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The Herpes Simplex Virus 1 Protein Kinase Encoded by the U_S3 Gene Mediates Posttranslational Modification of the Phosphoprotein Encoded by the U_L34 Gene

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Earlier studies have shown that a herpes simplex virus 1 (HSV-1) open reading frame, U_S3, encodes a novel protein kinase and have characterized the cognate amino acid sequence which is phosphorylated by this enzyme. This report identifies an apparently essential viral phosphoprotein whose posttranslational processing involves the viral protein kinase. Analyses of viral proteins phosphorylated in the course of productive infection revealed a phosphoprotein whose mobility was viral protein kinase and serotype dependent. Thus, the corresponding HSV-1 and HSV-2 phosphoproteins differ in their electrophoretic mobilities, and the phosphoprotein specified by the HSV-1 mutant deleted in U_S3 (R7041) differs from that of the corresponding HSV-1 and HSV-2 proteins. Analyses of HSV-1 × HSV-2 recombinants mapped the phosphoprotein between 0.42 and 0.47 map units on the prototype HSV-1 DNA map. Within this region, the U_L34 open reading frame was predicted to encode a protein of appropriate molecular weight which would also contain the consensus target site for phosphorylation by the viral protein kinase as previously defined with synthetic peptides. Replacement of the native U_L34 gene with a U_L34 gene tagged with a 17-amino-acid epitope from the α4 protein identified this gene as encoding the phosphoprotein. Finally, mutagenesis of the predicted phosphorylation site on U_L34 in the viral genome, and specifically the substitution of threonine or serine with alanine in the product of the U_L34 gene, yielded phosphoproteins whose electrophoretic mobilities could not be differentiated from that of the U_S3^- mutant. We conclude that the posttranslational processing of the U_L34 gene product to its wild-type phenotype requires the participation of the viral protein kinase. While the viral protein kinase is not essential for viral replication in cells in culture, the U_L34 gene product itself may not be dispensable.

The viruses comprising the subfamily *Alphaherpesvirinae* of the *Herpesviridae* family include herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus, and swine herpesvirus 1 (pseudorabies virus [PRV]). Among the genes conserved by members of this virus subfamily is one specifying a protein kinase (PK) (10, 21, 22). The PKs of both HSV-1 and PRV have been purified and assayed by using protamine as a suitable artificial substrate (9, 29, 30). The PRV PK is a dimer consisting of two 38,000-molecular-weight subunits, whereas the HSV-1 enzyme is a dimer consisting of two 68,000-molecular-weight subunits (15, 29, 39). The purified PK requires no effectors, and it transfers phosphate from ATP, but not GTP, to the seryl or threonyl residues of basic, but not acidic, synthetic peptides (13). The studies on synthetic oligopeptides have suggested that the phosphorylation site recognized by the viral PK has the consensus sequence $(R)_nX(S/T)YY$, where $n \geq 3$, R = Arg, X prefers Arg, Ala, Val, Pro, or Ser, Y shares the same preference except that acidic residues or proline is unacceptable, and (S/T) is the target residue, being either Ser or Thr (14, 28). In addition, the kinase is capable of autophosphorylation in vitro, although its autophosphorylation in the cell has not been observed (9, 29).

On the basis of the nucleic acid sequence of HSV-1, McGeoch and Davison (21) suggested that the open reading frame designated as U_S3 may encode a PK. It was subsequently shown that (i) deletion of the U_S3 open reading frame in the recombinant virus R7041 resulted in the loss of the novel PK activity from lysates of infected cells whereas restoration of the deleted sequences restored the activity (31) and (ii) antibody raised against a synthetic eight-amino-acid C-terminal U_S3 oligopeptide conjugated to bovine serum albumin reacted with purified preparations of the HSV-1 PK (9).

Interest in the HSV PK activity stems from the observation that while retroviruses are capable of transducing host PKs which may act as oncogenes (3, 6, 12, 34), virus-specific PKs are rare. In addition to the PK conserved among members of the subfamily *Alphaherpesvirinae*, PK activity has been ascribed to the transcriptional transactivator *hbx* of the *Hepadnaviridae* family (18, 35, 38). Comparisons of the sequences of HSV open reading frame U_L13, the Epstein-Barr virus open reading frame BGLF4, and the varicella-zoster virus open reading frame 47 with those of known PKs suggest that these genes may also encode novel PKs (36). The function of the HSV-1 PK encoded by U_S3 is not known. To determine its function, it is necessary to identify its natural substrates in the infected cell. To this end, we have compared the ^{32}P-labeled phosphoprotein profile of electrophoretically separated polypeptides from cells infected with the recombinant PK^- U_S3^- virus R7041 with those from cells infected either with its parent virus, HSV-1(F), or with a virus in which the deleted U_S3 sequences of R7041 have been restored. These studies led to the observation that in cells infected with the PK^- mutant, a slower-migrating phosphoprotein replaced a wild-type phosphoprotein. To determine whether the two phosphoproteins were genetically related, we first mapped the approximate location of the gene specifying the wild-type phosphoprotein to

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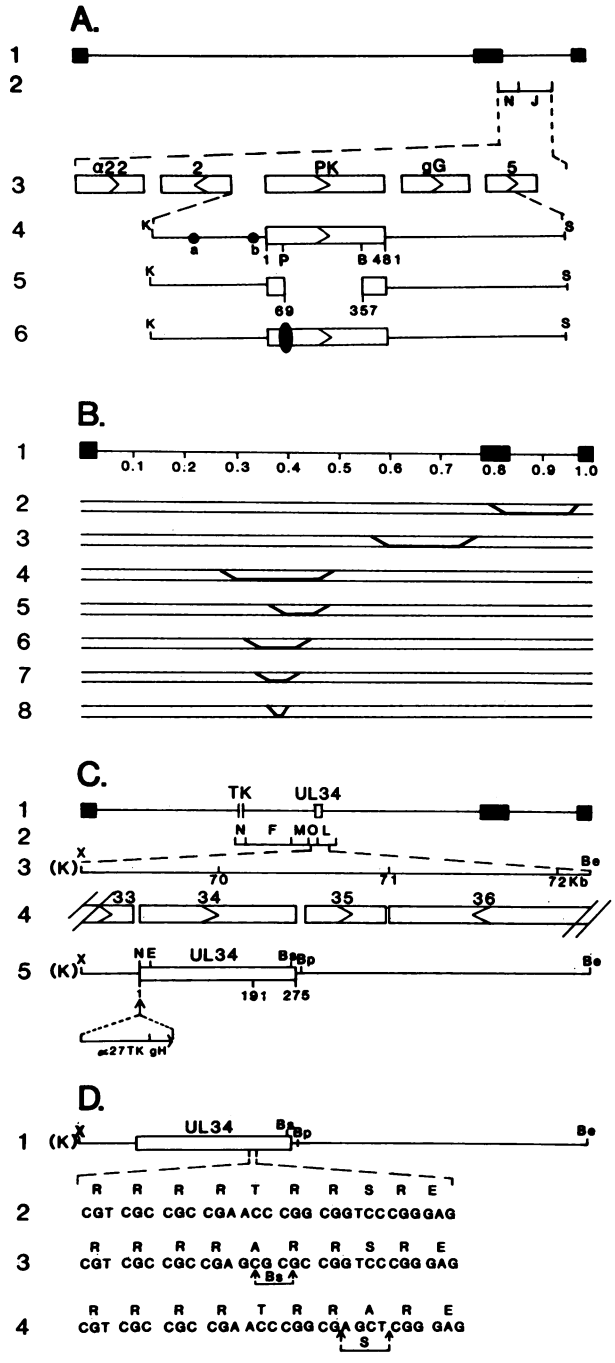

FIG. 1. Schematic representation of the DNA sequence arrangements in the genomes of HSV-1(F) and the various recombinant viruses. (A) Line 1, the sequence arrangement of the HSV-1 genome. The filled rectangles represent the inverted repeats flanking the unique sequences (represented by thin lines) of the long and short components. Line 2, the relevant HSV-1 *Bam*HI fragments which contain the open reading frames for α22, U_S2, glycoprotein G, the PK, and U_S5 as identified in line 3. Line 4, the *Kpn*I-*Sac*I DNA fragment containing the 481-amino-acid open reading frame U_S3 encoding the PK. Two alterative transcription initiation sites for the U_S3 mRNA are indicated by the circles labeled a and b (33). Line 5, the site of the deletion in R7041 spanning from the *Pst*I site located at amino acid 69 to the *Bam*HI site located at amino acid 357 of the PK. Line 6, the DNA arrangement of R7307, a recombinant virus in which a human CMV epitope (indicated by the solid oval) is inserted into the unique *Pst*I site located at amino acid 69 of the protein between 0.42 and 0.47 map units on the viral genome by using a set of characterized HSV-1 × HSV-2 intertypic recombinants. Within this region, the U_L34 gene was predicted to encode a protein of the approximate molecular weight of the mapped phosphoprotein. In addition, the product of this gene was predicted to contain an amino acid sequence identical to that of the consensus phosphorylation site of the viral PK determined earlier (14, 20, 28). Replacement within the viral genome of the native U_L34 gene with a U_L34 gene tagged at its amino terminus with a 17-amino-acid epitope from the α4 protein led to the unambiguous demonstration that the U_L34 gene encodes this phosphoprotein. Furthermore, mutations in the viral genome that led to substitution of either threonine or serine with alanine within the consensus phosphorylation sequence on the U_L34 gene product yielded phosphoprotein profiles which could not be differentiated from those of the PK^- U_S3^- virus. These results indicate that the posttranslational processing of the U_L34 gene product to its wild-type phenotype requires the participation of the viral PK.

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 (8). HSV-1(F)Δ305 and R7041 are genetically engineered deletion mutants (25). HSV-1(F)Δ305 lacks the 500-bp *Bgl*II-*Sac*I fragment in the thymidine kinase (*tk*) gene. R7041 lacks 860 bp of the coding sequences of the U_S3 open reading frame defined by the restriction endonuclease *Pst*I-*Bam*HI; the deleted sequences encode the predicted amino acids 69 to 357 of U_S3. The virus was produced by rescue of the *tk* gene of the recombinant R7040 (17, 23) in which deletions were introduced in both the *tk* and PK genes. Recombinants

kinase. (B) DNA sequence arrangements of HSV-1 × HSV-2 intertypic recombinants. Line 1, the genome arrangement of HSV divided into viral map units. Lines 2 to 8 indicate the DNA arrangements of the intertypic recombinants R7015, RS1G25, RHIG7, RHIG8, RHIG13, RHIG44, and RHIG48, respectively. The boldfaced line segments identify HSV-2 sequences present in these genomes, with the approximate crossover points falling within the indicated diagonal regions. (C) Arrangement of genes between 0.42 and 0.47 map units and position of the U_L34 gene on the viral genome. Line 1, the DNA sequence arrangement of HSV-1(F)Δ305, containing a 500-bp deletion in the *tk* gene. The position of the U_L34 gene is indicated by the open box. Line 2, relevant HSV-1 *Eco*RI fragments. Line 3, the *Xba*I-*Bst*EII fragment containing the U_L34 open reading frame used in construction of the recombinant viruses. Line 4, the open reading frames encoded within this fragment. Line 5, the DNA arrangement of recombinant virus R7309, which harbors an α27-*tk* gene inserted at the *Nco*I site located at the initiating methionine codon of the U_L34 gene such that the U_L34 gene is expressed and regulated by the gH promoter sequences. (D) Sequence arrangement at the consensus phosphorylation site of the U_L34 protein. Line 1, wild-type DNA sequence arrangement. Line 2, DNA and amino acid sequences between amino acids 191 and 200 of the wild-type U_L34 protein. Line 3, DNA arrangement of recombinant virus R7310, in which the threonine residue has been mutated to an alanine residue coincident with the introduction of a *Bss*HII site. Line 4, DNA arrangement of recombinant virus R7311, in which the serine residue has been mutated to an alanine, coincident with the introduction of a *Sac*I site. N, *Nco*I; K, *Kpn*I; P, *Pst*I; B, *Bam*HI; E, *Eco*RI; X, *Xba*I; Be, *Bst*EII; Bs, *Bss*HII; Bp, *Bsp*EI. Restriction sites shown in parentheses are derived from polylinker sequences.

TABLE 1. Genotypes of genetically engineered viruses[a]

Virus	Genotype
HSV-1(F)	PK^+ U_L34^+
R7041	PK^- U_L34^+
R7306	PK^+ U_L34^+
R7307	$PK(CMVtag)^+$ U_L34^+
R7310	PK^+ U_L34 Ala-195
R7311	PK^+ U_L34 Ala-198
R7314	PK^+ $U_L34\alpha4tag$

[a] Derivation of the viruses is described in Materials and Methods.

R7015, RH1G7, RH1G8, RH1G13, RH1G44, and RH1G48 were previously described (1, 4). Recombinant RS1G25 was derived by Conley and Roizman (5) by marker rescue of a mutant mapping at the terminus of the L component. The crossover maps of all HSV-1 × HSV-2 recombinants are shown in Fig. 1. All virus stocks were titered on Vero cells.

Construction of recombinant viruses. The recombinant viruses constructed for this study and their genotypes are shown in Table 1. In recombinant virus R7306, the sequences deleted in R7041 were restored by cotransfection of rabbit skin cells with intact R7041 DNA and plasmid pRB3446, which carries the HSV-1 4.89-kb *Sac*I fragment containing the entire U_S3 gene with flanking sequences. The progeny of this transfection was plated on Vero cells, and individual plaques were screened for the restoration of the *Bam*HI N fragment. In recombinant virus R7307, the R7041 deletion was repaired with a U_S3 gene (pRB4274) containing a 20-amino-acid epitope of human cytomegalovirus (CMV) (16) inserted in frame at nucleotide 69 of the coding sequence of the U_S3 gene.

To construct pRB4274, the 4.89-kb *Sac*I fragment from pRB3446 was cloned into the *Sac*I site of pGEM7Zf$^+$ to generate pRB4269. pRB4269 DNA was cleaved with *Kpn*I and religated to yield pRB4173; the *Kpn*I collapse deleted the sequences from the *Kpn*I site in the polylinker to the site located at position −435 relative to the initiation codon of the U_S3 gene and ensured that the *Pst*I site located at position +207 of the U_S3 coding sequence would provide a unique site into which a 70-bp oligonucleotide encoding a 20-amino-acid CMV epitope contained within a 10-base linker sequence could be inserted in-frame with the U_S3 coding sequences, thus generating pRB4274. DNAs extracted from plaque isolates of the recombinant viruses were screened for both the restoration of the *Bam*HI N fragment and the presence of the CMV epitope by Southern blotting (37) with the CMV oligonucleotide (Fig. 2B and C).

To construct recombinant viruses with mutations or deletions in the U_L34 gene, the α27-*tk* gene described elsewhere (17) was inserted between the U_L33 and U_L34 open reading frames to generate R7309. This recombinant was constructed by cotransfection of rabbit skin cells with intact HSV-1(F)Δ305 DNA and pRB4165, which contains an α27-*tk* gene inserted between the U_L33 and U_L34 genes. TK^+ progeny virus from this transfection were selected on $143tk^-$ cells under hypoxanthine-aminopterine-thymidine and further plaque purified on Vero cells as described previously (25). pRB4165 was constructed by inserting a chimeric α27-*tk* gene into the *Nco*I site located at the ATG of U_L34 in plasmid pRB4247 so that the initiation codon of the gene was restored and the U_L34 gene was driven by the gH promoter sequences located within the *tk* coding sequences (20). pRB4247 consists of the 3,066-bp *Xba*I-*Bst*EII fragment subcloned from the *Eco*RV E fragment cloned into pGEM3Z. Recombinant viruses were screened by detecting

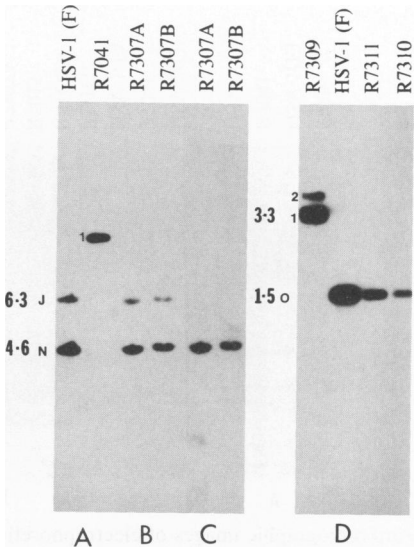

FIG. 2. Autoradiographic images of *Bam*HI or *Eco*RI digests of HSV-1 wild-type and recombinant mutant viral DNAs. Viral DNA digests were electrophoretically separated on 0.8% agarose gels, transferred to nitrocellulose membranes, and hybridized with specific radiolabeled probes. The viral DNA in panels A and B was digested with *Bam*HI and probed with radiolabeled pRB3446, which contains the viral PK gene. *Bam*HI N and J bands are indicated. The band designated 1 resulted from the deletion of U_S3 gene sequences (Fig. 1A, line 5), which caused the fusion of *Bam*HI-J with the remainder of *Bam*HI-N. The viral DNA shown in panel C was digested with *Bam*HI and probed with radiolabeled oligonucleotide specifying the human CMV epitope. The viral DNA shown in panel D was digested with *Eco*RI and probed with a radiolabeled HSV-1(F) *Eco*RI O fragment (identified by the letter O). The band designated 1 in the R7309 digests was generated by the insertion of a chimeric α27-*tk* gene into the *Eco*RI O fragment. A band designated 2 was generated as a result of the inversion of the DNA fragment spanning the native and inserted *tk* sequences. The genomes containing the inversions were present in relatively small amounts. The introduction of mutant U_L34 genes in viruses R7310 and R7311 restored the 1.5-kb *Eco*RI O fragment and eliminated the 3.3-kb fragment present in R7309.

the increase in size of the *Eco*RI O band from 1.5 to 3.3 kb resulting from the insertion of the α27-*tk* gene (Fig. 2D).

Recombinant virus R7314 contains a U_L34 gene which is tagged at its amino terminus with a 17-amino-acid epitope from the HSV-1 α4 protein in place of the native U_L34 gene. R7314 was constructed by cotransfection of rabbit skin cells with intact R7309 viral DNA and pRB4277, which consists of a 49-bp oligonucleotide encoding the α4 epitope cloned into the *Nco*I site located at the initiating methionine codon of the U_L34 gene in pRB4247. TK^- progeny virus from this transfection were selected on $143tk^-$ cells in the presence of bromodeoxyuridine. Recombinant viruses were plaque purified on Vero cells and screened by detecting the restoration of the *Eco*RI O band by the procedure of Southern (37).

Recombinant viruses R7310 and R7311 each contain a U_L34 gene in which the coding sequence has been altered to incorporate a specific single amino acid change (Fig. 1D). In recombinant virus R7310, the threonine residue at amino acid 195 has been changed to an alanine, whereas in recombinant virus R7311, the serine residue at amino acid 198 has been changed to an alanine. These viruses were constructed as follows. A 1,249-bp fragment of HSV-1(F) DNA containing U_L34 spanning from the *Xba*I site to the *Bsp*EI site

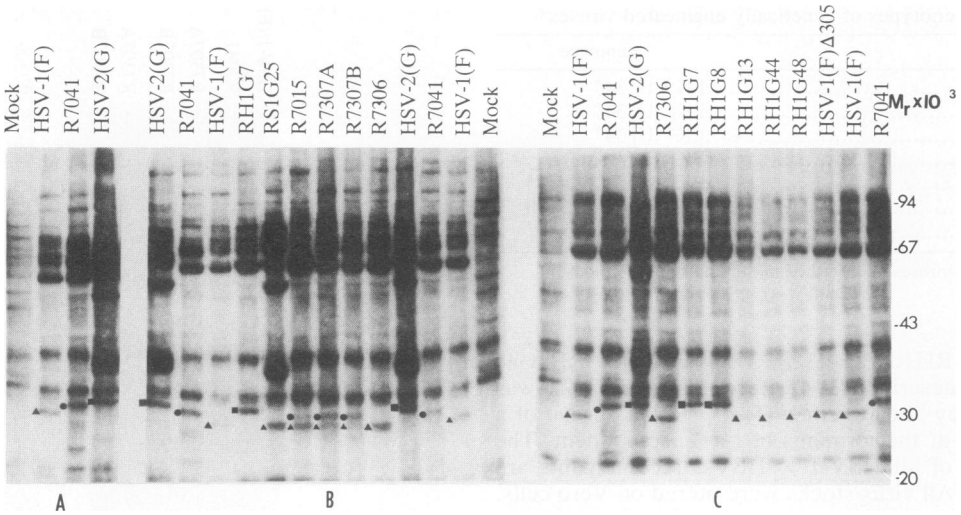

FIG. 3. Autoradiographic images of electrophoretically separated ^{32}P-labeled infected cell lysates from cells infected with wild-type and recombinant HSV-1. All of the cell lysates in each panel were electrophoretically separated concurrently. In panel A, the triangle identifies the wild-type HSV-1 U_L34 protein, the square identifies the HSV-2 U_L34 protein, and the circle represents the U_L34 phosphoprotein in lysates of cells infected with the R7041 ($PK^-U_S3^-$) virus. In other panels, the circle also identifies the U_L34 phosphoproteins which comigrate with the corresponding phosphoprotein from R7041-infected cells (e.g., the U_L34 phosphoprotein present in lysates of cells infected with the R7015 virus).

within *Eco*RV E fragment was excised as a *Kpn*I-*Bsp*EI fragment from pRB4247, blunt ended with T4 polymerase, and subcloned into the *Sma*I site of pGEM3Zf$^+$, generating pRB4164. With this construct, site-directed mutagenesis using synthetic oligonucleotides was performed to generate pRB4233, in which the threonine residue was mutated to an alanine residue coincident with the generation of a unique *Bss*HII restriction site. A similar procedure was used to generate pRB4234, in which the serine residue was mutated to an alanine residue coincident with the generation of a unique *Sac*I restriction site. Each of these mutated U_L34 genes was excised as a 682-bp *Nco*I-*Hin*cII fragment and subcloned back into pRB4247 in order to restore flanking sequences necessary for recombination into the viral genome, generating pRB4236, used in the construction of R7310, the U_L34 threonine mutant virus, and pRB4237, used in the construction of R7311, the U_L34 serine mutant virus. pRB4236 and pRB4237 were each cotransfected on rabbit skin cells with intact R7309 viral DNA. TK^- progeny virus were selected on 143*tk*$^-$ cells as previously described (26). Individual plaques were purified on Vero cells, and viral DNA was screened for the presence of the introduced mutations by amplifying a 600-bp fragment containing a portion of the U_L34 gene by the polymerase chain reaction and checking this amplified fragment for the presence of the genetically engineered restriction sites.

Preparation of ^{32}P-labeled infected cell lysates. BHK-C13 cells grown in 25-cm^2 flasks were infected with 10 PFU of the indicated virus per cell in medium 199V. After 13 h, the cells were preincubated in Eagle's minimal essential medium made without phosphate and supplemented with 1% dialyzed phosphate-free calf serum for 1 h. Cells were labeled with 100 to 200 μCi of $^{32}P_i$ (carrier free; New England Nuclear) in a final volume of 3 ml for 4 h. Because of the difficulty of obtaining revertant-free high-titer stocks of viruses R7310 and R7311, cells were infected with single-plaque isolates of these viruses and after 36 h were labeled with $^{32}P_i$ as described above. Cells were rinsed once and scraped into 1 ml of ice-cold PBS-A, subjected to centrifugation in a microcentrifuge for 5 min at 4°C, and resuspended in 350 μl of PBS-A containing 0.1 mM tosylsulfonyl phenylalanyl chloromethyl ketone, 0.1 mM tosylsulfonyl lysyl chloromethyl ketone, 1.0% (vol/vol) Nonidet 40, and 1.0% (wt/vol) sodium deoxycholate. Lysates were sonicated and frozen in aliquots at −70°C.

Polyacrylamide gel electrophoresis. Infected cell lysates containing approximately 50 μg of protein were electrophoretically separated in polyacrylamide (10%) gels containing 0.1% sodium dodecyl sulfate. The separated polypeptides were electrically transferred to nitrocellulose, stained with amido black, and subjected to autoradiography on Kodak X-Omat film for 12 h.

RESULTS

The phosphoprotein profile of R7041 differs from that of HSV-1(F). To identify protein substrates of the viral PK in infected cells, replicate cultures of BHK-C13 cells were infected with either HSV-1(F) or R7041. At 13 h postinfection, the cells were incubated in medium containing $^{32}P_i$ for 4 h. The cells were then harvested, solubilized, and electrophoretically separated in denaturing polyacrylamide gels as described in Materials and Methods. Comparison of the ^{32}P-labeled phosphoprotein profiles of HSV-1(F) and R7041 (Fig. 3A) showed that a novel phosphoprotein of approximately 33,000 in apparent molecular weight replaced a wild-type HSV-1(F) labeled phosphoprotein of approximately 30,000 in apparent molecular weight in the R7041-infected cell lysates.

To determine whether the observed differences in the phosphoprotein profiles of the HSV-1(F) and the R7041-infected cell lysates were due to the deletion of the U_S3 gene product in R7041 and not to some other unidentified mutation, two additional recombinant viruses, R7306 and R7307, were constructed as described in Materials and Methods. In recombinant R7306, the deleted sequences of U_S3 in R7041 were marker rescued with the authentic gene. In mutant R7307, the deleted sequences were repaired with a U_S3 gene containing an additional 20 amino acids inserted in frame at amino acid 69 and expressing a human CMV epitope.

Comparison of the ^{32}P-labeled phosphoprotein profiles of lysates from replicate cell cultures infected with R7306 and HSV-1(F), respectively (Fig. 3B), indicate that the two are indistinguishable and therefore the differences in the phosphoprotein profiles of HSV-1(F)- and R7041-infected cells reflect the deletion of the U_S3 gene in R7041.

The phosphoprotein profiles of lysates of replicate cell cultures infected with HSV-1(F), R7041, R7306, or two independent isolates of R7307 (R7307A and R7307B, respectively) (Fig. 3B) show that (i) in the infected cell lysates of R7306, the phosphoprotein profile was that of the wild-type HSV-1(F) and (ii) the phosphoprotein profile of the lysates of cells infected with R7307 contained both the 33,000-apparent-molecular-weight phosphoprotein characteristic of the R7041 deletion virus and the 30,000-apparent-molecular-weight phosphoprotein characteristic of the wild-type HSV-1(F)-infected cells. We conclude from these experiments that (i) the 30,000- and the 33,000-apparent-molecular-weight phosphoproteins are structurally related inasmuch as repair of the PK gene resulted in the replacement of the 33,000-apparent-molecular-weight phosphoprotein with the faster-migrating wild-type species and (ii) the two proteins may be related in that both phosphoproteins are present in cell lysates infected with R7307 virus. Thus, the 33,000-apparent-molecular-weight species may be either a precursor of the 30,000-molecular-weight protein or a by-product of the phosphorylation of a common precursor by an infected-cell PK. In the case of the R7307 virus, insertion of a sequence encoding the 20-amino-acid epitope into the U_S3 gene may have generated an impaired PK incapable of rapid processing of the 33,000-apparent-molecular-weight phosphoprotein into the wild-type phosphoprotein. It is noteworthy that the HSV-1 × HSV-2 intertypic recombinant virus R7015 yielded a similar pattern. This virus consists of the HSV-1(F) unique long region, which encodes the U_L34 gene, and the HSV-2(G) unique short region, which encodes the U_S3 gene. As seen in Fig. 3B, the phosphoprotein profiles of cells infected with R7015 exhibit both the 30,000- and the 33,000-molecular-weight forms of the U_L34 protein. We conclude either that in the recombinant the PK gene is defective or that the PK and U_L34 genes may have coevolved within serotypes to maximally interact with each other and therefore the recombinant PK is less efficient in phosphorylating its HSV-1 substrate. Differentiation between these hypotheses must await further study of the HSV-2 PK of the R7015 virus.

HSV-1 × HSV-2 intertypic recombinants map the gene specifying the 30,000-apparent-molecular-weight wild-type phosphoprotein to a region of the genome located between 0.42 and 0.47 map units. Earlier studies have shown that HSV-1 and HSV-2 fortuitously differ with respect to the electrophoretic mobilities of many of their proteins in denaturing gels (24). To determine whether the HSV-1 30,000-apparent-molecular-weight phosphoprotein differs from that of its HSV-2(G) counterpart, we compared the electrophoretic profiles of HSV-1(F)- and of HSV-2(G)-infected-cell polypeptides harvested 17 h postinfection. As illustrated in Fig. 3A, the corresponding HSV-2(G) phosphoprotein possessed an electrophoretic mobility distinctly lower than that of either the 30,000- or 33,000-apparent-molecular-weight HSV-1 phosphoprotein. The difference in the mobility of HSV-1 and HSV-2 phosphoproteins was in accord with our expectation that these phosphoproteins were specified by the virus and suggested that the gene specifying them might be mapped by analysis of HSV-1 × HSV-2 recombinants.

The analyses of HSV-1 × HSV-2 recombinants were done in two stages. The location of the gene specifying the 30,000-apparent-molecular-weight phosphoprotein was first approximated by comparing ^{32}P-labeled phosphoprotein profiles of lysates from cells infected with those of the three intertypic recombinants, RH1G7, RS1G25, and R7015 (Fig. 3B). Of these, the lower-mobility HSV-2 form of the phosphoprotein was observed only in lysates of cells infected with RH1G7. On the basis of this result, the location of the phosphoprotein was further mapped by using four more intertypic recombinants, i.e., RH1G8, RH1G13, RH1G44, and RH1G48. As shown in Fig. 3C, the phosphoprotein with the characteristic slower mobility of HSV-2(G) was present in lysates of cells infected with RH1G8. These results, interpreted with respect to the crossover points illustrated in Fig. 1B, indicate that the genes specifying the 30,000-apparent-molecular-weight HSV-1(F) phosphoprotein and its HSV-2(G) counterpart map between 0.42 and 0.47 map units on the viral genome.

The U_L34 open reading frame encodes the phosphoprotein which displays altered electrophoretic mobility in the presence of the viral PK. The region between 0.42 and 0.47 map units includes part of the open reading frame for U_L30, the viral DNA polymerase, and the entire open reading frames for genes U_L31 to U_L35. Of these genes, only U_L31 and U_L34 encode polypeptides with predicted molecular weights in the range of that of the mapped phosphoprotein. Inspection of the predicted amino acid sequence of the U_L34 gene product revealed a potential viral PK target site (Arg-Arg-Arg-Arg-Thr-Arg-Arg-Ser-Arg-Glu) located between amino acids 191 and 200. Within this sequence, both the serine and the threonine were potential target residues of the viral PK. To determine whether the U_L34 gene encoded the phosphoprotein, we constructed the recombinant virus R7314, in which the native U_L34 gene was replaced with a U_L34 gene tagged at its amino terminus with a 17-amino-acid epitope from the HSV-1 α4 protein as diagrammed in Fig. 4A. As illustrated in Fig. 4B, the phosphoprotein present in the two independently derived R7314-infected cell lysates possessed a mobility distinctly lower than that of either the 30,000- or 33,000-apparent-molecular-weight HSV-1 phosphoprotein found in either HSV-1(F)- or R7041-infected cell lysates. In addition, this lower-mobility phosphoprotein reacted with monoclonal antibody H943, directed against the 17-amino-acid epitope present on the tagged U_L34 gene product (11). These results positively identified the phosphoprotein as being encoded by the U_L34 gene. It is important to note that the decrease in electrophoretic mobility of the tagged protein expressed by R7314 was greater than that predicted by the insertion of the 17-amino-acid epitope into the 30,000-apparent-molecular-weight phosphoprotein specified by HSV-1(F) and exactly that predicted by the insertion of the epitope into the 33,000-apparent-molecular-weight phosphoprotein specified by the U_S3^- recombinant R7041. These results suggest that the presence of the amino-terminal α4 epitope tag on the U_L34 protein may prevent it from converting to the higher-mobility species seen in the wild-type virus in the presence of the PK.

Site-specific mutagenesis of the U_L34 open reading frame indicates that the U_S3 PK is involved in posttranslational processing of the U_L34 gene product. To determine whether single amino acid changes within the predicted consensus target site of the viral PK could affect the migration of the U_L34 phosphoprotein, we constructed recombinant viruses R7310 and R7311, each of which contained site-specific mutations in the U_L34 gene within this region. In recombinant virus R7310, the threonine residue of the target site was changed to an alanine residue, whereas in R7311, the serine residue was changed to an alanine residue. The ^{32}P-labeled phosphoprotein profiles of the lysates of cells infected with

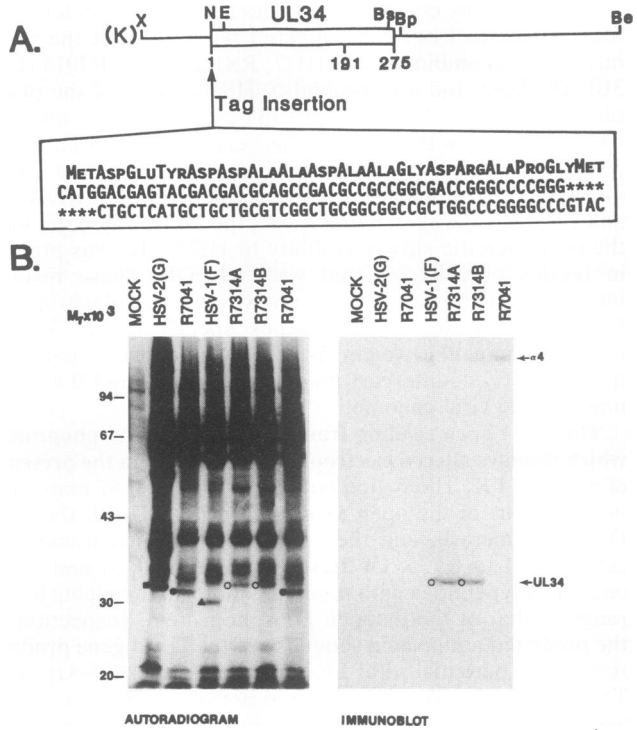

FIG. 4. Autoradiographic and photographic images of electrophoretically separated phosphoproteins for identification of the mapped phosphoprotein as the product of the U_L34 gene. (A) DNA sequence arrangement of the U_L34 gene present in recombinant virus R7314, in which a 17-amino-acid epitope from the HSV-1 α4 protein was inserted at the amino terminus of the U_L34 gene. Be, *Bst*EII; Bp, *Bsp*EI; Bs, *Bss*HII; E, *Eco*RI; K, *Kpn*I; N, *Nco*I; X, *Xba*I. Restriction sites shown in parentheses are derived from polylinker sequences. (B) ^{32}P-labeled phosphoprotein profile and corresponding immunoblot of lysates of cells infected with the indicated viruses, electrophoretically separated in denaturing gels, and transferred to nitrocellulose. The nitrocellulose was reacted with monoclonal antibody H943 to the 17-amino-acid α4 epitope. The autoradiogram illustrates the three electrophoretically distinct phosphoprotein species present in HSV-2(G)- R7041-, and HSV-1(F)-infected cell lysates (indicated by squares, filled circles, and triangles, respectively), as well as the the more slowly migrating phosphoprotein present in R7314 infected cell lysates (indicated by open circles). This phosphoprotein reacts with the α4 monoclonal antibody. Note that at 17 h postinfection, the amount of U_L34 protein exceeds the amount of α4 protein present in the lysates of the infected cells.

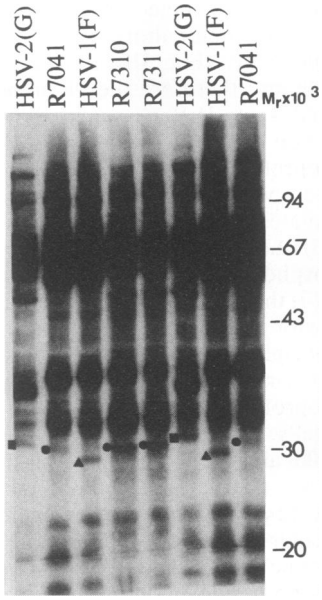

FIG. 5. Autoradiographic images of electrophoretically separated ^{32}P-labeled infected cell lysates from cells infected with recombinant viruses R7310 and R7311, which harbor specific mutations within the putative target site for the viral PK on the U_L34 protein. Labeled lysates were prepared from cells infected with single-plaque isolates of each virus. The squares, circles, and triangles represent the HSV-2(G), R7041, and HSV-1(F) forms of the U_L34 gene product, respectively.

either of these viruses are displayed in Fig. 5. In each case, the 30,000-apparent-molecular-weight wild-type phosphoprotein was replaced by a phosphoprotein whose electrophoretic mobility cannot be differentiated from that of the 33,000-apparent-molecular-weight phosphoprotein present in lysates of cells infected with the PK^- U_S3^- R7041 recombinant. The observations that the mutations confer upon the product of the U_L34 gene in a PK^+ virus the phenotype of the PK^- U_S3^- virus indicate that the viral PK is required for the posttranslational processing of the U_L34 gene product.

DISCUSSION

The studies described in this report show the following. (i) In cells infected with the PK^- virus, R7041, a wild-type phosphoprotein with an apparent molecular weight of 30,000 is replaced by a phosphoprotein with an apparent molecular weight of 33,000. (ii) The 30,000-molecular-weight phosphoprotein maps to the open reading frame U_L34. (iii) The protein specified by U_L34 is predicted to contain an amino acid sequence which corresponds to the idealized substrate of the viral PK determined previously. (iv) Direct evidence of the association of the 33,000-apparent-molecular-weight phosphoprotein with the U_L34 gene product and of the involvement of the viral PK with the replacement of the 33,000-apparent-molecular-weight phosphoprotein with the 30,000-molecular-weight phosphoprotein is based on the experimental evidence that mutagenesis of the threonine or serine within the sequence corresponding to the idealized substrate of the viral PK results in the expression of the 33,000-apparent-molecular-weight phosphoprotein in place of the faster-migrating wild-type phosphoprotein. These data indicate that the posttranslational processing of the U_L34 gene product involves the function of the viral PK.

The results presented in this report raise four significant issues: (i) the relationship between the two products of the U_L34 open reading frame, i.e., the 33,000- and the 30,000-molecular-weight phosphoproteins, (ii) the localization and function of the product of the U_L34 gene, (iii) the role of the U_L34 gene product in the reproductive cycle, and (iv) the role of the U_S3 gene encoding the PK in the reproductive cycle of the virus.

Relationship between the 33,000- and 30,000-apparent-molecular-weight phosphoprotein products of the U_L34 gene. Several observations reported in this paper are significant. (i) The product of the U_L34 open reading frame is phosphorylated in the absence of the viral PK encoded by the U_S3 open reading frame. This observation implies that the product of the U_L34 open reading frame is, or can be, phosphorylated by another PK present in the infected cell. (ii) If the viral PK

phosphorylates the U_L34 phosphoprotein at the viral PK phosphorylation consensus site, then the observation that replacement of either the serine or threonine in the predicted phosphorylation site led to accumulation of the 33,000-apparent-molecular-weight phosphoprotein rather than of the wild-type 30,000-molecular-weight phosphoprotein indicates that the sites of phosphorylation of the 33,000- and the 30,000-apparent-molecular-weight phosphoproteins are not identical. (iii) Because both the 33,000- and the 30,000-apparent-molecular-weight proteins are phosphorylated, it is not known whether the 33,000-molecular-weight phosphoprotein is a precursor or a by-product of the wild-type 30,000-apparent-molecular-weight phosphoprotein encoded by U_L34. Thus, the U_L34 translation product could be phosphorylated by a cellular PK to yield the 33,000-apparent-molecular-weight protein, which in turn serves as a substrate for the viral PK, the product of the U_S3 open reading frame. Alternatively, the translation product of the U_L34 gene is phosphorylated by either the viral or cellular PK, but not at identical sites. Either phosphorylation enables the function of the U_L34 gene product.

In either case, the increase in electrophoretic mobility of wild-type U_L34 protein may reflect either a change in the charge and shape of the phosphoprotein or a proteolytic cleavage event induced by the viral PK. The latter hypothesis is formally supported by the predicted structure of the protein described below, although definitive evidence is still lacking.

Localization and function of the U_L34 gene product during the replicative cycle of the virus. The U_L34 gene product has been reported to be a virion component, but the data upon which this is based have not been reported or cited (19, 20). Analysis of its predicted amino acid sequence indicates that the N-terminal domain of the U_L34 polypeptide has properties similar to those of signal sequences, lending support to the hypotheses that the protein is targeted to membranes and that under certain circumstances this sequence might be cleaved (27). Hydrophobicity analyses (Fig. 6) predict that the protein contains an extremely hydrophilic domain which coincides precisely with the amino acid sequence corresponding to the idealized substrate of phosphorylation by the viral PK. In addition, the U_L34 polypeptide is predicted to have a remarkably hydrophobic C terminus, which is highly conserved (2, 7). This hydrophobic sequence could conceivably anchor the U_L34 protein in membranes. The predicted site of phosphorylation, surprisingly, is not as well conserved and may not be present in either VZV or EBV.

Role of the product of the U_L34 open reading frame in the viral reproductive cycle. Whereas the viral PK is dispensable, numerous attempts to delete the U_L34 gene have not been successful (32). Additional support of the hypothesis that the U_L34 gene is not dispensable rests on the observation that the recombinants with the single amino acid substitutions, i.e., R7310 and R7311, are impaired in their growth properties. These mutants grow so slowly that revertants capable of normal growth rates frequently arise, which quickly outgrow the mutant viruses. The revertants exhibit U_L34 gene products of wild-type phenotype, i.e., characterized by a faster electrophoretic mobility corresponding to 30,000 in apparent molecular weight (data not shown).

The necessary conclusions are that (i) U_L34 plays a significant, probably essential role in viral replication, (ii) while the posttranslational modification of U_L34 mediated by the U_S3 PK is not essential for viral replication in cells in culture, mutations in the PK phosphorylation consensus sequence may interfere with viral replication, and (iii) if the phosphorylation of the U_L34 gene product is essential for its function, then either the 33,000- or the 30,000-apparent-molecular-weight phosphoprotein is essential for viral replication, at least in cells in culture.

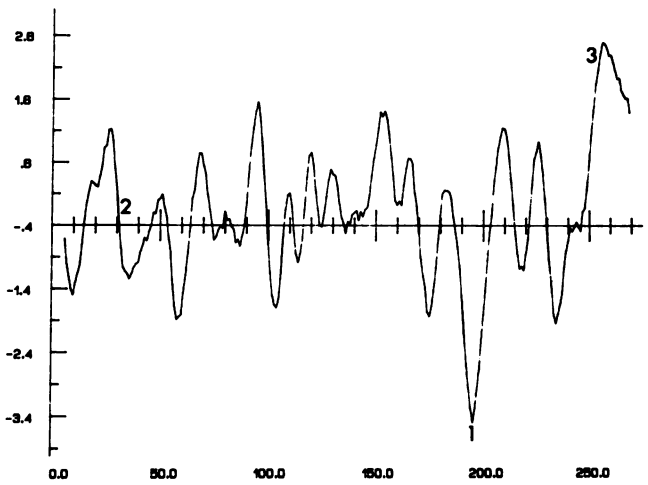

FIG. 6. Hydropathic analysis of the predicted amino acid sequence for the U_L34 protein from HSV-1 strain 17. The hydropathic plot was obtained by using the algorithm of Kyte and Doolittle with a moving window of 7. The subsequent profile was smoothed for plotting by taking its average in a moving 5-residue window. The plot is constructed such that points lying above the *x* axis represent regions of above-average hydrophobicity while points lying below the axis represent regions of above-average hydrophilicity. Positions of the putative kinase target site, the observed signal sequence consensus, and the extremely hydrophobic C-terminal tail are indicated by 1, 2, and 3, respectively.

Role of the PK in the replicative cycle of the virus. Our results demonstrate that replacement of the 33,000-apparent-molecular-weight phosphoprotein by the wild-type 30,000-molecular-weight phosphoprotein requires the participation of the viral PK, and in this sense the product of the U_L34 gene is a target of the PK and the first such target identified to date. The possibility that additional targets exist are based on two observations. The first relates to the morphology of infected cells in culture. Specifically, the plaques formed by R7041 are characteristically small and consist largely of shriveled cells which tend to float off their solid substrate very early after infection, in contrast to cells infected by the wild-type parent.These properties are accentuated in cultures infected with high ratios of virus per cell. Marker rescue of R7041 results in the restoration of the wild-type properties. It is therefore highly significant that cells infected with recombinant viruses R7310 and R7311, in which the PK target site on the U_L34 gene has been mutated, while exhibiting a U_L34 gene product with the decreased electrophoretic mobility characteristic of the R7041 virus, do not display the highly distinctive plaque morphology of cells infected with the R7041 virus. It also noteworthy that while the viral PK gene is nonessential for the growth of HSV-1 in tissue culture (17), the R7041 virus displays decreased neurovirulence in mice when tested by intracranial injection (23). The search for additional targets of the viral PK gene is in progress.

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ADDENDUM IN PROOF

After the proofs were received, we tested newly made antibody to U_L34 protein. These studies indicated that, in cells infected with PK^- virus, the U_L34 protein is not phosphorylated, that is, its phosphorylation totally depends on viral PK. These studies also indicated that the phosphoprotein in the upper ^{32}P-labeled band in autoradiograms of electrophoretically separated lysates of cells infected with PK^- virus is not structurally related to the U_L34 protein although it may be functionally related to it.

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