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Herpes Simplex Virus 1 Protein Kinase Is Encoded by Open Reading Frame US3 Which Is Not Essential for Virus Growth in Cell Culture

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Earlier reports have described a novel protein kinase in cells infected with herpes simplex or pseudorabies viruses. These novel enzymes were characterized by their acceptance of protamine as a substrate and by their differential chromatographic behavior in anion-exchange chromatography. We report that this activity was not present in extracts of uninfected cells or of cells infected with a mutant constructed so as to contain a deletion in the US3 open reading frame mapping in the small component of herpes simplex virus 1 DNA. The activity was present in extracts of cells infected with wild-type virus and with a recombinant in which the US3 open reading frame had been rescued. Our results are consistent with the observation reported earlier that the coding sequences predict an amino acid motif common to protein kinases and lead to the conclusion that the US3 open reading frame encodes a virus-specific protein kinase that is not required for virus growth in cells in culture.

We report that a novel protein kinase previously shown to be present in extracts of cells infected with herpes simplex virus 1 (HSV-1; 27, 28) is a product of a viral gene. The enzyme is encoded by the open reading frame designated US3, which maps in the unique sequences of the S component of HSV-1 DNA (23). The enzyme and sequences encoding the enzyme are not essential for growth of the virus in cells in culture (20). Relevant to this report are the following findings. Reversible phosphorylation of proteins is a major mechanism for the regulation of the activity of cellular enzymes (for reviews, see references 4 and 17) and has also been implicated in the replicative cycle of several viruses (11, 15, 29). During lytic infection of cells by herpesviruses, the phosphorylation of both viral (21, 24, 34) and cellular (14) proteins occurs, although the functional significance of this remains to be established. Attempts to identify protein kinases that might be encoded by herpesviruses have used three main approaches. One is to examine the phosphorylating ability of immune-precipitated viral antigens, the substrate being either the putative kinase itself (autophosphorylation reaction), the immunoglobulin, or added exogenous protein. Although this approach was applied successfully to the v-*src* kinase (5), its main drawback is the possible contamination of the antigen with minute amounts of catalytically active cellular protein kinases (6, 33).

A second approach centers on the reports that herpesvirus virions contain a protein kinase activity (18). Subsequent studies, however, have shown that the virions of HSV-1 and pseudorabies virus (PRV) contain a spectrum of cellular protein kinases and that one of these, cellular casein kinase II, may be responsible for the phosphorylation of the major virion phosphoproteins of HSV-1 and PRV (30).

A third approach is to screen extracts of infected cells for new protein kinase activities by using artificial substrates, such as histones, protamine, casein, and phosvitin. Although this approach does not detect an enzyme with a very narrow substrate specificity, it led to the identification of a possible candidate for herpesvirus protein kinase (12, 13, 28). This enzyme, designated as virus-induced protein kinase, can utilize ATP but not GTP to phosphorylate substrates with seryl or threonyl residues on the C-terminal side of multiple arginyl residues, such as protamine, but not acidic substrates, such as casein and phosvitin (27). It has been purified to homogeneity from cells infected with PRV (F. C. Purves, M. Katan, and D. P. Leader, Eur. J. Biochem., in press) and highly purified from cells infected with HSV-1 (27). The substrate specificity and other properties of the enzyme clearly differentiate it from known cellular protein kinases (13) and from another activity reported in cells infected with HSV-1 and catalyzing the phosphorylation of the acidic protein phosvitin in vitro (3). Furthermore, the protein kinases induced in cells infected with HSV-1 and PRV could be chromatographically resolved from one another, suggesting, although not proving, that they are viral gene products.

The protein kinases responsible for the phosphorylation of proteins constitute a large and diverse family of enzymes, differing in their substrate specificities, regulatory control, and the amino acids that they phosphorylate (9, 16). Nevertheless, the amino acid sequences of all eucaryotic protein kinases so far examined share a number of highly conserved motifs that are unique to, and diagnostic of, the catalytic domain of this class of enzymes (2, 10, 31). Strong evidence that HSV-1 indeed encodes a protein kinase came from the finding that the amino acid sequences of the predicted products of the US3 gene of HSV-1 and the US2 gene of varicella-zoster virus contain most of the sequence motifs of protein kinases mentioned above (22). In the case of varicella-zoster virus, for which the sequence of the entire genome is known, this is the only gene predicted to encode a protein kinase (7). To test the hypothesis that the HSV-1-induced protein kinase is encoded by the open reading frame US3, we took advantage of a mutant with a genetically engineered deletion in the US3 open reading frame (20). We

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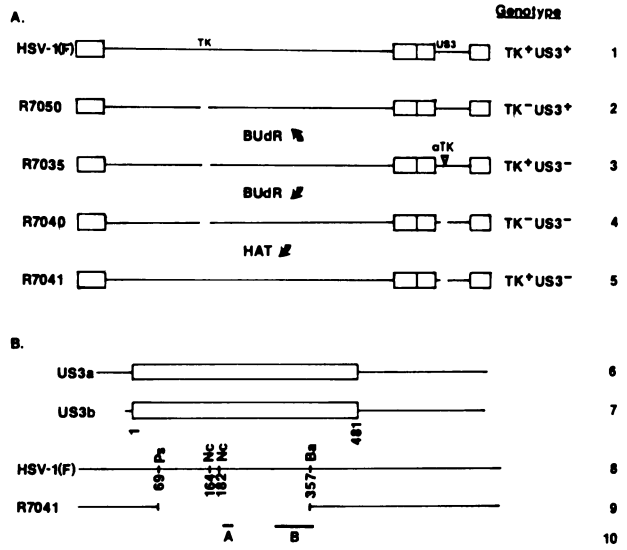

FIG. 1. Schematic representations of the DNA sequence arrangements in the genomes of HSV-1(F) and the HSV-1(F) recombinants R7035, R7040, and R7041. (A) The boxes in lines 1 to 5 represent the terminal sequences that are repeated internally and divide the HSV-1(F) genome into the long (L) and short (S) components. Line 1, sequence arrangement in the HSV-1(F) genome. TK and US3 indicate the locations of the thymidine kinase gene and the US3 open reading frame, respectively. Lines 2 to 5, sequence arrangement of the various viral recombinants. The genotype of each of the viral recombinants is indicated. (B) Lines 6 and 7, the two species of mRNA encoded by the US3 open reading frame (22, 23). The boxes indicate the translated portion of the mRNA. Line 8, restriction endonuclease sites located within the US3-coding region pertinent to this report (Ps, *Pst*I, Nc, *Nco*I, and Ba, *Bam*HI). The *Nco*I sites indicate the insertion site of the α27-*tk* gene. The sequences intervening between the *Pst*I and *Bam*HI sites were deleted in the R7040 and R7041 viral genomes. As a result of the deletion, the protein encoded by the US3 mRNA is out of frame downstream (3′) of the *Bam*HI site. Numbers in lines 7 and 8 correspond to the predicted amino acid number in the predicted translation product of the US3 open reading frame. Line 10, A indicates the position of the nucleotide-binding site (10) and B indicates the previously reported position of the domain shared by the known protein kinases (22).

report that the mutant with a deletion in the US3 open reading frame lacked the ability to express the HSV-1-induced protein kinase. The activity was regained when the US3 open reading frame was restored.

MATERIALS AND METHODS

Viruses and cells. The properties of HSV-1(F) and the HSV-1(F) recombinants R7035, a virus containing an insertion of a chimeric thymidine kinase gene within the coding region for the US3 gene, and R7040, a virus carrying a deletion within the coding region for the US3 gene, were reported elsewhere (8, 20). Vero cells were used in all experiments except when noted below. Cotransfections were done in rabbit skin cells originally obtained from J. McLaren, and selection of recombinants was done as previously described (19, 20). Growth medium for cells consisted of Dulbecco modified Eagle minimal essential medium supplemented with 10% calf serum. The maintenance medium 199-V consisted of mixture 199 supplemented with 1% calf serum.

DNAs. The purification of plasmid and viral DNAs was as described elsewhere (20, 32). Clones pRB103 and pRB3446, carrying *Bam*HI-Q and a *Sac*I fragment containing the entire US3 open reading frame, respectively, were described elsewhere (20, 25).

Polyacrylamide gel electrophoresis and immunoblots. Electrophoresis in denaturing polyacrylamide gels, solubilization of proteins, electrical transfer of electrophoretically separated proteins to nitrocellulose, and reaction with monoclonal antibodies in an immunoperoxidase-coupled reaction were done as described elsewhere (1). Protein concentrations were determined with the Bio-Rad assay (Bio-Rad Laboratories, Richmond, Calif.).

Preparation of cellular cytoplasmic extracts. Vero cells (15 roller bottles containing a total of approximately 3×10^9 cells) were infected with HSV-1(F), R7041, or R7050 or were mock infected and harvested 17 h after infection. All manipulations were done at 4°C. The cells were scraped into phosphate-buffered saline, washed twice with phosphate-buffered saline, and then suspended in an equal volume of a solution containing 10 mM KCl, 1.5 mM magnesium acetate, 10 mM Tris hydrochloride (pH 7.6), 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 40 μg of phenylmethylsulfonyl fluoride per ml and allowed to stand on ice for 10 min. The cells were then broken open by 20 strokes of a Teflon (Du Pont Co.)-glass homogenizer, and the ionic composition was adjusted to 125 mM KCl, 5 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 25 mM Tris hydrochloride (pH 7.5). The homogenate was centrifuged at 30,000 × *g*, and the supernatant fluid was cleared of mitochondria and ribosomes by centrifugation at 160,000 × *g* for 2.5 h in a Beckman Ti75 rotor. The supernatant fluid (approximately 4 to 5 mg/ml) was dialyzed overnight against buffer A (20 mM Tris hydrochloride [pH 7.5], 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 40 μg of phenylmethylsulfonyl fluoride per ml, 10% glycerol) and either subjected to immediate anion-exchange chromatography or shock frozen and stored at −80°C.

Anion-exchange chromatography. The cytoplasmic extract from each preparation (30 mg of protein) was applied to preequilibrated DEAE-cellulose (DE-52; Whatman Ltd., Maidstone, England) packed in a column (5 by 1.6 cm). The column was washed with 165 ml of buffer A and eluted with a 500-ml linear gradient of 0 to 0.4 M KCl in the same buffer at a flow rate of 66 ml/h. The fractions (5.5 ml) were collected and assayed immediately.

Assay of protein kinase activity. The assay mixture for protein kinase activity contained, in a total volume of 0.12 ml, 20 mM Tris hydrochloride (pH 7.6), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.1 mM ATP containing 1 μCi of [γ-^{32}P]ATP, 96 μg of protamine sulfate, and KCl in the range of 50 to 150 mM. The reaction was started by the addition of the fraction containing protein kinase (80 μl), and incubation was at 30°C for 30 min. At the end of the incubation, 100-μl samples were applied to P81 paper (2 by 2.5 cm; Whatman), washed for 15-min periods, twice in 10% (wt/wt) trichloroacetic acid and four times in 5% (wt/wt) trichloracetic acid, rinsed in absolute alcohol, and dried. Radioactivity was measured as Cherenkov radiation in the ^{3}H channel of a scintillation spectrometer.

RESULTS

Construction and properties of the HSV-1 mutants. In addition to the wild-type HSV-1(F), we tested three mutants

for virus-induced protein kinase activity, i.e., R7041, R7035, and R7050 (Fig. 1). The mutants and their derivations were as follows.

HSV-1(F)Δ305 was derived by recombination replacement of the wild-type thymidine kinase (*tk*) gene with a fragment from which 700 base pairs within the domain of the *tk* gene had been deleted (26).

R7035 was constructed by cotransfection of HSV-1(F)Δ305 intact TK^- viral DNA with a DNA fragment in which a chimeric *tk* gene replaced a small *Nco*I fragment within the coding region for US3 (Fig. 1, lines 3 and 8) (20). The chimeric *tk* gene was constructed by fusion of the promoter-regulatory domain of the α27 gene to the transcribed noncoding and coding sequences of the *tk* gene. Analyses of plaque-purified progeny selected for thymidine kinase activity verified the site of insertion of the chimeric *tk* gene (20).

R7040 was constructed by cotransfection of intact R7035 viral DNA with plasmid pRB3696 DNA. The HSV-1(F) DNA fragment cloned in plasmid pRB3696 contains a deletion within the US3 coding region from the *Pst*I site located at the predicted amino acid 69 at the 5′ end of US3 to the *Bam*HI site located at amino acid 357 of US3 (Fig. 1, line 9) (20, 23). As a result of the deletion, the US3 coding sequences downstream of the deletion were frameshifted (Fig. 1, line 9). The domains of the coding sequences deleted from the open reading frame include the motifs common to protein kinases discussed above and located in positions shown in Fig. 1B, line 10, A and B. This plasmid was cotransfected with intact R7035 DNA into rabbit skin cells as previously described (19). TK^- progeny were selected in $143TK^-$ cells in the presence of bromodeoxyuridine as previously described, and the plaque-purified R7040 DNA was shown to lack the sequences deleted from pRB3696 DNA (19, 20).

R7041 is identical to R7040 except that the 700-base-pair deletion in the *tk* gene was restored by cotransfection of intact R7040 DNA and *Bam*HI-Q DNA cloned as pRB103 into rabbit skin cells. The progeny of the transfection were plated on $143TK^-$ cells, and TK^+ progeny were selected as previously described (19), plaque purified, and tested for the presence of an intact *Bam*HI Q fragment with restriction endonucleases (data not shown). R7041 is expected to differ from the wild-type HSV-1(F) solely with respect to the deletion in the US3 open reading frame (Fig. 1, line 5).

As described below, R7041 was shown to lack the virus-induced protein kinase. To establish that lack of activity is due to the deletion in US3 and not to a fortuitous mutation introduced into the domain of the *tk* gene during the rescue of the *tk* gene and, concurrently, a mutation in the genes which partially overlap the *tk* gene, we repaired the US3 open reading frame of recombinant virus R7035. Recombinant R7050 was constructed by cotransfecting on rabbit skin cells intact R7035 DNA with a cloned HSV-1(F) *Sac*I fragment (pRB3446) containing the entire US3 coding region and flanking DNA. The progeny of the transfection were plated on $143TK^-$ cells, and TK^- virus was selected, plaque purified, and tested for the presence of an intact *Bam*HI N DNA fragment containing an intact US3 open reading frame (data not shown).

Analyses of the cell extracts for the HSV-1-induced protein kinase. Earlier studies have shown that the novel protein kinase activity present in infected but not in uninfected BHK cells can utilize protamine as a substrate and can be resolved from uninfected-cell protein kinases by anion-exchange chromatography (28). A similar resolution of protein kinases from Vero cells mock infected or infected with HSV-1 is

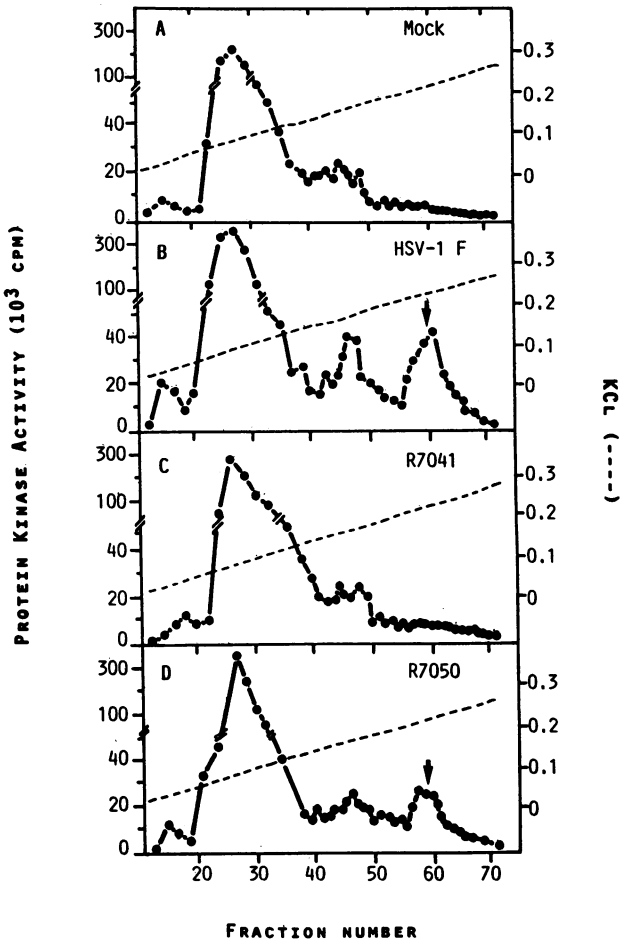

FIG. 2. DEAE-cellulose chromatography of protein kinase from cytoplasmic extracts of Vero cells infected with different strains of HSV-1 (10 to 15 PFU per cell). Extracts were prepared, absorbed onto DE-52, and eluted with a gradient of KCl (------), and fractions were assayed for the ability to phosphorylate protamine (●——●) as described in Materials and Methods. Cells were either mock infected (A) or infected with HSV-1(F) (B), R7041 (C), or R7050 (D). The arrows indicate the protein kinase activity found only in infected cells.

shown in Fig. 2A and B. The differences between the results obtained in these and previous studies are that the novel protein kinase activity was better resolved and eluted with 0.22 M KCl rather than with 0.20 M KCl as in previous studies. The difference in the elution profile is probably a reflection of the chromatographic medium used in the study (DEAE-cellulose); DEAE-Sephacel was used in the previous studies. Identical results were obtained with infected BHK cells (data not shown). Another factor contributing to the better resolution of the novel protein kinase activity was the addition of protease inhibitor, phenylmethylsulfonyl fluoride, and the Ca^{2+} chelator, EGTA, to decrease the proteolytic conversion of protein kinase C eluting at approximately 0.1 M KCl to an activity which eluted at approximately 0.18 M KCl.

The results of infection with the mutant viral constructs are shown in Fig. 2C and D. It can be seen that the elution profile of the protein kinases from extracts of Vero cells infected with R7041 (Fig. 1) lacks the protein kinase activity present in the extracts of cells infected with wild-type virus

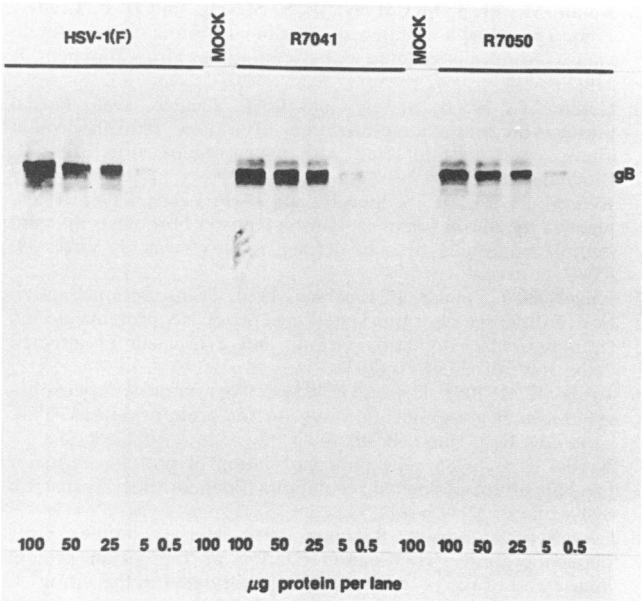

FIG. 3. Titration of gB made in Vero cells mock infected or infected with the wild-type parent strain HSV-1(F) and the recombinant viruses R7041 and R7050. Mock-infected or infected cell proteins were solubilized, separated in a denaturing polyacrylamide gel by electrophoresis, electrically transferred to nitrocellulose, and reacted with monoclonal antibody H1163 to gB in an immunoperoxidase-coupled reaction as described elsewhere (1). Protein concentrations were determined by Bio-Rad assay, and the amount loaded in each lane is indicated.

and eluting at 0.22 M KCl. The profile obtained with this mutant is almost identical to that of the mock-infected cells. Similar differences in the elution patterns were obtained in two independent experiments with extracts of infected Vero cells and in one experiment with extracts of infected BHK cells (data not shown). Infection of cells with R7050, in which the US3 gene had been rescued (Fig. 1), resulted in the reappearance of the protein kinase activity induced by the wild-type virus (Fig. 2D).

To control for the possibility that the results described above reflect poor growth of R7041 in Vero cells, two experiments were done. The results of the first show that similar virus yields were obtained from duplicate Vero roller-bottle cultures 18 h postinfection with R7041, R7050, and HSV-1(F) (titers of 7.9, 3.6, and 4.8 [10^7 PFU/ml], respectively) under conditions similar to those used for the preparation of extracts tested for protein kinase activity. In the second experiment, we tested the infected-cell extracts used for the determination of the protein kinase activity for the presence of glycoprotein B (gB). In these experiments, identical amounts of total protein from cell lysates were electrophoretically separated in denaturing polyacrylamide gels, transferred to nitrocellulose, and reacted with monoclonal antibody H1163 specific for HSV-1(F) gB. The results indicate that the cell extracts of R7041, R7050, and HSV-1(F) contained similar amounts of gB (Fig. 3).

DISCUSSION

US3 open reading frame encodes the protein kinase previously identified as virus induced. The conclusion that HSV-1 specifies a protein kinase and that it is encoded by DNA sequences within the domain of the open reading frame US3 rests on the following evidence. (i) The novel protein kinase in infected cells differs from that in uninfected cells with respect to substrate specificity and chromatographic properties (12, 13, 28). (ii) The novel protein kinase activity in HSV-1-infected cells differs from that induced in PRV-infected cells with respect to size (28). (iii) The novel enzyme activity was absent from cells infected with a virus mutant from which a major portion of the US3 open reading frame sequences was deleted. (iv) The predicted amino acid sequence of the protein encoded by the US3 open reading frame contains sequences shared by all protein kinases whose sequences are known (22).

Taken together, these studies argue against the possibility that US3 encodes a protein which activates a hitherto unidentified host protein kinase and suggest that the enzyme is specified by the virus. To obtain evidence stronger than that obtained to date, in a procedure which has been applied to very few viral genes, the amino acid sequence of the purified protein must be matched with that predicted by the open reading frame. Pending this evidence, we shall refer to the novel protein kinase as virus coded (HSV-1 PK) rather than virus induced.

Significance of the protein kinase encoded by herpesviruses. Several comments should be made regarding the significance of our finding that HSV-1 encodes a protein kinase. It is now well established that certain transforming retroviruses carry genes that specify protein kinases. These enzymes, however, are the products of various transduced cellular genes. Although such genes may confer a selective advantage to the transformed cell, there is no evidence that any advantage accrues to the ability of the virus to sustain itself in its ecological niche, as a consequence of the acquisition of the *pk* gene, or that the gene satisfies a viral requirement for specific phosphorylation of the virus proteins. In contrast, the protein kinase gene in HSV-1 and by extension in other α-herpesviruses is an integral and evolutionarily conserved part of the genetic repertoire of the virus. Furthermore, HSV-1 PK is the first and only eucaryotic protein kinase shown to be an authentic product of a viral gene. The fact that this enzyme has already been purified and characterized to a considerable extent (27, 28) should greatly facilitate attempts to identify its substrate and function.

Conserved and divergent protein kinase domains in HSV-1 PK. As indicated above, the impetus for testing cells infected with the $US3^-$ mutant for the novel protein kinase rested on the report that the predicted gene product shares an amino acid sequence motif (Fig. 1B, line 10, B) with known protein kinases (22). One aspect of the conserved amino acid homology not given sufficient emphasis previously is that the predicted products of the US3 gene of HSV-1 and of the corresponding gene of varicella-zoster virus deviate from the protein kinase consensus sequences in a second amino acid motif (Fig. 1B, A 10, A) in the nucleotide-binding domain of the enzyme. All protein kinases whose amino acid sequences are known contain the sequence Gly-Xaa-Gly-Xaa-Xaa-Gly (or Ala or Ser) followed 11 to 28 amino acids later by Ala-Xaa-Lys (10). In the predicted US3 gene product, however, the corresponding sequences are Thr-Xaa-Gly-Xaa-Xaa-Gly and Ile-Xaa-Lys, respectively. It is not clear at this stage whether the altered amino acid sequence accounts for any of the differential properties of HSV-1 PK, such as its activity at high ionic strength (28). Identification of the gene will make it possible to express the wild-type and specifically mutagenized genes in biochemically transformed cells to identify the functions of the various domains of HSV-1 PK.

Function of the *pk* gene. In addition to R7041, from which a major portion of the US3 open reading frame was deleted, Longnecker and Roizman (20) also reported a recombinant virus (R7036) from which the entire domain of US3 and that of glycoprotein G (US4) are deleted. The viability of both mutants excludes the possibility that the viability of R7041 is due to the expression of a partially active, truncated enzyme that was undetected in our assay system. Although these recombinant viruses grow in a variety of cells in culture and establish latency in experimental animals (B. Meignier, R. Longnecker, and B. Roizman, manuscript in preparation), it is conceivable that in these host systems the natural substrate of HSV-1 PK is either not phosphorylated or is processed by a host protein kinase. In principle, we must start with the premise that the open reading frame and particularly the enzyme activity of its gene product would not have been conserved had their function been dispensable. It is both reasonable and necessary to regard the enzyme as playing a role in viral replication in the repertoire of the cells in which the virus grows in the human host. The availability of mutant viruses like R7041 should prove invaluable in the attempts to identify the substrate and function of this intriguing viral enzyme.

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