Herpes Simplex Virus Type 1 UL46 and UL47 Deletion Mutants Lack VP11 and VP12 or VP13 and VP14, Respectively, and Exhibit Altered Viral Thymidine Kinase Expression

YINGZE ZHANG AND JENNIFER L. C. MCKNIGHT*

*Department of Infectious Diseases and Microbiology, University of Pittsburgh,
Graduate School of Public Health, Pittsburgh, Pennsylvania 15261*

Received 28 September 1992/Accepted 11 December 1992

The gene products of herpes simplex virus type 1 UL46 and UL47 enhance the efficiency of α TIF (VP16)-mediated α gene expression through an unknown mechanism of action. To further characterize the function of the UL46- and UL47-encoded proteins during virus infection, a series of isogenic herpes simplex virus type 1 strain F-derived UL46 and UL47 single-deletion mutants and a UL46/47 double-deletion mutant were constructed and compared with the wild type. Analysis of purified virions obtained from the UL46 deletion mutant showed for the first time that UL46 encoded the virion tegument phosphoproteins VP11 and VP12 (VP11/12). Similar analyses of the UL47 deletion mutants confirmed an earlier report by McLean et al. that UL47 also encoded two virion tegument phosphoproteins, VP13 and VP14 (VP13/14) (G. McLean, F. Rixon, N. Langeland, L. Haarr, and H. Marsden, *J. Gen. Virol.* 71:2953–2960, 1990). Kinetic analysis demonstrated a delay of approximately 2 h in the appearance of thymidine kinase (TK) activity in all of the UL46 and UL47 single-deletion mutants. In the UL46/47 double-deletion mutant, the delay in TK activity increased twofold, suggesting that the proteins encoded by UL46 and UL47 may act at the same level. Since the delay in TK expression occurred within the first 4 h of infection, the actions of VP11/12 and VP13/14 resulted from their virion association and not from their *de novo* synthesis as late ($\beta\gamma$ and γ) genes. Densitometric analysis of purified virions showed that the levels of VP11/12 and VP13/14 in the virion tegument were near the molar ratios of α TIF. On the basis of these observations, we predict that the abilities of UL46 and UL47 to enhance α TIF-mediated transcription could result from a stoichiometric association of VP11/12 and VP13/14 with α TIF within the infecting virion.

The herpes simplex virus type 1 (HSV-1) genome encodes more than 70 open reading frames, most of which are expressed during lytic infection (19, 26; reviewed in reference 37). Approximately half of the genes encode virion proteins which constitute the capsid, tegument, and envelope of a mature infectious virion (18, 19, 26, 37, 42). The remaining genes encode nonvirion proteins involved in viral gene expression, genome replication, and virion assembly (19–21, 26, 37). There is an increasing body of evidence which demonstrates that the tegument contains both structural and regulatory proteins, the latter of which act immediately postinfection to increase the efficiency of the lytic cycle (3, 18, 36, 41, 48–50). At least one tegument protein, α TIF (VP16), has both a structural and a regulatory role (3, 5, 18, 32, 45). In addition to being a powerful transcriptional inducer of the α or immediate-early genes of HSV-1, α TIF is an essential structural component of the virion (3, 5, 18, 32, 45). α TIF-mediated induction occurs immediately postinfection, it does not require the concomitant synthesis of either cellular or viral proteins, and it results from the presence of 1,000 to 2,000 molecules of the protein in each infecting virion (3, 18). Thus, although α TIF is synthesized as a $\beta\gamma$ or late gene (17), its virion location enables the protein to effect the induction of α genes during each round of infection prior to its own *de novo* synthesis.

Other HSV-1 regulatory proteins, including ICP0, ICP4, VP13 and VP14 (VP13/14), and the virion host shutoff protein, VHS, can be found in the tegument (29, 36, 39, 41,

48–50). The potential structural roles of these proteins, if any, are unknown and cannot be predicted on the basis of copy number alone. For example, only a small number (100 to 150) of molecules of ICP0 and ICP4 are present in the tegument of each virion; however, previous characterization of mutants with deletions in the genes encoding each of these proteins has shown that ICP4 is absolutely essential for viral growth, whereas mutants with deletions in ICP0 are severely attenuated (4, 7, 11, 14, 38, 44, 48–50). Thus, on the basis of numbers alone, it is more likely that the requirements for ICP0 and ICP4 are regulatory and not structural. In contrast, VHS and VP13/14, like α TIF, are present in numbers high enough to be stoichiometrically significant (~ 400 and $\sim 1,700$ molecules, respectively), yet unlike mutants with deletions in α TIF, mutants with deletions in VHS and VP13/14 do not exhibit significant attenuation of their lytic growth cycle, demonstrating that neither VHS nor VP13/14 is essential for virion structure (1, 2, 18, 31, 36, 45, 51). Clearly, more information on tegument assembly and function is needed in order to determine the precise structural and regulatory contributions of the tegument proteins vis-à-vis the viral life cycle and host range.

The UL46 and UL47 open reading frames of HSV-1 are members of a gene cluster that encodes known or putative tegument proteins, including UL48 and UL49, which encode α TIF and VP22, respectively (13, 29, 30, 47). It is interesting that in addition to their genomic association, at least three members of this gene cluster interact functionally. The proteins expressed by UL46 and UL47 modulate α TIF-mediated transcriptional induction in transient expression assays and in α TK transfectant cell lines infected with TK[−]

* Corresponding author.

UL46 or UL47 or a UL46/47 double-deletion mutant (27, 28, 51). Also, the mRNAs of the nonoverlapping UL46 and UL47 open reading frames are 3' coterminal (17). Whether the common 3' terminus contributes to the temporal offset in the expression of the two genes (UL46 is a $\beta\gamma$ gene, whereas UL47, residing 5' of UL46, is expressed slightly later as a γ gene) is unknown (17, 29).

Until this report, there was virtually no information regarding the protein product(s) of UL46 and only limited information regarding the physical properties and functions of the proteins encoded by UL47. UL47 encodes two related proteins identified as virion proteins VP13 and VP14 (29, 42). VP13 and VP14 have observed molecular masses of 79 and 81 kDa, respectively, and both proteins are found in infected-cell extracts as well as in purified virions (29, 42). Recent reports suggest that the observed difference in molecular mass between VP13 and VP14 could result from differential phosphorylation and/or glycosylation (29, 30, 47). Alternatively, the difference could result from a second in-frame AUG that resides 99 amino acids downstream of the primary translational initiation site (28, 29). Although the DNA sequence of UL47 does not predict the presence of consensus-specific DNA binding domains in the proteins, VP13 was previously reported to be associated with the nuclear matrix in HSV-1-infected cells (34). The latter observation could reflect a nonspecific DNA binding activity conferred by a hydrophilic arginine-rich domain near the N terminus (28). Similarly, the DNA sequence of UL46 is of little predictive value, and aside from a number of proline repeats present within the C-terminal domain, it is unremarkable (28). Both genes have homologs in other herpesviruses. For example, UL47 shares homology with both gp10 of equine herpesviruses 1 and 4 and VP8 of bovine herpesvirus (6, 47). There is less information on UL46, but this gene, UL47, and UL48 are conserved in a similar varicella-zoster virus gene cluster corresponding to open reading frames 12, 11, and 10, respectively (10, 28). UL46, like UL47, is a nonessential gene (2, 51).

The goals of the current study were (i) to further characterize the regulatory functions of the UL46 and UL47 gene products within the context of virus infection and (ii) to determine whether UL46, like UL47, encodes a tegument protein. Toward this end, a series of isogenic UL46 and UL47 single-deletion mutants and a UL46/47 double-deletion mutant were constructed. The deletion mutants were used to demonstrate that (i) UL46 encodes two tegument phosphoproteins, VP11 and VP12 (VP11/12), (ii) UL46, UL47, and UL46/47 deletion mutants exhibit altered kinetics of viral thymidine kinase (TK) induction, (iii) the effect on β gene expression is directly related to the absence of each or both proteins in the tegument of the infecting virion, and (iv) both VP11/12 and VP13/14 are present in sufficiently high numbers to predict a stoichiometric association with α TIF in the tegument.

MATERIALS AND METHODS

Mutant viruses. All viruses are derived from HSV-1 (F), whose properties and propagation are described elsewhere (12). The construction strategy is outlined in Fig. 1. The UL46 Δ 3, UL47 Δ 2, and UL46/47 Δ 2 deletion mutants were described previously (51). UL47 Δ 4 and UL47 Δ 10 contain ~1.0-kb and ~300-bp deletions, respectively, of the UL47 open reading frame. The deletions were constructed by *Bal* 31 exonuclease digestion of a plasmid containing the UL47 gene linearized at a unique *Msr*II site 599 bp downstream of

FIG. 1. Schematic representation of construction strategy for and locations of the UL46, UL47, and UL46/47 deletions used in this study. (A) The location of the TK deletion (Δ TK) and the locations of UL46, UL47, and UL48 (α TIF) within the *Bam*HI F and I' regions of the viral genome in R Δ 305 are depicted (35, 51). (B) Expansion of the *Bam*HI F and I' regions of the viral genome showing the sites of the α TK insertions in R3787 and R3622, two previously described virus strains used for TK deletion mutagenesis (51). The arrows indicate the transcriptional orientation and location of the mRNAs in the HSV-1 genome, and the rectangles represent the open reading frames of UL46, UL47, and UL48. (C) Locations of the UL46, UL47, and UL46/47 deletions in RUL46 Δ 3, RUL47 Δ 10, RUL47 Δ 4, RUL47 Δ 2, and RUL46/47 Δ 2, respectively, are shown. (D to F) Repair strategies for TK (D and E) and for UL46, UL47, and UL46/47 (E and F) are shown. Corresponding isogenic viral strain nomenclature is also shown. Relevant restriction sites are indicated. A, *Asu*II; B, *Bam*HI; N, *Not*I; Nc, *Nco*I; S, *Sca*I.

the initiating ATG (28). All deletion mutants were constructed by using the TK insertion-deletion technique described previously (35, 51) (Fig. 1A to D). The endogenous TK locus was subsequently restored by marker rescue with the intact HSV-1 TK gene (Fig. 1D and E). The marker-rescued viruses were plaque purified in the presence of hypoxanthine, aminopterin, and thymidine, and the physical repair of the TK locus was confirmed by Southern blot analysis (51). The functional repair of the TK locus was confirmed by measuring TK activity (23). The TK⁺ UL46 and UL47 single- and UL46/47 double-deletion mutants were designated R(F)UL46 Δ 3, R(F)UL47 Δ 2, R(F)UL47 Δ 4,

R(F)UL47Δ10, and R(F)UL46/47Δ2. All TK⁺ deletion mutants subsequently underwent recombination repair of the UL46 and UL47 loci with plasmids containing the wild-type UL46 and UL47 genes (Fig. 1E and F). In the absence of selection, repair was determined by Southern blot analysis of randomly selected plaques over multiple rounds of plaque purification with a UL46- and UL47-specific probe (51). Once judged to be pure by Southern blot analysis, the repaired viruses underwent three additional rounds of plaque purification. The repaired viruses were designated R(F)UL46Δ3r, R(F)UL47Δ2r, R(F)UL47Δ4r, and R(F)UL46/47Δ2r. Viral stocks were prepared and titers were determined as described previously (51).

Cell lines. The source and growth conditions of the Vero and 143TK⁻ cells used in these studies have been described previously (51). Human epithelioid cervix carcinoma (HeLa) cells were gifts from P. Sharp and T. Kristie and were maintained in Dulbecco modified Eagle's medium (DMEM) (GIBCO) containing 10% newborn calf serum (GIBCO) at 37°C in either a humidified atmosphere containing 5% CO₂ and 95% air or airtight roller bottles.

Virus infection. For each virion preparation, 1.5×10^8 HeLa cells were grown to a density of 3×10^5 cells per ml in spinner culture and infected with 7 PFU of virus per cell in a total volume of 7.5 ml of medium 199 (Hazleton) containing 1% fetal calf serum (199V). Virus was adsorbed for 1.5 h at room temperature with gentle shaking, the inoculum was removed, and the cells were washed once with 199V, resuspended in 90 ml of a mixture of 30% 199V and 70% methionine-deficient Dulbecco modified Eagle's medium (DMEM Met⁻; Sigma), and returned to 37°C for 18 to 20 h. Flasks (25 cm²) containing approximately 4×10^6 Vero cells were used for the pulse-label time course experiments. Cells were infected with 5 PFU of virus per cell at 4°C with gentle shaking. The inoculum was removed after 1 h, the cells were washed once with cold 199V, and 2 ml of fresh 199V was added. The cells were returned to 37°C, and the 199V was replaced with 2 ml of 199V containing 0.1% pooled human immunoglobulin (Calbiochem) at 2 h postinfection to prevent a secondary infection. Mock infections were carried out in parallel in all cases.

Metabolic labeling with [³⁵S]methionine. To achieve steady-state labeling of HSV-1 proteins, mock-infected and virus-infected HeLa cells were labeled from 1.5 to 18 h postinfection in a mixture of 30% 199V and 70% DMEM Met⁻ containing 11.1 μCi of [³⁵S]Met (Tran³⁵S-label; ICN) per ml. The pulse-labeling conditions have been described previously (51).

Purification of cell-associated virions. Cell-associated virions were purified according to the technique described by Spear and Roizman (42), with the following modifications. Briefly, at 18 h postinfection, cells were transferred to 50-ml-volume tubes, cooled on ice, and harvested by centrifugation at $10,000 \times g$ for 10 min at 4°C. All subsequent steps postharvest were carried out at 4°C. The cell pellet was resuspended in 2 volumes of 1.0 mM phosphate buffer, pH 7.4, and transferred to a 5-ml-volume Dounce homogenizer, and the cell membranes were disrupted with 15 strokes of the loose (B) pestle. The disrupted cells were adjusted to a final concentration of 0.25 M sucrose with 60% sucrose in phosphate buffer. Nuclei were removed by centrifugation at $10,000 \times g$ for 25 min. Approximately 1.0 ml of supernatant remained, and this was layered over a 10 to 30% continuous dextran T10 (Pharmacia) gradient prepared in a polyallomer tube (15 by 100 mm). The gradient was centrifuged at $74,000 \times g$ for 80 min in an AH-629 rotor (Sorvall), and 1.0-ml

fractions were collected from the bottom of the tube with a peristaltic pump. The A_{280} gradient profile was recorded with a continuous-flow UV monitor, and the peak fractions containing the purified virions were pooled and brought to a concentration of 0.5 M urea by using 5 M urea in 0.01 M Tris-HCl, pH 7.4. The virions were sonicated for 5 s with a Branson 250 Sonifier outfitted with a microtip and were brought to a final concentration of 50% sucrose by using solid sucrose. The volume was increased to 4 ml with 50% sucrose, and the virion mixture was transferred to a polyallomer tube (15 by 100 mm) and overlaid sequentially with 3.5 ml (each) of 40, 30, and 20% sucrose in phosphate buffer. The step gradient was centrifuged at $116,000 \times g$ for 18 h in an AH-629 rotor. The purified virions were found at the 40 to 50% interface and were harvested as described above for the continuous gradients. The virions were diluted fourfold (to approximately 11.5 ml) with 10 mM Tris-HCl (pH 7.4), layered over 4.5 ml of 10% sucrose in 2 M urea, and centrifuged at $116,000 \times g$ for 2 h as described above. The virion pellet, containing 10 to 30 μg of protein (20 to 80 cpm/ng), was resuspended in 100 to 300 μl of 10 mM Tris-HCl (pH 7.4) and stored at -80°C. Approximately 2 μg of protein was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The apparent molecular weights of VP11/12 and VP13/14 were determined relative to those of other virion proteins, specifically, VP5, VP16, VP19, VP21, and VP23 (8, 16, 18), by using external molecular weight markers (Bio-Rad).

Purification of cell-free virions. Cell-free virions were purified from the growth medium by using a modification of the procedure described by Frame et al. (15). All steps were carried out at 4°C. The culture medium from the cell-associated virion preparation described above was transferred to 50-ml-volume tubes and cleared of remaining large cell debris by centrifugation at $14,500 \times g$ for 5 min. Cleared medium (35 ml) was transferred to 40-ml-volume oak-ridge tubes, underlayered with 5 ml of 10% sucrose in 0.1× normal phosphate-buffered saline, and centrifuged at $39,000 \times g$ for 2 h in an SS-34 rotor (Sorvall). The virion-containing pellet was resuspended in 1.0 ml of phosphate buffer and layered over the top of a 15 to 60% continuous sucrose density gradient prepared in a polyallomer tube (15 by 100 mm). The gradient was centrifuged at $60,000 \times g$ for 1.0 h and harvested as described for the cell-associated virion preparations. The virion-containing peaks were pooled and centrifuged at $39,000 \times g$ for 2 h to pellet the virions. The virion pellet, containing 20 to 60 μg of protein (20 to 80 cpm/ng), was resuspended in 100 to 300 μl of 10 mM Tris-HCl (pH 7.4) and stored at -80°C. Approximately 2 μg of protein was used for SDS-PAGE analysis.

Virion fractionation. Freshly purified virions (12 μg) were sequentially stripped of their envelopes or envelopes and teguments with NaCl and/or detergents as described by others (29, 30, 48). Virions were treated with 0 to 1.0% Nonidet P-40 (NP-40) in the presence or absence of 1 M NaCl or with a mixture of 5% Triton X-100 and 5% sodium deoxycholate for 1.0 h at room temperature, diluted 10-fold with 10 mM Tris-HCl (pH 7.5), and recovered by centrifugation at $39,000 \times g$ for 2 h. The pelleted virions were resuspended to an equivalent (prespin) volume in 10 mM Tris-HCl (pH 7.4). Equal volumes of each fraction, representing approximately 4 μg of intact virion protein, were used for SDS-PAGE analysis. Remaining extracts were stored at -20°C.

In vitro phosphorylation assays. Assays were performed essentially as described by LeMaster and Roizman (24).

Briefly, ~0.5 μ g of purified virions was treated with 1.0% NP-40 at room temperature for 30 min in a final volume of 100 μ l. NP-40 solubilization was terminated by diluting the samples eightfold with 50 mM Tris-HCl (pH 8.0). Thirty to 80 ng of the NP-40-treated virions was subsequently reacted for 30 min at 37°C in 50 μ l of 50 mM Tris-HCl (pH 8.0) containing 50 mM magnesium acetate, 1.0 mM dithiothreitol, 0.1% NP-40, and 1 to 5 μ Ci of [γ - 32 P]ATP (ICN). The reaction was stopped by boiling the sample in gel loading buffer. Ten to 30 ng of protein was used for SDS-PAGE analysis. Remaining extracts were stored at -20°C.

SDS-PAGE and Western blot (immunoblot) analysis. SDS-PAGE, autoradiography, Western blots, and α TIF-specific antibodies have been described previously (51). The polyclonal rabbit antisera specific for HSV-1 ICP8 was kindly provided by P. R. Kinchington. Sera were used at dilutions of 1:200.

TK assays. TK assays of mock- and HSV-1-infected 143TK⁻ cells were done as described previously (23).

Densitometric analysis. Autoradiograms of SDS-PAGE-separated virion proteins, metabolically labeled to steady-state levels with [35 S]methionine, were scanned with a Gilford Response I spectrophotometer programmed for peak area integration and baseline correction. A minimum of two exposures of each autoradiogram were scanned to control for possible film saturation. The peak values obtained for VP11/12, VP13/14, and α TIF were adjusted for the number of methionine residues present in each protein and to their predicted minimum molecular weights. These values were subsequently normalized to those obtained similarly for the major capsid protein, VP5, by using a value of 960 copies per virion (18, 40). The amino acid content of each protein was obtained by a computer-based translation of the nucleic acid sequence of each open reading frame (IntelliGenetics PC-Genie) (26, 28, 32).

RESULTS

UL46, UL47, and UL46/47 deletion mutants exhibit delayed expression of the viral thymidine kinase gene at early times postinfection. A series of UL46 and UL47 single-deletion mutants and a UL46/47 double-deletion mutant were constructed as previously reported by Zhang et al. by following the strategy presented in Fig. 1 (35, 51). Since this strategy utilizes TK activity as a selectable marker (35), the first generation of UL46 and UL47 deletion mutants were TK⁻ (Fig. 1A to D). The TK gene was subsequently restored in all of the deletion mutants by marker rescue. Figure 2 depicts a representative Southern blot analysis of parallel sets of the isogenic TK⁻ and TK⁺ UL46, UL47, and UL46/47 deletion mutants. The top panel shows purified viral DNA digested with *Bam*HI and probed with the UL46- and UL47-containing *Bam*HI F fragment of the HSV-1 genome. The decrease in the size of the *Bam*HI F fragment was the same in each pair of isogenic mutants, confirming that the TK marker-rescued mutants still harbored the previously mapped UL46 and/or UL47 deletion (51). The bottom panel depicts the same blot after it was stripped and reprobed with a plasmid containing the TK gene. Note that for each virus, TK marker rescue was accompanied by an increase in the size of the *Bam*HI fragment that hybridized to the TK gene (Δ 305 to *Bam*HI Q). This shift corresponded to the repair of the 700-bp Δ 305 deletion in the TK gene (35). The isogenic series of TK⁺ deletion mutants retained the same nomenclature but were distinguished from their TK⁻ counterparts by the prefix "R(F)-" (Fig. 1).

FIG. 2. Southern blot analysis of the isogenic TK⁻ and TK⁺ UL46, UL47, and UL46/47 deletion mutants. (Top panel) Viral DNA (0.2 to 1.0 μ g) was digested overnight with *Bam*HI, electrophoresed through a 0.8% agarose gel, transferred to GeneScreen Plus, and hybridized to the *Bam*HI F fragment of HSV-1 (51). Lane 1 contains pRB158 marker DNA, digested with *Sal*I (51). The fragment sizes are indicated in kilobases. The remaining paired lanes (except for lane 47 Δ 10.1) contain sets of isogenic TK⁻ (left lanes) and TK⁺ (right lanes) UL46, UL47, and UL46/47 deletion mutants. Numbers after the decimal point indicate independent isolates of the same deletion mutant. Lanes: wt, R Δ 305 and strain F; 46/47 Δ 2.1, RUL46/47 Δ 2.1 and R(F)UL46/47 Δ 2.1; 46 Δ 3.1, RUL46 Δ 3.1 and R(F)UL36 Δ 3.1; 46 Δ 3.2, RUL36 Δ 3.2 and R(F)UL46 Δ 3.2; 47 Δ 2.1, RUL47 Δ 2.1 and R(F)UL47 Δ 2.1; 47 Δ 2.2, RUL47 Δ 2.2 and R(F)UL47 Δ 2.2; 47 Δ 4.1, RUL47 Δ 4.1 and R(F)UL47 Δ 4.1; 47 Δ 4.2, RUL47 Δ 4.2 and R(F)UL47 Δ 4.2; 47 Δ 10.2, RUL47 Δ 10.2 and R(F)UL47 Δ 10.2; 47 Δ 10.1, RUL47 Δ 10.1. (Bottom panel) The membrane in the top panel was stripped and reprobed with the 1.5-kb *Bg*/II-*Nco*I fragment of the HSV-1 (F) TK gene in pUC9 (51). Lanes are as indicated for the top panel.

To confirm the functional restoration of the TK gene, levels of viral TK activity were compared after infection of 143TK⁻ cells with the marker-rescued deletion mutants and with strain F. Since UL46 and UL47 modulate the efficiency of α TIF-mediated α gene induction, early time points were taken to determine whether the deletion mutants would exhibit an altered TK activity as a secondary phenotype arising from the effect of UL46 and UL47 on α gene induction (27, 28, 51). Figure 3 shows a series of representative TK inductions. Figure 3A depicts a 0- to 24-h time course which shows that whereas each mutant was able to achieve wild-type TK levels by 8 h postinfection, the TK induction curves differed among the deletion mutants and the wild type (strain F) at early times postinfection. The most significant difference between strain F and the deletion mutants was that the UL46 and UL47 single-deletion mutants exhibited a 2-h delay and the UL46/47 double-deletion mutant exhibited a 4-h delay in the induction of TK activity. This result was confirmed in the 0- to 4-h time course shown in Fig. 3B. In this experiment, several multiplicities of infection (MOIs) were used for strain F to control for possible MOI-dependent temporal shifts in TK induction. In addition, each virus was retitrated at the time of infection to control for variations in input MOIs. The titers obtained at the time of infection are presented as PFU per cell. All mutants subsequently underwent repair of the UL46 and UL47 deletions to confirm that the observed 2- to 4-h delays in TK induction were specific to the UL46 and UL47 gene products (data not shown). Figure 3C shows a 0- to 4-h TK induction experiment using the repaired mutants. In each

FIG. 3. Induction of endogenous viral TK during infection of 143 TK⁻ cells. (A) Six-well cluster dishes containing 0.5×10^6 to 1.0×10^6 cells per well were infected with approximately 5 PFU of strain F or the indicated TK⁺ UL46, UL47, or UL46/47 deletion mutant per cell and harvested at 2, 4, 6, 8, 11, 12, and 24 h postinfection for TK assays. Shown are relative TK levels, obtained as described previously (51). (B) The assay is as described in the legend to panel A. Input multiplicities of infection in PFU per cell are shown in brackets. (C) All conditions are as described for panel B, except that UL46-, UL47-, and UL46/47-repaired TK⁺ deletion mutants were used.

FIG. 4. Viral protein synthesis profiles for cells infected with strain F or the TK⁺ UL46, UL47, or UL46/47 deletion mutants. Mock- and virus-infected Vero cells were pulse-labeled with [³⁵S]Met 30 min prior to being harvested at the indicated times postinfection, and extracts were prepared and proteins were separated by SDS-PAGE as described previously (51). Shown are autoradiograms of SDS-PAGE gels containing [³⁵S]Met-labeled infected Vero cell extracts after electrophoretic transfer to nitrocellulose. Lanes: Mock, mock infected; F, wild-type strain F; Δ 46, R(F)UL46 Δ 3.1; Δ 47, R(F)UL47 Δ 4.1; Δ 46/47, R(F)UL46/47 Δ 2.1. The locations of ICP4, ICP8, UL46, UL47, and α TIF are as indicated.

case, the induction profiles of the repaired mutants paralleled those of strain F at the comparable MOI, confirming that the 2- to 4-h delays in TK induction shown in Fig. 3A and B resulted from the UL46 and UL47 deletions.

UL46 and UL47 are expressed late in infection and encode virion proteins VP11/12 and VP13/14. The observation that the UL46 and UL47 deletion mutants exhibited altered TK inductions shortly after infection suggested that the proteins encoded by the two genes could function as virion-associated regulatory factors. There are three levels of precedent for this argument. First, characterization of the UL46 and UL47 mRNAs by Hall et al. placed UL46 and UL47 into the $\beta\gamma$ and γ kinetic classes, respectively (17). Thus, it is unlikely that either gene is synthesized early enough to produce the observed change in TK expression. Second, α TIF functions by virtue of its location in the virion, and both UL46 and UL47 modulate this activity, suggesting a possible physical association (3, 27, 28, 51). Third and last, the majority of viral proteins synthesized late in infection either are associated with virion assembly or are themselves virion components (20; reviewed in reference 37). In support of the last argument, UL47 had been previously shown by McLean et al. to encode a virion protein (29).

Figure 4 shows a pulse-label experiment comparing protein synthesis in the UL46, UL47, and UL46/47 deletion mutants with that in strain F over a 17.5-h time course. Confluent monolayers of Vero cells were infected with 5 PFU of strain F, R(F)UL46 Δ 3, R(F)UL47 Δ 4, or R(F)UL46/47 Δ 2 and were pulse-labeled with [³⁵S]Met for 30 min prior to being harvested for SDS-PAGE analysis at the indicated time postinfection. The locations of the putative UL46- and UL47-encoded proteins are marked at the 10-h point and are readily apparent by comparing the deletion mutants with strain F. Neither the UL46- nor UL47-encoded proteins were detected at or before 4 h postinfection in the infected-cell extracts. However, at 8 h postinfection, both the UL46- and UL47-encoded proteins were easily detected. Expression of α TIF, also a $\beta\gamma$ gene, paralleled that of the UL46- and UL47-encoded proteins at 8, 10, and 17.5 h postinfection, supporting the previous observations that UL46 and UL47 are expressed as $\beta\gamma$ and γ genes, respectively (17, 29). The

FIG. 5. Virion protein profiles of HSV-1 (F) and the TK⁺ UL46 and UL47 deletion mutants. Virions were purified and extracts were prepared as described in Materials and Methods. (A) Autoradiogram of [³⁵S]methionine-labeled purified virions and infected-cell extracts separated by SDS-PAGE and transferred to nitrocellulose. (B) Western blot analysis was done as described previously (51) with the same nitrocellulose filter as in panel A with antisera for ICP8 and αTIF. Lanes: Mock, mock infected; F, HSV-1 (F); Δ47, R(F)UL47Δ4.1; Δ46, R(F)UL46Δ3.1; Δ46/47, R(F)UL46/47Δ2.1; CE, infected-cell extract; CA, virions purified from infected cells; CF, virions purified from infected-cell media. The locations of known virion proteins are marked (8, 16, 18, 24).

appearance and accumulation of αTIF were also measured in parallel Western blot analysis of the same nitrocellulose filter, and the levels of the unlabeled protein consistently reflected the intensity of the ³⁵S-pulse-labeled profile (data not shown). Similar results were reported by us previously during the analysis of the isogenic TK⁻ strains (51). Interestingly, the pulse-labeled levels of ICP4 in strain F and the deletion mutants did not appear to be significantly different.

To determine whether the gene product of UL46 is a component of the virion and to confirm the earlier report that UL47 encodes a tegument protein, virions were purified from HeLa cells infected with strain F, R(F)UL46Δ3, R(F)UL47Δ4, and R(F)UL46/47Δ2 after being labeled to steady-state levels with [³⁵S]Met. To control for the quality of our virion preparations (or, in other words, to rule out the possibility that significant amounts of cellular debris were present as contaminants), virions were purified in parallel from infected cells (cell associated) and from the growth medium (cell free) by two completely different methods (15, 42). The cell extracts and purified virions were separated by SDS-PAGE and transferred to nitrocellulose, and autoradiograms were prepared. Figure 5A depicts an example of the comparative analysis showing a set of triplet lanes, each of which contains infected-cell extracts (lanes CE), purified cell-associated virions (lanes CA), and purified cell-free virions (lanes CF) obtained from the same infection with the indicated virus. We were unable to detect significant differences between the virion profiles obtained by either technique. The purity of the virions was monitored by Western blot analysis of the same nitrocellulose membrane by using antibodies against ICP8, a known nonstructural protein, and αTIF, a major structural protein (Fig. 5B) (19). The Western blot clearly showed that high levels of ICP8 were present in the infected-cell extracts but not in the purified virions. This difference was even greater when the ratios of ICP8 and αTIF in the purified virions were compared with those present in the infected-cell extracts, demonstrating that little cellular debris was associated with the purified virion preparations. Note, however, that the UL47 and UL46/47 deletion mutants have more ICP8 associated with the purified

virions than either strain F or the UL46 single-deletion mutant. On the basis of data presented later in this report, it is conceivable that ICP8 was present in the tegument of UL47 deletion mutants rather than being copurified as an exterior contaminant.

Comparison of the wild-type virion protein profiles present in strain F with those obtained from each of the deletion mutants clearly showed that UL46 and UL47 encode VP11/12 and VP13/14, since these virion proteins were absent from their corresponding deletion mutants. The virion protein profile for the UL46 deletion mutant, R(F)UL46Δ3, was missing an 87- and 93-kDa doublet band corresponding to VP11/12 in the wild-type (F) strain (Fig. 5A, VP11/12 [UL46]) (18). Similarly, the virion protein profiles for the UL47 deletion mutants, R(F)UL47Δ4 and R(F)UL47Δ2, were also missing a doublet band that was clearly visible in strain F (Fig. 5A, VP13/14 [UL47]; data from 47Δ2 not shown). The UL47-specific doublet migrated at apparent molecular masses of 79 and 81 kDa, corresponding to the VP13/14 protein bands in strain F (18). As predicted from the results obtained with the single-deletion mutants, both the VP11/12 and VP13/14 bands were missing in the double-deletion mutant, R(F)UL46/47Δ2. Note that proteins corresponding to VP11/12 and VP13/14 were also absent from the corresponding infected-cell extracts (lanes CE), confirming that the absence of the proteins from the virion profiles resulted from a lack of synthesis and not from a defect in virion assembly.

UL46 and UL47 deletion mutant virions lack virion phosphoproteins corresponding to VP11/12 and VP13/14. LeMaster and Roizman previously reported that VP11/12 and VP13/14 are phosphorylated *in vitro* by a virion-associated protein kinase (24). To confirm the relationship between UL46, UL47, and the two sets of virion proteins, a similar *in vitro* assay was performed to compare the phosphorylation patterns among strain F and the UL46, UL47, and UL46/47 deletion mutants. Consistent with an earlier report (24), a subset of proteins corresponding to those previously identified as VP11/12, VP13/14, VP16, VP18.8, and VP23 were heavily phosphorylated in the purified virions (Fig. 6). As

FIG. 6. Phosphorylation of proteins present in purified virions by a virion-associated protein kinase. The cell-free virion preparations depicted in Fig. 5 were used for the virion-associated kinase assay as described in Materials and Methods. Shown is an autoradiogram of SDS-PAGE-separated ^{32}P -labeled virion proteins after they were transferred to nitrocellulose. Lanes: ^{35}S , wild-type HSV-1 (F) ^{35}S -labeled virion profile shown in Fig. 5; 1, equivalent volume of a mock-infected preparation corresponding to the virion peak fraction from the gradient, assayed for kinase activity; 2, heat-inactivated (60°C for 20 min) purified F virions assayed for kinase activity; 3, mixture of contents of lanes 1 and 2 assayed for kinase activity; $\Delta 46/47$, R(F)UL46/47 $\Delta 2.1$; $\Delta 46$, R(F)UL46 $\Delta 3.1$; $\Delta 47$, R(F)UL47 $\Delta 4.1$; F, HSV-1 (F). The locations of VP5 (^{35}S lanes only), VP11/12 (46), VP13/14 (47), and αTIF are indicated. An asterisk marks the location of VP18.8, which is heavily phosphorylated in the wild-type virion kinase assay but not in the deletion mutants. VP23 (not indicated) is the major phosphoprotein band at the very bottom of the gel. Other minor phosphoproteins are not identified.

would be predicted if UL46 encodes VP11/12, the virion phosphoprotein doublet designated VP11/12 (46 in Fig. 6) was missing in both the R(F)UL46 $\Delta 3$ and R(F)UL46/47 $\Delta 2$ deletion mutants. The same was true for the virion phosphoprotein VP13/14 (47 in Fig. 6), which was absent in both R(F)UL47 $\Delta 4$ and R(F)UL46/47 $\Delta 2$. Taken together, the most straightforward interpretation of these data would be that VP11/12 and VP13/14 are the gene products of UL46 and UL47.

VP11/12 and VP13/14 are tegument proteins. Recently, McLean et al. reported that VP13 and VP14 are both found in the tegument of the HSV-1 17 strain SYN⁻ (29). Data presented in Fig. 7 confirmed this observation with strain F and demonstrated for the first time that VP11 and VP12 are also tegument proteins. In these experiments, virions were extracted with increasing stringency by using NP-40 plus or minus NaCl or Triton X-100 and sodium deoxycholate to sequentially remove the envelope and tegument compartments of each virion (29, 30, 48). The treated virions were pelleted, and both the capsid-containing pellets (P) and the supernatants containing the extracted noncapsid virion proteins (S) were separated by SDS-PAGE. Under mild extraction conditions, that is, NP-40 concentrations below 1.0%, the envelope glycoproteins and a portion of αTIF were extracted (compare 1% NP40 lanes S and P in Fig. 7). Increasing the extraction stringency by adding 1 M NaCl to 1.0% NP-40 resulted in a more efficient removal of both the glycoproteins and αTIF from the virion (lanes 1M NaCl 1% NP40). However, neither VP11/12 nor VP13/14 was detected in the S fractions extracted under these conditions. When the virions were treated with a mixture of 0.5% Triton X-100

FIG. 7. Detergent fractionation of purified wild-type F virions. Virions purified from infected-cell medium (CF in Fig. 5) were sequentially stripped of their envelopes or envelopes and teguments with the indicated combinations of detergent and 1 M NaCl as described in Materials and Methods. The specific detergents and concentrations are indicated above each set of lanes. P, pellet; S, supernatant; V, unreacted virion protein profile. Control lanes are virions subjected to the entire procedure in the absence of both salt and detergent. The locations of relevant virion proteins are marked (8, 16, 18, 24).

and 0.5% sodium deoxycholate, the majority of the VP13/14 and αTIF proteins were released from the virion into the supernatant (lanes 0.5% DOC 0.5% X-100). In contrast, only about half of the VP11/12 protein was removed from the virion (P) to the supernatant (S). Note that under these conditions, the capsid proteins, VP5 and VP19, were not extracted. These results provide conclusive evidence that both VP11/12 and VP13/14 are tegument proteins.

VP11/12 and VP13/14 are major virion components. Table 1 summarizes the results of densitometric analysis of autoradiograms of SDS-PAGE-separated virion protein profiles. Several exposures of a series of wild-type and deletion mutant virion protein profiles, including those depicted in Fig. 5, were analyzed. Since glycoprotein E (gE) comigrates in the region of VP11/12 and VP13/14, autoradiograms of the glycoprotein-stripped virion preparations depicted in Fig. 7 were also analyzed as controls. Levels of VP11/12, VP13/14, and αTIF were corrected for molecular weight and methionine content (12 methionine residues in VP11/12, 7 in VP13/14, and 16 in αTIF) and normalized to those of VP5 as described in Materials and Methods (26, 28, 32). The ratios of VP5 to αTIF were consistent in all of the F strain virion protein profiles scanned. Comparison of these results with those obtained previously by Heine et al. showed that they were, for the most part, proportionately consistent, but in these studies, the absolute numbers of αTIF and VP11/12 molecules per virion were higher, whereas the numbers of VP13/14 molecules remained the same (18). This may reflect differences in methionine content and in our use of the minimum molecular weights obtained from computer-based translations of the nucleic acid sequence (26, 28, 32). VP11/12, VP13/14, and αTIF were present in close to equimolar ratios (1,270:1,846:1,647, respectively) in F virions. Interest-

TABLE 1. Stoichiometries of VP11/12 and VP13/14 in purified virions^a

Virion component	No. of molecules of specific virion proteins in:				
	HSV-1 (F)		Δ UL46 ^b	Δ UL47 ^b	Δ UL46/47 ^b
	Previous ^c	Current ^b			
gC-gB-gH	1,220–1,440 (1,330)	1,600–1,874 (1,750)	1,649–2,433 (2,047)	2,168–3,399 (2,822)	1,978–2,771 (2,485)
VP11/12	500–600 (550)	1,097–1,690 (1,270)	0	2,391–3,764 (3,073)	0
VP13/14	1,590–1,880 (1,735)	1,683–2,286 (1,846)	3,097–4,508 (3,613)	0	0
VP16 (α TIF)	850–1,010 (930)	1,242–2,095 (1,647)	1,425–1,959 (1,676)	1,917–2,509 (2,198)	1,920–2,350 (2,180)
VP5	850–1,000 (960) ^d				

^a Densitometric scans were carried out on autoradiograms of SDS-PAGE-separated [³⁵S]Met-labeled virion proteins as described in Materials and Methods and as shown in Fig. 5.

^b Δ UL46, Δ UL47, and Δ UL46/47 refer to the isogenic deletion mutants R(F)UL46 Δ 3.1, R(F)UL47 Δ 4.1, and R(F)UL46/47 Δ 2.1, respectively. The range of values obtained for a minimum of four and up to eight independent scans is shown, with the average values presented in parentheses.

^c As reported by Heine et al. (18).

^d As reported by Schrag et al. (40).

ingly, the number of VP11/12 molecules present in the virion increased nearly 2.5-fold in the UL47 deletion mutant (from 1,270 to 3,073), and similarly, the numbers of VP13/14 increased approximately 1.5- to 2-fold in the UL46 deletion mutant (from 1,846 to 3,613). It also appeared that numbers of α TIF were increased in the UL47 and UL46/47 deletion mutants (from 1,647 to 2,198 and 2,180, respectively) but not in the UL46 deletion mutant relative to strain F. Numbers of gC-gB-gH were also increased in the UL47 and UL46/47 deletion mutants (1,750 to 2,822 and 2,485, respectively).

DISCUSSION

The analysis of a series of deletion mutants in the UL46 and UL47 open reading frames of HSV-1 (F) provides the first genetic evidence for the direct association between UL46, UL47, and two sets of related virion proteins, VP11/12 and VP13/14. Virion proteins absent in the UL46 deletion mutant corresponded to VP11 and VP12, two virion proteins originally described by Spear and Roizman (42) that were later shown to be genetically linked and located either to the left of 0.18 map units or to the right of 0.65 map units (24). Subsequent studies by Weller et al. (46) provided evidence that the locus mapped to the right of 0.65 map units, placing the gene(s) encoding VP11/12 within the *Bam*HI I' and F regions, the location of the UL46, UL47, UL48, and UL49 gene cluster (17, 26, 28). The UL46-specified virion proteins migrated at observed molecular masses of 87 and 93 kDa, in agreement with the previously published molecular masses of VP11 and VP12 (18, 24). The virion proteins missing in the UL47 deletion mutants corresponded to the VP13/14 doublet in strain F, also shown to be genetically linked and located to the right of 0.65 map units, again within the *Bam*HI I' and F regions (18, 24, 26, 28). The UL47-specified proteins migrated at apparent molecular masses of 79 and 81 kDa, values that are consistent with the published molecular masses of VP13 and VP14 and with the size of the UL47-encoded proteins identified by McLean et al. (29) and by Whittaker et al. (47) with the HSV-1 strain 17 SYN⁻ (16). Moreover, these data are in agreement with those of an earlier report by Pereira et al., who first pointed out a possible association between VP13 and VP14 (33). Since neither VP11/12 nor VP13/14 was detected in cells or virions after infection with the relevant UL46 or UL47 deletion mutant, the absence of these proteins in the purified virions could not be attributed to a defect in tegument packaging.

Nineteen virion phosphoproteins were identified by Le-

Master and Roizman, five of which are heavily phosphorylated in vitro, including VP11/12, VP13/14, VP16, VP18.8, and VP23 (24). Our in vitro protein kinase analysis of the UL46, UL47, and UL46/47 deletion mutants showed that phosphoproteins with mobilities corresponding to the mobilities published for VP11/12 and VP13/14 were absent from purified virions prepared from UL46 and UL47 deletion mutants, respectively, providing further evidence that these proteins are indeed encoded by UL46 and UL47. Phosphorylation would also provide an explanation for the relationship between VP11 and VP12 and that between VP13 and VP14, that is, the two forms of each protein most likely result from differential phosphorylation. This would be consistent with results of earlier reports and with the predicted phosphorylation sites in the translated DNA sequence of each gene (1, 26, 28). Alternatively, UL47 is reported to be glycosylated and also contains a second in-frame ATG 99 bp downstream of the predicted translational start site, providing additional explanations for the size difference observed between VP13 and VP14 (28–30). It is likely, therefore, that a combination of translational or posttranslational modifications of the UL46 and UL47 gene products results in the two sets of related virion proteins, VP11/12 and VP13/14.

Taken together, the data presented above provide conclusive evidence that UL46 and UL47 are the structural genes for VP11/12 and VP13/14. However, there is the remote possibility that UL46 and UL47 do not directly encode VP11/12 and VP13/14 but instead that the expression of other genes encoding VP11/12 and VP13/14 is somehow inhibited in response to the deletion of UL46 and/or UL47. This is unlikely for several reasons. First, McLean et al. showed that antibodies against synthetic peptides derived from the UL47 DNA sequence react specifically with VP13 and VP14 (29). Second, the UL47 deletion mutant, R(F)UL47 Δ 10, which contains a small in-frame deletion in the UL47 open reading frame, expressed a novel protein doublet which migrated just below and in lieu of the wild-type VP13/14 (data not shown). Third, UL46 was expressed normally in all of the UL47 deletion mutants and, conversely, UL47 was expressed normally in the UL46 deletion mutants. To clarify the latter argument, if the deletion of either UL46 or UL47 resulted in a nonspecific transcriptional interference with other genes, this event would most likely include the adjacent transcription units. Last, the double-deletion mutant lacked the entire promoter and coding regions of both genes, it was transcriptionally inactive in this region, and it expressed neither VP11/12 nor VP13/14. Altogether, these

observations render an argument of transcriptional interference and/or secondary effect tenuous. Thus, on the basis of the direct correlation between the deletions of UL46 and UL47 and the absence of the virion proteins VP11/12 and VP13/14, we concluded that UL46 encoded VP11/12, while UL47 encoded VP13/14.

In this report, we have demonstrated that the proteins encoded by UL46 and UL47 belong to a class of tegument proteins which exhibit regulatory functions. Detergent extraction of purified virions showed that both VP11/12 and VP13/14 colocalized with the α TIF-containing tegument fraction. VP13/14 was released from the virion more readily than VP11/12, suggesting that VP13/14 may associate more closely with the envelope. In contrast, VP11/12 required more stringent conditions for its release that were similar to those for the UL49-encoded capsid-associated tegument protein VP22 (13, 18, 42). Hydropathic analysis of the predicted amino acid sequence of VP13/14 shows a hydrophilic arginine-rich region in the N terminus and a hydrophobic C terminus (28). The amphipathic character of VP13/14 may enable the C terminus of the protein(s) to interact with the envelope, while the N terminus interacts with other tegument proteins. Along these lines, it is interesting that VP13/14 shares significant homology with the gp10 glycoprotein of equine herpesvirus 1 and 4 (47). In contrast, analysis of the VP11/12 sequence predicts no obvious hydrophobic or hydrophilic region (28).

Although both VP11/12 and VP13/14 are major constituents of the tegument and may represent between 15 and 18% of the total virion protein mass, neither protein is essential for growth in vitro; in fact, all of the UL46 and UL47 single- and double-deletion mutants examined to date grow either as well as or to within 0.5 to 1.0 log titer of that of the wild type (2, 18, 42, 51). The ratios of VP11/12 and VP13/14 to α TIF, which range from 0.5:1 to 1:1 in the tegument, suggested a stoichiometric association among the three proteins in the virion, which may reflect a functional association during the early stages of infection. In light of the observed stoichiometry combined with the nonessential nature of VP11/12 and VP13/14, it is tempting to speculate that essential tegument proteins, such as α TIF, are required for a stoichiometrically fixed tegument framework around which the remaining nonessential tegument proteins assemble. In wild-type virions, VP11/12 and VP13/14 may be coordinately packaged in stoichiometries which reflect their synthesis within the cell. Whether this is linked to a coordinate transcription of UL46 and UL47 remains to be seen.

Notably, VP11/12 and VP13/14 appear to be able to substitute for one another; that is, virions purified from infections with UL46 deletion mutants contained approximately twice the levels of VP13/14, and, similarly, those obtained after infection with UL47 deletion mutants contained approximately twice the levels of VP11/12. The UL46/47 double-deletion mutant represented a bit of a paradox, because the protein mass contributed by VP11/12 and VP13/14 does not appear to be entirely compensated for by other known tegument proteins, although numbers of α TIF do increase by approximately 30%. Interestingly, levels of gB, gC, and gH increased by approximately 50%, and there was more ICP8 present in these virions. That this was due primarily to the absence of VP13/14 is supported by similar increases in α TIF, gB, gC, gH, and ICP8 observed in virions obtained from the UL47 single-deletion mutant. Thus, whereas VP13/14 alone seemed capable of substituting for VP11/12, the converse does not occur, suggesting that both competition and specificity may play roles in tegument

packaging. We have not determined whether the increased levels of gB, gC, and gH can be attributed to their presence in the envelope or tegument or both. Schrag et al. reported that purified, undamaged virions can have diameters which vary from 1,875 to 2,370 Å (187.5 to 237 nm) (40). The different diameters could reflect significant variations in the numbers of nonessential tegument proteins. We are currently comparing the virion diameters and morphology of HSV-1 (F) with those of the deletion mutants. The question remains, however, why such high levels of nonessential and apparently nonstructural proteins are packaged into the virion tegument. The answer could lie in their regulatory properties, particularly if they function stoichiometrically with α TIF.

Regarding the potential functions of VP11/12 and VP13/14, all of the known regulatory proteins found within the tegument are phosphoproteins, either in vivo or in vitro, an observation which is not surprising in light of the evidence which associates regulatory functions with phosphorylation states (9, 13, 24, 25, 30, 41; reviewed in reference 22). Protein kinase activity is present in HSV-1 virions and is associated with the gene product of UL13 (9, 24, 43). Although the bovine herpesvirus homolog of VP13/14, VP8, is also a virion protein and is reported to be associated with a protein kinase activity (6), it is unlikely that either UL46 or UL47 encodes a protein kinase. The UL46, UL47, and UL46/47 deletion mutants exhibited the abilities to phosphorylate the remaining major virion phosphoproteins, notably VP11/12 (in the UL47 deletion mutants), VP13/14 (in the UL46 deletion mutants), α TIF, and VP23. The one apparent exception was VP18.8, which was underphosphorylated in all of the deletion mutants. VP18.8 aside, the UL47 deletion mutants appeared to increase the overall phosphorylation levels, whereas the UL46 deletion mutants exhibited reduced phosphorylation of the major phosphoproteins. The increased phosphorylation levels observed in the UL47 deletion mutants, however, must be interpreted with caution, since the numbers of VP11/12 molecules in the tegument of this mutant are doubled. Nevertheless, it is reasonable to conclude that a virion kinase activity is still present in the deletion mutants but that it may exhibit altered specificities and levels of activity. Whether VP11/12 and/or VP13/14 is involved in virion-associated phosphorylation or dephosphorylation events remains to be seen, but if so, such a mechanism could be particularly relevant if α TIF function is found to be regulated by its phosphorylation state. The caveat in these observations is the physiological relevance of the in vitro phosphorylation assays to events occurring in vivo.

As demonstrated by the present study, compared with strain F-infected cells, cells infected with either UL46 or UL47 single-deletion mutants exhibited a 2-h delay in the appearance of viral TK activity. The delay increased to 4 h when both UL46 and UL47 were deleted from the viral genome. The specificity of this effect to VP11/12 and VP13/14 was confirmed in subsequent assays using the repaired deletion mutants. Since the effect of the UL46 and UL47 deletions on viral TK gene expression occurred during the first 4 h of infection, the delayed TK phenotype cannot be attributed to the de novo synthesis of either VP11/12 or VP13/14, considering their $\beta\gamma$ and γ kinetic classes, respectively (17). Therefore, the most likely explanation for the observed phenotype is that both VP11/12 and VP13/14 act in a manner similar to that described for both α TIF and VHS, that is, within the context of the infecting virion and prior to their de novo synthesis.

Although the precise molecular mechanism(s) responsible for the delayed TK phenotype is not clear at this time, several predictions can be made. During lytic infection, transcriptional induction of TK depends on the prior expression of specific α genes (minimally ICP4), the expression of which, in turn, is dependent on efficient transinduction by α TIF (37). VP11/12 and VP13/14 enhance the efficiency of α TIF-mediated transinduction of α genes, an event most likely mediated through their virion association (28, 51). Thus, if deleting UL46 and/or UL47 reduces the efficiency of α gene induction, delayed TK induction may simply reflect a subsequent delay in the transition from α to β gene expression. Alternatively, VP11/12 and VP13/14 themselves may individually (or in combination) modulate the expression of the viral TK gene. This effect could be either TK gene specific or β gene specific. Notably, the pulse-labeled levels of ICP4 did not appear to be significantly reduced in the deletion mutants (Fig. 4). However, preliminary kinetic analyses (data not shown) suggest that the pulse-labeled ICP4 protein profiles may not reflect alterations in the levels of α gene mRNAs in the deletion mutants. Together, these observations introduce the possibility of a mechanism of action more indirect or complex than a simple delay in α 4 transcription. Further studies to quantify levels and specificities of HSV-1 α and β gene mRNAs and protein levels in the UL46, UL47, and UL46/47 deletion mutants are underway, and they should distinguish among the various possibilities. In any event, since the TK activities in cells infected with any of the deletion mutants reached the same level as in the wild type within 6 to 8 h postinfection, the delay must result from a mechanism operating within the first 4 h of infection. The possibility that the delay was due to differences in infection rates is not consistent with the reported function of the two genes in transient expression assays (28). On the other hand, it does not preclude the possibility that VP11/12 and/or VP13/14 could act as a transporter or chaperonin-like molecule.

Taken together, these and previous observations are consistent with the conclusion that both VP11/12 and VP13/14 are tegument-transported regulatory proteins which have an effect on both HSV-1 α and HSV-1 β gene expression. This effect most likely involves, but may not be limited to, a stoichiometric association with α TIF in the virion tegument.

ACKNOWLEDGMENTS

We thank S. Sridharan for her excellent technical assistance and Richard Courtney and Neal DeLuca for their helpful discussions and critical review of the manuscript.

This work has been supported in part by BRSG 2 S07 RR05451-26, BRSG 2 S07 RR05451-27, and BRSG 2 S07 RR05451-28, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health; in part by a Basil O'Connor Starter Scholar Research Award from the March of Dimes Birth Defects Foundation; and in part by R01 AI26539, awarded by the National Institute of Allergy and Infectious Diseases to J.L.C.M.

REFERENCES

1. **Bairoch, A.** 1991. PROSITE: a dictionary of sites and patterns in proteins. *Nucleic Acids Res.* **19**:2241-2245.
2. **Barker, D. E., and B. Roizman.** 1990. Identification of three genes nonessential for growth in cell culture near the right terminus of the unique sequences of long component of herpes simplex virus 1. *Virology* **177**:684-691.
3. **Batterson, W., and B. Roizman.** 1983. Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. *J. Virol.* **46**:371-377.
4. **Cai, W., and P. Schaffer.** 1992. Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells. *J. Virol.* **66**:2904-2915.
5. **Campbell, M. E. M., J. W. Palfreyman, and C. M. Preston.** 1984. Identification of herpes simplex virus DNA sequences which encode a *trans*-acting polypeptide responsible for stimulation of immediate early transcription. *J. Mol. Biol.* **180**:1-19.
6. **Carpenter, D. E., and V. Misra.** 1991. The most abundant protein in bovine herpes 1 virions is a homologue of herpes simplex virus type 1 UL47. *J. Gen. Virol.* **72**:3077-3084.
7. **Chen, J., and S. Silverstein.** 1992. Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. *J. Virol.* **66**:2916-2927.
8. **Cohen, G. H., M. Ponce de Leon, H. Diffelmann, W. C. Lawrence, S. K. Vernon, and R. J. Eisenberg.** 1980. Structural analysis of the capsid polypeptides of herpes simplex virus types 1 and 2. *J. Virol.* **34**:521-531.
9. **Cunningham, C., A. J. Davison, A. Dolan, M. C. Frame, D. J. McGeoch, D. M. Meredith, H. W. Moss, and A. C. Orr.** 1992. The UL13 virion protein of herpes simplex virus type 1 is phosphorylated by a novel virus-induced protein kinase. *J. Gen. Virol.* **73**:303-311.
10. **Davison, A. J., and J. E. Scott.** 1986. The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* **67**:1759-1816.
11. **DeLuca, N. A., A. M. McCarthy, and P. A. Schaffer.** 1985. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J. Virol.* **56**:558-570.
12. **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. Virol.* **3**:357-364.
13. **Elliott, G. D., and D. M. Meredith.** 1992. The herpes simplex virus type 1 tegument protein VP22 is encoded by gene UL49. *J. Gen. Virol.* **73**:723-726.
14. **Everett, R. D.** 1989. Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate-early gene 1. *J. Gen. Virol.* **70**:1185-1202.
15. **Frame, M. C., D. J. McGeoch, F. J. Rixon, A. C. Orr, and H. S. Marsden.** 1986. The 10K virion phosphoprotein encoded by gene US9 from herpes simplex virus type 1. *Virology* **150**:321-332.
16. **Gibson, W., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. VIII. Characterization and composition of multiple capsid forms of subtypes 1 and 2. *J. Virol.* **10**:1044-1052.
17. **Hall, L. M., K. G. Draper, R. H. Costa, and E. K. Wagner.** 1982. Herpes simplex virus mRNA species mapping in *EcoRI* fragment I. *J. Virol.* **43**:594-607.
18. **Heine, J. W., R. W. Honess, E. Cassai, and B. Roizman.** 1974. Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains. *J. Virol.* **14**:640-651.
19. **Honess, R. W., and B. Roizman.** 1973. Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and nonstructural herpes virus polypeptides in infected cells. *J. Virol.* **12**:1347-1365.
20. **Honess, R. W., and B. Roizman.** 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.* **14**:8-19.
21. **Honess, R. W., and B. Roizman.** 1975. Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional polypeptides. *Proc. Natl. Acad. Sci. USA* **71**:1276-1280.
22. **Hunter, T., and M. Karin.** 1992. The regulation of transcription by phosphorylation. *Cell* **70**:375-387.
23. **Kristie, T. M., and B. Roizman.** 1984. Separation of sequences defining basal expression from those conferring α gene recognition within the regulatory domains of herpes simplex virus 1 α genes. *Proc. Natl. Acad. Sci. USA* **81**:4065-4069.
24. **LeMaster, S., and B. Roizman.** 1980. Herpes simplex virus phosphoproteins. II. Characterization of the virion protein kinase and of the polypeptides phosphorylated in the virion. *J. Virol.* **35**:798-811.
25. **MacLean, C. A., A. Dolan, F. E. Jamieson, and D. J. McGeoch.**

1992. The myristylated virion proteins of herpes simplex virus type 1: investigation of their role in the virus life cycle. *J. Gen. Virol.* **73**:539–547.
26. 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.* **61**:992–1001.
27. McKnight, J. L. C., T. M. Kristie, S. Silver, P. E. Pellett, P. Mavromara-Nazos, G. Campadelli-Fiume, M. Arsenakis, and B. Roizman. 1986. Regulation of herpes simplex virus 1 gene expression: the effect of genomic environments and its implication for model systems. *Cancer Cells* (Cold Spring Harbor) **4**:163–173.
28. McKnight, J. L. C., P. E. Pellet, F. J. Jenkins, and B. Roizman. 1987. Characterization and nucleotide sequence of two herpes simplex virus 1 genes whose products modulate α -*trans*-inducing factor-dependent activation of α genes. *J. Virol.* **61**:992–1001.
29. McLean, G., F. Rixon, N. Langeland, L. Haarr, and H. Marsden. 1990. Identification and characterization of the virion protein products of herpes simplex virus type 1 gene UL47. *J. Gen. Virol.* **71**:2953–2960.
30. Meredith, D. M., J. A. Lindsay, I. W. Halliburton, and G. R. Whittaker. 1991. Posttranslational modification of the tegument proteins (VP13 and VP14) of herpes simplex virus type 1 by glycosylation and phosphorylation. *J. Gen. Virol.* **72**:2771–2775.
31. Oroskar, A. A., and G. S. Read. 1987. A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-early (alpha) mRNAs. *J. Virol.* **61**:604–606.
32. Pellet, P. E., J. L. C. McKnight, F. J. Jenkins, and B. Roizman. 1985. Nucleotide sequence and predicted amino acid sequence of a protein encoded in a small herpes simplex virus DNA fragment capable of *trans* inducing α genes. *Proc. Natl. Acad. Sci. USA* **82**:5870–5874.
33. Pereira, L., E. Cassai, R. W. Honess, B. Roizman, M. Terni, and A. Nahmias. 1976. Variability in the structural polypeptides of herpes simplex virus 1 strains: potential application in molecular epidemiology. *Infect. Immun.* **13**:211–220.
34. Pinard, M.-F., R. Simard, and V. Bibor-Hardy. 1987. DNA-binding proteins of herpes simplex virus type 1-infected BHK cell nuclear matrices. *J. Gen. Virol.* **68**:727–735.
35. Post, L. E., and B. Roizman. 1981. A generalized technique for deletion of specific genes in large genomes: α gene 22 of herpes simplex virus 1 is not essential for growth. *Cell* **25**:227–232.
36. Read, G. S., and N. Frenkel. 1983. Herpes simplex virus mutants defective in the virion-associated shutoff of host polypeptide synthesis and exhibiting abnormal synthesis of α (immediate early) viral polypeptides. *J. Virol.* **46**:498–512.
37. Roizman, B., and A. Sears. 1990. Herpes simplex viruses and their replication, p. 1795–1841. *In* B. N. Fields (ed.), *Virology*. Raven Press, Ltd., New York.
38. Sacks, W. R., and P. A. Schaffer. 1987. Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. *J. Virol.* **61**:829–839.
39. Schek, N., and S. L. Bachenheimer. 1985. Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. *J. Virol.* **55**:601–610.
40. Schrag, J. D., B. V. V. Prasad, F. J. Rixon, and W. Chiu. 1989. Three-dimensional structure of the HSV-1 nucleocapsid. *Cell* **56**:651–660.
41. Smibert, C. A., D. C. Johnson, and J. R. Smiley. 1992. Identification and characterization of the virion-induced host shutoff product of herpes simplex virus gene UL41. *J. Gen. Virol.* **73**:467–470.
42. Spear, P. G., and B. Roizman. 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. *J. Virol.* **9**:143–159.
43. Stevely, W. S., M. Katan, V. Stirling, G. Smith, and D. Leader. 1985. Protein kinase activity associated with the virions of pseudorabies and herpes simplex virus. *J. Gen. Virol.* **66**:661–673.
44. Stow, N. D., and E. C. Stow. 1986. Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *J. Gen. Virol.* **67**:2571–2585.
45. Weinheimer, S. P., B. A. Boyd, S. K. Durham, J. L. Resnick, and D. R. O'Boyle II. 1992. Deletion of the VP16 open reading frame of herpes simplex virus type 1. *J. Virol.* **66**:258–269.
46. Weller, S. K., E. P. Carmichael, D. P. Aschman, D. J. Goldstein, and P. A. Schaffer. 1987. Genetic and phenotypic characterization of mutants in four essential genes that map to the left half of HSV-1 U_L DNA. *Virology* **161**:198–210.
47. Whittaker, G. R., M. P. Riggio, I. W. Halliburton, R. A. Killington, G. P. Allen, and D. M. Meredith. 1991. Antigenic and protein sequence homology between VP13/14, a herpes simplex virus type 1 tegument protein, and gp10, a glycoprotein of equine herpesvirus 1 and 4. *J. Virol.* **65**:2320–2326.
48. Yao, F., and R. J. Courtney. 1989. A major transcriptional regulatory protein (ICP4) of herpes simplex virus type 1 is associated with purified virions. *J. Virol.* **63**:3338–3344.
49. Yao, F., and R. J. Courtney. 1991. Association of a major transcription regulation protein, ICP4, of herpes simplex virus type 1 with the plasma membrane of virus-infected cells. *J. Virol.* **65**:1516–1524.
50. Yao, F., and R. J. Courtney. 1992. Association of ICP0 but not ICP27 with purified virions of herpes simplex virus type 1. *J. Virol.* **66**:2709–2716.
51. Zhang, Y., D. A. Sirko, and J. L. C. McKnight. 1991. Role of herpes simplex virus type 1 UL46 and UL47 in α TIF-mediated transcriptional induction: characterization of three viral deletion mutants. *J. Virol.* **65**:829–841.