

recombinant virus, R7202, unable to express glycoprotein gE (1a). Specifically, we observed strong surface immunofluorescence in unfixed cells. In fixed cells, there was also weak perinuclear and cytoplasmic immunofluorescence but no antigen was detected in nuclei (data not shown).

In the last series of experiments, BHK-C13 cells were infected with either HSV-1(F); R7314, in which the UL34 gene had been tagged with an α4 epitope; or R7041 and overlaid with medium containing either $^{32}P_i$ or [^{35}S]methionine. The infected-cell monolayers were then incubated simultaneously with both rabbit anti-U_L34 serum and a mouse monoclonal anti-US11 antibody (CL28), described elsewhere (23). After incubation for 2 h, the cells were extensively washed to remove unreacted antibody and solubilized and the extract was divided into two fractions. Antibody-bound U_L34 protein was immunoprecipitated from the first fraction on protein A-conjugated Sepharose beads. Figure 7A illustrates that the U_L34 protein could be immunoprecipitated by the polyclonal anti-U_L34 serum from the surface of cells infected with HSV-1(F), R7041, or R7314. U_S11 protein, which is known not to be present on the surface of infected cells and which served as a control for cell leakiness, could not be immunoprecipitated from the second fraction with goat anti-mouse immunoglobulin G conjugated to agarose (Fig. 7B, lane 3), although it was readily precipitable from solubilized, disrupted cell lysates (Fig. 7B, lane 1).

The ^{32}P- and [^{35}S]methionine-labeled proteins immunoprecipitated with anti-U_L34 serum from disrupted infected cell lysates are shown in Fig. 8. The salient features of the results were as follows. (i) The U_L34 gene product was present in immune complexes formed both before (Fig. 7, lanes 1 to 4) and after (Fig. 8A, lanes 1 to 4, and 8B, lanes 2, 3, and 5) disruption of the infected cells. The U_L34 gene product can be readily identified on the basis of its electrophoretic mobility inasmuch as the product of the U_L34 gene in R7314 migrated more slowly because of the additional 17 amino acids in the inserted epitope. (ii) While the unphosphorylated form of the U_L34 protein present in R7041-infected cells was not seen in the autoradiograms of the ^{32}P-labeled profiles (Fig. 8B, lane 1, and 8C, lane 4), the protein was readily visualized in autoradiograms of electrophoretically separated proteins labeled with [^{35}S]methionine (Fig. 8A, lane 3). (iii) An unexpected significant finding was that the antibody to the U_L34 protein also precipitated the A, B, and C phosphoprotein species present in cells infected with U_S3^- virus. Although these proteins were not detected in precipitates of cells infected with HSV-1(F), it is not clear whether they were not present or remained undetected because of lack of phosphorylation with ^{32}P. While the association of these proteins with the U_L34 gene product requires further investigation, the observation that clear differences exist in the immunoprecipitated phosphoprotein profiles of HSV-1(F)- and R7041-infected cells (Fig. 8A, B, and C) lends support to the hypotheses that these proteins interact specifically with the U_L34 gene product and that their phosphorylation state or migration is dependent on U_S3-PK.

DISCUSSION

The salient features of the results reported here are as follows. (i) The U_L34 protein, which migrates with an apparent molecular weight of 30,000, is not phosphorylated in cells infected with recombinant viruses which do not express U_S3-PK. (ii) The 33,000-M_r protein, which appears as a phosphorylated species only in cells infected with either U_S3-PK$^-$ virus or viruses containing mutations in the U_S3-PK target site of the U_L34 gene product (22), is a member of a group of at least four phosphoproteins present in infected cells under conditions in which U_L34 is not phosphorylated. These phosphoproteins are not genetically related to the U_L34 protein inasmuch as they did not react with the polyclonal anti-U_L34 rabbit serum. (iii) This group of phosphoproteins may be associated with the U_L34 gene

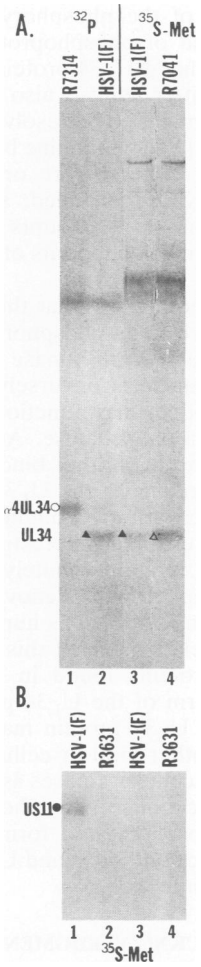

FIG. 7. Autoradiographic images of electrophoretically separated phosphoproteins precipitated from infected cells with rabbit polyclonal anti-U_L34 serum. Intact monolayers of cells were incubated simultaneously with anti-U_L34 serum and a monoclonal antibody to U_S11 (CL28). The cells were then rinsed to remove the excess unreacted antibody and solubilized with Nonidet P-40. The U_L34 protein was precipitated with protein A, whereas the U_S11 protein was immunoprecipitated with an anti-mouse antibody conjugated to agarose. (A) Immunoprecipitates of ^{32}P-labeled cells infected with HSV-1(F) and R7314. In lanes 3 and 4 the cells were infected with HSV-1(F) and R7041, respectively, and labeled with [^{35}S]methionine (^{35}S-MET). Symbols: ▲, HSV-1(F) form of the U_L34 protein; △, unphosphorylated U_L34 protein in U_S3-PK$^-$-infected cells; ○, more slowly migrating U_L34 protein tagged with the 17-amino-acid α4 epitope in R7314-infected cells. (B) For lanes 1 and 2, cells infected with HSV-1(F) and R3631 (a recombinant virus from which U_S11 had been deleted [13, 17]) were treated with Nonidet P-40 before addition of rabbit polyclonal anti-U_L34 serum and the monoclonal antibody to U_S11. In this instance, the U_S11 protein present in infected cells was precipitated by the monoclonal antibody. Lanes 3 and 4 were the same as lanes 1 and 2, except that intact cells were exposed to the anti-U_S11 monoclonal antibody and the cells were rinsed to remove the excess anti-U_S11 antibody prior to solubilization with Nonidet P-40 before precipitation of the antibody-antigen complexes. As U_S11 does not appear on the surface of infected cells, it is not detected in precipitates. The precipitation shown in panel B, lane 3, was done with the same starting material as that shown in panel A, lane 3.

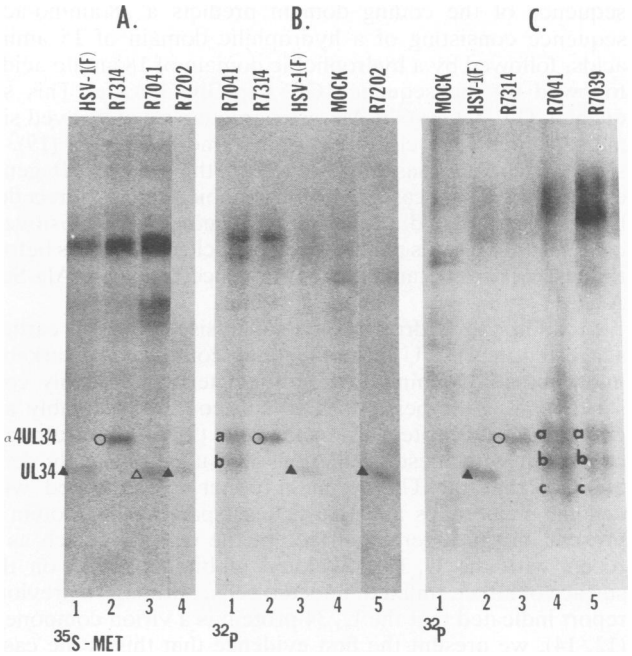

FIG. 8. Autoradiographic images of [^{35}S]methionine (^{35}S-MET) (A)- and ^{32}P (B and C)-labeled proteins immunoprecipitated with polyclonal anti-U_L34 serum and electrophoretically separated in denaturing gels. Symbols: ○, U_L34 protein tagged with a 17-amino-acid epitope from the α4 protein; △, unphosphorylated form of the U_L34 protein; ▲, wild-type phosphorylated form of the U_L34 protein. The letters A to C identify the novel phosphoproteins detected by ^{32}P labeling in infected cells in which the U_L34 protein is not phosphorylated. Infection with recombinant virus R7202 (panels A, lane 4, and B, lane 5), which contains an insertion in the U_S8 open reading frame that encodes glycoprotein gE, demonstrates that the high-molecular-weight proteins immunoprecipitated from cells infected with the other viruses shown here are probably due to the Fc receptor activity of this glycoprotein when it is complexed with glycoprotein gI (7) and are not specific to the U_L34 antiserum. Panels B and C differ in that deoxycholate was omitted from the lysates and immunoprecipitation mixtures shown in panel C, allowing increased detection of the highest-mobility phosphoprotein, identified by the letter C, immunoprecipitated from infected cells in which the U_L34 protein is not phosphorylated.

product inasmuch as they can be immunoprecipitated together with unphosphorylated U_L34 protein by polyclonal anti-U_L34 serum from lysates of cells infected with U_S3-PK$^-$ virus. (iv) Several lines of evidence indicate that the U_L34 gene product is a membrane protein. (v) The U_L34 protein could be detected in small amounts in preparations containing purified virions. The results presented in this study address two significant issues; they are (i) the localization and function of the U_L34 gene product during the replicative life cycle of the virus and (ii) the relationship between the U_S3-PK-dependent phosphorylation state of the U_L34 protein and the appearance of the novel phosphoproteins (A, B, C, and D in Fig. 3) apparent only in cells in which the U_L34 protein is not phosphorylated.

Localization and function of the U_L34 gene product during the replicative cycle of the virus. The hypothesis that the U_L34 protein is targeted to membranes was based on the observation that the predicted amino acid sequence of the protein encodes an N-terminal domain characteristic of signal sequences (14, 19). Specifically, the nucleic acid

sequence of the coding domain predicts a 38-amino-acid sequence consisting of a hydrophilic domain of 15 amino acids, followed by a hydrophobic domain of 18 amino acids, followed by the sequence Gly-Asp-Gly-Glu-Ala. This sequence, Gly-X-Gly-Glu-Ala, is characteristic of cleaved signal sequences; the cleaved peptide bond is Gly-Glu (19). A similar sequence has been found in the HSV-1 gB gene, except that in this case, the hydrophobic region is preceded by a 10-amino-acid sequence containing three positively charged amino acids and the predicted cleavage site is before the last Ala in the amino acid sequence sequence Ala-Ser-Ala-Ala (18).

In addition, hydrophobicity analysis, reported earlier, suggested that the U_L34 polypeptide contains a remarkably hydrophobic 35-amino-acid carboxyl terminus, highly conserved among herpesviruses, which could conceivably anchor the U_L34 protein in membranes (22). Our results are consistent with these predictions and unambiguously demonstrate that the U_L34 gene product is associated with cellular membranes and that at least part of the protein is present on the exterior surface of the cell, inasmuch as it reacts with the U_L34 polyclonal rabbit antiserum on the surface of intact, unfixed, infected cells. Although a previous report indicated that the U_L34 protein is a virion component (12, 14), we present the first evidence that this is the case. We should note, however, that the amount of U_L34 protein contained in the virion appears to be relatively small. As we have demonstrated that the U_L34 protein associates with plasma membranes, we suggest that in virions it is located in the virion envelope. Inasmuch as the amino acid sequence of the U_L34 protein does not predict sites for N-linked glycosylation, the protein fails to bind wheat germ agglutinin resins (data not shown), and the protein forms a sharp band in denaturing polyacrylamide gels, we have no evidence indicating that the U_L34 protein is glycosylated.

The function of the U_L34 gene product in the viral replication cycle is unknown. As noted previously, while the U_S3-PK gene and the consequent phosphorylation of the U_L34 gene product are dispensable for growth of the virus in tissue culture, the U_L34 gene product itself may not be dispensable, inasmuch as numerous attempts to delete the U_L34 gene from the viral genome have been unsuccessful. Construction of U_L34 protein-producing cell lines, which can be used to delete the U_L34 gene from the virus, is in progress and may eventually help elucidate the functions of this protein.

Relationship between the U_S3-PK-dependent phosphorylation state of the U_L34 protein and the novel 25,000- to 35,000-M_r phosphoproteins present in infected cells lacking phosphorylated U_L34 protein. A key and very interesting finding is that the four 25,000- to 35,000-M_r novel phosphoproteins appear only in lysates of cell infected with U_S3-PK$^-$ virus or with mutants containing a functional U_S3 gene but with mutations in the U_L34 gene such that its gene product cannot be phosphorylated. Inasmuch as these proteins failed to react with the anti-U_L34 polyclonal rabbit serum in immunoblots, they are not genetically related to the U_L34 gene product. The hypothesis that these phosphoproteins are functionally related to the U_L34 gene product is supported by the observation that they can be immunoprecipitated by the anti-U_L34 serum together with unphosphorylated U_L34 protein in cells infected with U_S3-PK$^-$ virus. The significant observation is that the phosphorylated form of these proteins is absent from lysates of cells infected with virus in which the phosphorylated form of the U_L34 protein is present. It seems, therefore, that the presence of the phosphorylated tetrad of proteins is dependent on the absence of functional U_S3-PK and that the presence of detectable amounts of the phosphorylated form of the B member of the tetrad of phosphoproteins and that of the wild-type phosphorylated U_L34 protein are mutually exclusive. Whether this latter point is also true for phosphoproteins A, C, and D remains to be resolved. Attempts to label proteins A to D with [^{35}S]methionine by adding [^{35}S]methionine to the medium either before or after infection with wild-type or U_S3-PK$^-$ virus failed, suggesting that these proteins are present in small amounts during viral infection or that they lack significant amounts of methionine (data not shown).

One attractive hypothesis is that the U_L34 gene product acts a regulator which in its phosphorylated form either (i) inhibits another infected-cell kinase or (ii) activates an infected-cell phosphatase. Conversely, the unphosphorylated U_L34 gene product may function to activate another kinase or inactivate a phosphatase. Any of these functions could be mediated through either binding or release of the target enzyme, depending on the U_L34 protein phosphorylation state, or by constitutive binding of the U_L34 gene product to the target enzyme. Interestingly, a protein kinase activity with an M_r of approximately 33,000 has recently been found in a complex with adenovirus E1A protein (8). The enzyme has been identified as human cyclin-dependent kinase 2 (25). The relationship of this kinase to any of the tetrad of phosphoproteins found in association with the unphosphorylated form of the U_L34 protein is not known. The function of the U_L34 protein may be to regulate the phosphorylation of other viral or cellular proteins by regulating the function of cellular kinases associated with cellular membranes. Identification of the phosphoproteins associated with the unphosphorylated form of U_L34 may help resolve the function of both U_S3 and U_L34 gene products in the biology of HSV infection.

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