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Processing of the herpes simplex virus regulatory protein α22 mediated by the U_L13 protein kinase determines the accumulation of a subset of α and γ mRNAs and proteins in infected cells

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ABSTRACT **We reported previously that the posttranslational processing associated with phosphorylation of the herpes simplex virus 1 infected-cell protein 22 (ICP22), a regulatory protein, is encoded by U_L13, a gene encoding a structural protein of the virion. We now report the following. (*i*) In cells infected with a mutant lacking U_L13 (ΔU_L13), restricted infected cells accumulate reduced levels of the regulatory protein ICP0 and several late viral proteins. Identical reductions have been observed in the same cell lines infected with a mutant from which the α22 gene, encoding ICP22, had been deleted (Δα22). We conclude that the U_L13-mediated processing of ICP22 is essential for its gene-regulatory function. (*ii*) The reduced accumulations of specific viral protein in cells infected with either ΔU_L13 or Δα22 viruses correlate with reduced levels of specific mRNAs for both ICP0 and the affected late genes. (*iii*) ICP22 is not modified by the U_L13 protein introduced into cells during infection. (*iv*) ICP22 is also modified by the protein kinase encoded by U_S3, but this modification is different from that of the U_L13 protein kinase. These results predict that U_L13 encodes a protein kinase or phosphotransferase which is expressed late in the replicative life cycle and which directly or indirectly phosphorylates ICP22. This modification is essential for stabilization or increased transcription of a specific subset of viral RNAs and, ultimately, for the accumulation of corresponding viral proteins.**

The expression of the known 77 genes of herpes simplex virus 1 (HSV-1) (1–6) is regulated in two ways. At a global level, these genes form at least four kinetic classes, designated as α, β, γ_1, and γ_2, whose expression is coordinately regulated and sequentially ordered in a cascade fashion (7, 8). Thus the virion protein VP16 encoded by the gene U_L48 and also designated as α trans-inducing factor (αTIF) induces the five α genes (9–14). At least four of the α proteins, designated as infected-cell proteins (ICPs) 0, 4, 22, and 27, regulate the transition from α to β and ultimately γ gene expression (15). However, the expression of specific genes and their gene products is also regulated at a posttranscriptional (16) and translational (17) level. One example illustrating both posttranscriptional and posttranslational regulation concerns the product of the gene, U_L34. At the posttranslational level, in the absence of the protein kinase (PK) encoded by U_S3, the U_L34 protein is not phosphorylated but becomes associated with four infected-cell phosphoproteins (18, 19). At the posttranscriptional level, in the absence of the gene product of U_S11, a truncated 5′ portion of the U_L34 mRNA accumulates in infected cells (16).

The effect of phosphorylation of the U_L34 protein on its interaction with other proteins is but one example of the regulation of the function of an essential viral protein by a viral kinase. Other viral phosphoproteins include αTIF and four (ICP0, -4, -22, and -27) of the α proteins (20, 21). The preponderance of phosphoproteins among viral gene products raises questions regarding the role of the two viral genes U_S3 and U_L13. U_S3 encodes a PK (22, 23). The U_L13 protein has been reported to contain sequences common to PKs (24), and recent studies from this laboratory demonstrated that the posttranslational processing of ICP22 associated with phosphorylation is mediated by the U_L13 protein (25). In this report, we show that a set of functions associated with ICP22 requires processing of the protein by the U_L13 product.

Relevant to this report are the functions of two regulatory proteins, ICP0 and ICP22, the products of the α0 and α22 genes. ICP0 appears to be a promiscuous transactivator of genes introduced into cells by infection or transfection (26, 27). ICP22 is required for optimal viral replication in primary human cell strains and in cell lines of rodent derivation. In these cell lines the yields of virus and the production of late (γ) proteins are greatly reduced (28).

MATERIALS AND METHODS

Cells and Virus. All cell lines were from the American Type Culture Collection. The isolation and properties of HSV-1(F), the prototype HSV-1 strain used in this laboratory, have been described (29). In recombinant R325 (Δα22*tk*$^-$) 821 bp of the coding domain of the α22 gene had been deleted (30). The construction of the recombinant viruses HSV-1(F)Δ305 (*tk*$^-$), R7041 (ΔU_S3), R7351 ($\Delta U_S3\Delta U_L13tk^-$), R7352 ($\Delta U_S3\Delta U_L13Rtk^-$), and R7355 ($\Delta U_L13tk^-$) has been described (9, 25, 31). Recombinant viruses R7353, R7359, and R7356 correspond to R7351, R7352, and R7355, respectively, except that the thymidine kinase gene (*tk*) had been restored.

RESULTS

The Phenotype of ΔU_L13 Viruses Is Similar to That of the Δα22 Mutant Virus. The gene U_L13 encodes the functions for processing associated with phosphorylation of ICP22 (25). Specifically, in cells infected with ΔU_L13 mutants, ICP22 accumulated in a higher-mobility phosphospecies which was not processed to the typical lower-mobility species observed in wild-type-infected cells (Fig. 1, lanes C and D). In addition, ΔU_L13 viruses exhibited impaired growth characteristics on rabbit skin cells and baby hamster kidney (BHK) cells when compared with either wild-type virus or viruses in which the U_L13 gene had been restored. Similar growth characteristics had been reported for a recombinant virus R325 (Δα22), which harbors an 821-bp deletion in the α22 gene (28). Thus, in rabbit skin cells, BHK and RAT-1 cell lines, and a human embryonic lung (HEL) cell strain infected with Δα22, the synthesis of late protein was significantly reduced. This effect was less pronounced in infected HEp-2 human epidermoid

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Abbreviations: HSV-1, herpes simplex virus 1; ICP, infected-cell protein; αTIF, α trans-inducing factor; *tk*, thymidine kinase gene; PK, protein kinase.

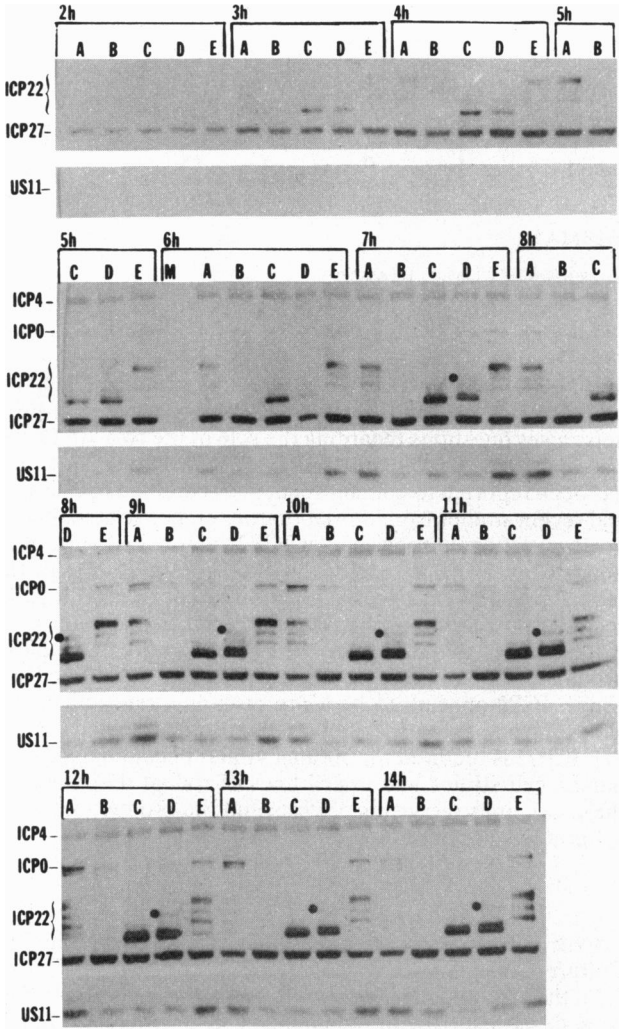

FIG. 1. Lysates of BHK cells harvested at various times (2–14 hr) postinfection were electrophoresed in denaturing gels, and the proteins were transferred to nitrocellulose sheets for reaction with antibodies to specific antibodies to viral proteins. The cells were infected with HSV-1(F)Δ305 (*tk*$^-$, lanes A), R325 (Δα22, lanes B), R7351 (ΔU_S3ΔU_L13*tk*$^-$, lanes C), R7355 (ΔU_L13*tk*$^-$, lanes D), or with R7352 (ΔU_S3ΔU_L13R*tk*$^-$), in which the sequences deleted from U_L13 had been restored (lanes E). Lanes M show lysates from mock-infected BHK cells. The infected cells were harvested at the times indicated, and total cell extracts were prepared as described (18, 19). Approximately 50 μg of protein was loaded in each lane and subjected to electrophoresis in denaturing 8.5% polyacrylamide gels. The nitrocellulose sheets to which the electrophoretically separated proteins were transferred were blocked with phosphate-buffered saline containing 5% skim milk and were reacted sequentially with a polyclonal antiserum to ICP22 (R77) and monoclonal antibodies to U_S11 protein (CL28), ICP27 (H1117), ICP0 (H1083), and ICP4 (H943), as described (18, 19). The filled circle identifies a U_S3-dependent phosphorylation of ICP22 in lanes D. In this and all other immunoblots shown the reactivity of the antibody to ICP27 serves as a control for equal loading of ICPs.

carcinoma or Vero monkey cell lines, suggesting that a complementing host-cell factor existed in these cell lines.

To determine whether the posttranslational modification of ICP22 mediated by U_L13 was necessary for increased levels of late protein synthesis, we compared the levels of the U_S11 protein that accumulated in BHK cells infected with either ΔU_L13, Δα22, or wild-type virus. U_S11 is expressed as a late (γ_2) gene. Fig. 1 shows the immunoblots of lysates of BHK cells infected with either HSV-1(F) (lanes A), Δα22 (lanes B), ΔU_L13 (lanes C and D), or ΔU_L13(R) (lanes E) viruses, harvested at intervals from 2 to 14 hr postinfection and reacted with antibodies to specific viral proteins. To control for equal infectivity and protein concentration, the immunoblots were also reacted with monoclonal antibody to ICP27, whose accumulation is not affected by absence of the genes deleted in these viruses. Significantly reduced levels of U_S11 protein were present in cells infected with ΔU_L13 virus (lanes C and D) or Δα22 (lane B) compared with those of cells infected with the other viruses.

Analyses of electrophoretically separated proteins from lysates of BHK cells labeled for 30 min with [^{35}S]methionine at 14 hr postinfection with ΔU_L13 or Δα22 viruses (Fig. 2) indicate that a number of proteins were underproduced. These include protein bands (E and F), identified with specific monoclonal antibodies (data not shown) as U_S11 protein, a γ protein with an apparent M_r of 70,000 (band B) tentatively identified as the product of U_L47, a γ protein with an apparent M_r of 38,000 (band C) tentatively identified as the product of U_L49, and two additional γ proteins (bands A and D) whose identity is not known. A similar pattern of reduced late protein synthesis was also observed in rabbit skin cells infected with the ΔU_L13 viruses (data not shown).

Fig. 3 represents an immunoblot of electrophoretically separated lysates of rabbit skin cells infected with the indicated viruses and reacted sequentially with mouse monoclonal antibodies to ICP0, ICP27, the U_S11 protein, and the viral capsid scaffolding protein ICP35 and with the polyclonal serum to ICP22. The results show reductions in accumulation of ICP0, ICP22, ICP35, and U_S11 protein but, as expected from earlier data, not of ICP27. The results obtained with the Δα22 virus were similar to those obtained with ΔU_L13 viruses

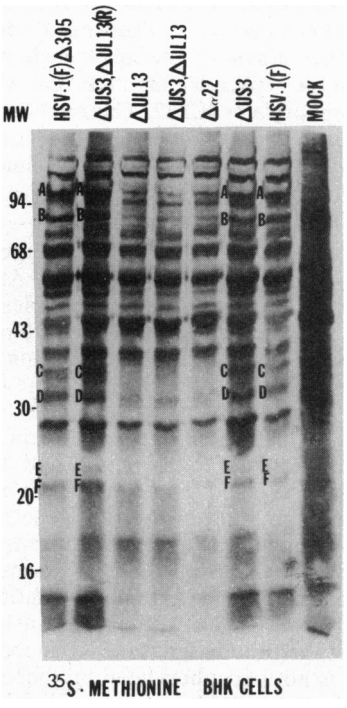

FIG. 2. Autoradiographic image of electrophoretically separated ^{35}S-labeled lysates of BHK cells labeled for 30 min in methionine-free medium supplemented with 100 μCi of [^{35}S]methionine (1300 Ci/mmol, Amersham; 1 Ci = 37 GBq) just prior to harvest at 14 hr after infection with viruses indicated at the top. The labeled ICPs were separated in denaturing gels containing 10% acrylamide, transferred to nitrocellulose, and subjected to autoradiography. Molecular weight standards are indicated at left ($M_r \times 10^{-3}$), and the letters A–F indicate ICPs which were synthesized at reduced levels in cells infected with Δα22 or ΔU_L13 viruses. The cells were infected with HSV-1(F)Δ305 (*tk*$^-$), R7041 (ΔU_S3), R325 (Δα22), R7351 (ΔU_S3ΔU_L13*tk*$^-$), R7355 (ΔU_L13*tk*$^-$), or R7352 (ΔU_S3ΔU_L13R*tk*$^-$).

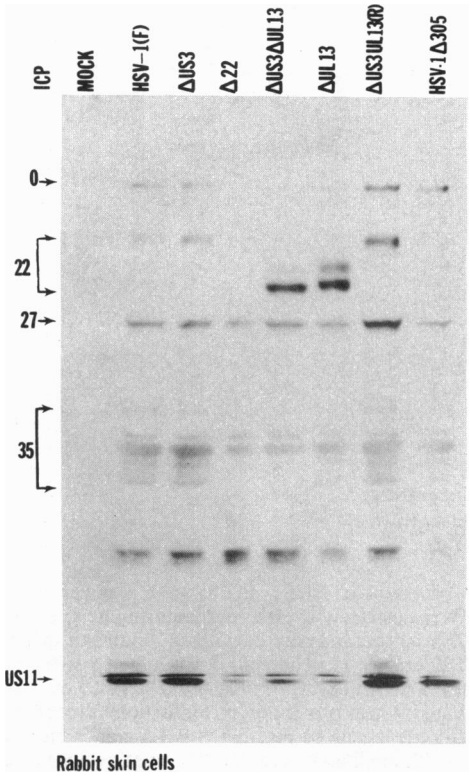

FIG. 3. Rabbit skin cells harvested at 18 hr after infection with the viruses indicated were lysed, and the lysate proteins were electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet and reacted with antibodies to specific viral proteins. Procedures were as described in the legend to Fig. 1. The separated polypeptides were transferred to nitrocellulose and reacted sequentially with monoclonal antibody to the U_S11 protein (CL28), to the scaffolding proteins constituting the ICP35 family of proteins encoded by $U_L26.5$ (H725), to ICP0 (H1083), and to ICP27 (H1117) and with the rabbit polyclonal antibody to ICP22 (R77). The immunoreactive proteins are identified by their ICP numbers and arrows at left. The viruses used in this experiment were HSV-1(F), HSV-1(F)Δ305 (tk^-), R7041 (ΔU_S3), R325 ($\Delta\alpha22$), R7351 ($\Delta U_S3\Delta U_L13tk^-$), R7355 ($\Delta U_L13tk^-$), or R7352 ($\Delta U_S3\Delta U_L13Rtk^-$).

except that cells infected with this virus make a truncated form of ICP22 which is unstable and not detectable in lysates of cells infected at 37°C (32). Not all late viral proteins were diminished in cells infected with the ΔU_L13 and $\Delta\alpha22$ viruses. For instance, the products of genes U_L44 (glycoprotein C), U_L48 (αTIF), and U_L34 accumulated in normal amounts in $\Delta\alpha22$- or ΔU_L13-infected cells (data not shown). The remarkable observation that accumulation of ICP0, the product of the $\alpha0$ gene, was reduced in rabbit skin cells infected with $\Delta\alpha22$ (Fig. 3, lanes B) or ΔU_L13 (lanes C and D) viruses was reproduced in BHK cells infected with these mutants (Fig. 4). In this instance too, cells infected with the wild type (Fig. 4, lanes A) and the virus in which the U_L13 gene was repaired (lanes E) maintained equivalent levels of ICP0 as late as 14 hr postinfection.

Levels of Both $\alpha0$ and U_S11 mRNAs Are Reduced in Cells Infected with ΔU_L13 or $\Delta\alpha22$ Recombinant Viruses. The levels of U_S11 (Fig. 5 *A* and *D*) and $\alpha0$ (Fig. 5*B*) RNAs hybridizing to specific probes were decreased in the cytoplasm of BHK cells infected with ΔU_L13 or $\Delta\alpha22$ relative to the levels contained in the cytoplasms of cells infected with wild-type and repaired viruses. The *tk* RNA hybridizing to its probe (Fig. 5*C*) served as a control for proper infection and for extraction of RNA.

Cycloheximide Reversal Experiments Indicate That U_L13 May Mediate Posttranslational Modification of ICP22 Late Rather Than Early in Infection. It was reported that U_L13 is contained in the virion (33) and that one of the PKs contained in the virion (34) has properties similar to those of a nuclear PK that copurifies with the UL13 protein (35). To resolve the question whether the U_L13 contained in the virion mediates the posttranslational processing of the newly synthesized ICP22, BHK cells were infected and maintained for 6 hr in the presence of cycloheximide. At that time actinomycin (10 μg/ml) was added to the medium, the cycloheximide was withdrawn, and the cells were incubated for an additional 4 hr. The design of the experiment allowed the synthesis of α-gene mRNA and proteins but not of gene products dependent on α proteins for their synthesis. If the U_L13 kinase introduced during infection were available for posttranslational modification of the nascent ICP22, the posttranslational processing should have been evident in Fig. 6. The immunoblots indicate that the processing of ICP22 was apparent as early as 4 hr after infection (Fig. 6*B*) and was most pronounced at 10 hr postinfection (Fig. 6*C*) in cells infected with wild-type virus. ICP22 translated from RNA transcribed in the presence of cycloheximide was not processed to slower migrating forms (Fig. 6*A*).

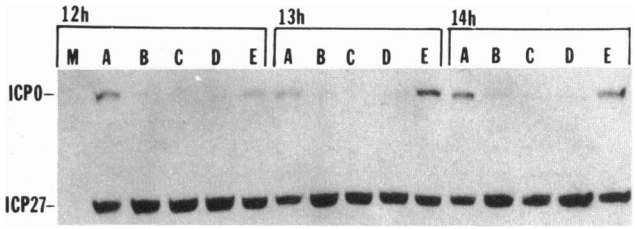

FIG. 4. Lysates of BHK cells harvested at 12, 13, and 14 hr postinfection were electrophoretically separated in denaturing gels, transferred to a nitrocellulose sheet, and reacted with the monoclonal antibody to ICP0 (H1083) and ICP27 (H1117). Procedures were as described in the legend to Fig. 1. The immunoreactive polypeptide bands species are identified by their ICP numbers at left. The viruses were HSV-1(F)Δ305 (tk^-, lanes A), R325 ($\Delta\alpha22$, lanes B), R7351 ($\Delta U_S3\Delta U_L13tk^-$, lanes C), R7355 ($\Delta U_L13tk^-$, lanes D), and R7352 ($\Delta U_S3\Delta U_L13Rtk^-$, lanes E). Lanes M, lysates from mock-infected BHK cells.

U_S3 PK May Also Phosphorylate ICP22. Comparison of the pattern of ICP22 phosphospecies present in BHK cells infected with the $\Delta U_S3\Delta U_L13$ double-deletion virus (Fig. 1, lane C) with that present in cells infected with the ΔU_L13 single-deletion virus (Fig. 1, lane D) revealed an additional ICP22 phosphospecies (indicated by a filled circle) present only in cells infected with the single-deletion virus. This ICP22 phosphospecies was first detected at about 7 hr postinfection, correlating with the appearance of the U_S3 viral PK. We expect that the PK encoded by U_S3 (U_S3 PK) phosphorylates ICP22 directly. In fact, the $\Delta\alpha22$ protein has three potential target sites for the U_S3 PK (1, 36). It is important to note that in cells infected with the $\Delta U_S3\Delta U_L13R$ virus (Fig. 1, lane E), another ICP22 phosphospecies comigrates with the band marked with the filled circle, which is obviously not due to U_S3 phosphorylation but which must represent an ICP22 isomer possessing an equivalent number of phosphates.

DISCUSSION

In this article we show that the processing of ICP22, a HSV-1 regulatory protein, mediated by the U_L13 PK determines the accumulation of a specific subset of α and γ mRNAs and proteins in infected cells. Relevant to the studies we reported are the following.

(*i*) The U_L13 coding sequence predicts amino acid sequences characteristic of the catalytic domains of eukaryotic

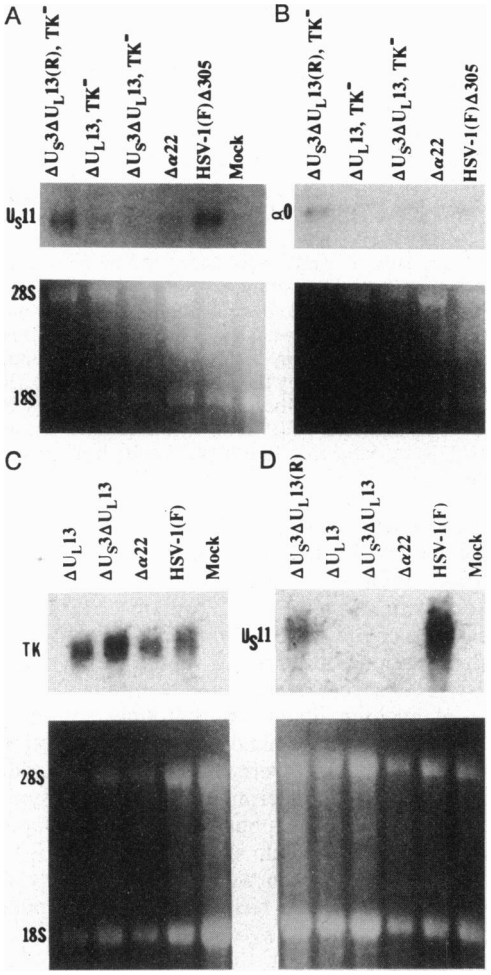

FIG. 5. Electrophoretically separated cytoplasmic RNAs from BHK cells harvested 12 hr after mock infection or infection with recombinant viruses. The RNAs were electrophoretically separated in 1% agarose gels containing formaldehyde, stained with ethidium bromide, and transferred to nitrocellulose, and specific mRNA species were detected by hybridization with ^{32}P-labeled DNA probes. (*A*) RNAs were stained with ethidium bromide (*Lower*), transferred to nitrocellulose, and hybridized to ^{32}P-labeled pRB3910, a plasmid containing a HSV-1 DNA fragment containing the gene U_S11 (*Upper*). (*B*) RNAs were stained with ethidium bromide (*Lower*), transferred to nitrocellulose, and hybridized to ^{32}P-labeled pRB3710, which contains $\alpha0$ gene sequences (*Upper*). (*C*) RNAs stained with ethidium bromide (*Lower*) were transferred to nitrocellulose and hybridized to ^{32}P-labeled pRB173, which contains HSV-1 *tk* (*Upper*). (*D*) The same preparation of RNA analyzed in *C* was electrophoretically separated in agarose gels and the RNAs were stained with ethidium bromide (*Lower*) transferred to nitrocellulose, and probed with ^{32}P-labeled pRB3910 to detect the level of U_S11 mRNA produced (*Upper*). The recombinant viruses used were HSV-1(F), HSV-1(F)Δ305 (*tk*$^-$), R325 ($\Delta\alpha22$ *tk*$^-$), R7351 ($\Delta U_S3\Delta U_L13tk^-$), R7355 ($\Delta U_L13tk^-$), R7352 ($\Delta U_S3\Delta U_L13Rtk^-$), R7353 ($\Delta U_S3\Delta U_L13$), R7356 ($\Delta U_L13$), and R7359 ($\Delta U_S3\Delta U_L13R$).

PKs and possibly of bacterial phosphotransferases (24). The gene U_L13 is highly conserved: homologs of U_L13 have been identified in representative members of all three herpesvirus subfamilies (37–40). Interestingly, the homolog encoded by the cytomegalovirus (CMV) U_L97 gene has been shown to mediate the phosphorylation and activation of the antiviral nucleoside analog ganciclovir (41, 42). It remains to be determined whether the CMV U_L97 gene product phosphorylates nucleosides directly or whether it encodes a PK which secondarily activates a nucleoside kinase.

(*ii*) We have established that the posttranslational processing associated with the phosphorylation of ICP22 is encoded

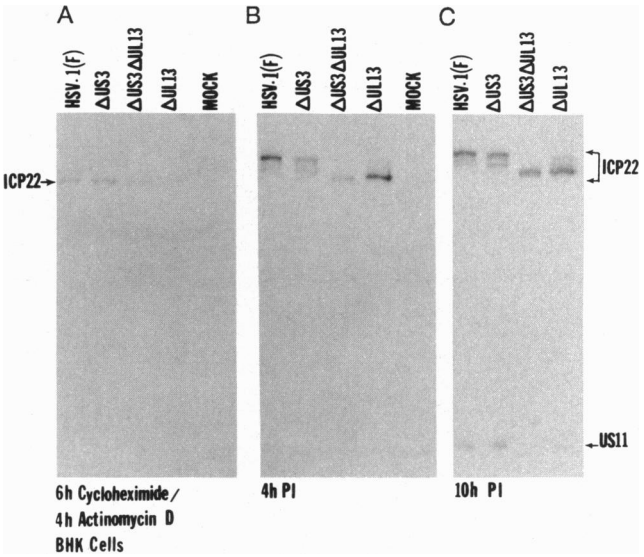

FIG. 6. Lysates of BHK cells harvested at various times postinfection were electrophoresed in denaturing gels, transferred to a nitrocellulose sheet, and reacted with antibodies to specific antibodies to ICP22 and U_S11. The cells were infected with the indicated viruses in the presence (*A*) or absence (*B* and *C*) of cycloheximide and harvested at various times postinfection. Procedures were as described in the legend to Fig. 1. (*A*) BHK cells were preincubated for 30 min with medium containing 100 μg of cycloheximide per ml, then infected and maintained for 6 hr in medium containing cycloheximide. The infected monolayers of cells were then extensively washed with medium lacking cycloheximide and the cells were overlaid with medium containing 10 μg of actinomycin D per ml for an additional 4 hr. (*B*) Electrophoretically separated polypeptides from BHK cells infected for 4 hr with the indicated recombinant viruses. (*C*) Electrophoretically separated polypeptides from cells infected for 10 hr with the indicated recombinant viruses. The separated infected-cell polypeptides were transferred to nitrocellulose and reacted first with polyclonal antiserum R77 and then with monoclonal antibody to the U_S11 protein (CL28). Viruses used were HSV-1(F), R7041 (ΔU_S3), R7351 ($\Delta U_S3\Delta U_L13tk^-$), and R7355 ($\Delta U_L13tk^-$).

by U_L13. The hypothesis that ICP22 is the substrate of U_L13 protein is supported by several lines of evidence. The ICP22 protein is highly phosphorylated, and the stepwise increases in apparent molecular weight and negative charges observed between the five ICP22 subspecies in one- and two-dimensional separations are consistent with successive phosphorylations of the protein (25, 32). We should note that although we could not demonstrate that the virion U_L13 protein phosphorylates newly made ICP22, the conditions of our tests are not physiological and do not exclude the possibility that by the time ICP22 was available for post-translational modification, the U_L13 PK introduced into cells during infection may have been degraded.

(*iii*) We have established that processing of ICP22 is required for the maintenance of appropriate levels of ICP0 and of a subset of late proteins in infected cell lines which we have designated as restrictive. In nonrestrictive cell lines (e.g., Vero and HEp-2), neither the $\alpha22$ nor the U_L13 gene is required although, interestingly, in the absence of the U_L13 protein, the fully processed forms of ICP22 are underrepresented (25). We predict that U_L13 encodes a kinase which either directly phosphorylates the ICP22 as its major substrate or induces the phosphorylation of ICP22 via another protein.

The phenotype of $\Delta\alpha22$ virus—and by extension, of ΔU_L13 virus—suggests that restrictive cells lack a host factor which performs the functions of ICP22. The function of ICP22 or of the host factor is not known. The reduced accumulation of a

selected group of γ proteins correlates with decreased rates of synthesis, with decreased levels of γ gene mRNAs, and with decrease in both ICP0 and its mRNA. ICP0 has been implicated in transient expression assays in the transactivation of certain viral late genes (43) and is important *in vivo* for the efficient expression of β and γ genes at low multiplicity of infection (26, 27). It is conceivable that the decreased accumulation of late proteins mediated by the posttranslational modification of ICP22 by the U_L13 PK is partially or wholly the consequence of a decrease in the levels of ICP0 and that the reduced levels of both ICP0 and of selected late gene mRNAs are due to decreased transcription or stability of these mRNAs. These two possibilities are not mutually exclusive. For instance, ICP22, once it is posttranslationally modified by the U_L13 protein, might act to stabilize α0 mRNA, resulting in increased ICP0 levels and consequent increased transcription rates for selected late viral genes. A similar function might be performed by the putative host factor in the nonrestrictive cells.

Parenthetically, reduction in the amounts of ^{32}P label in a protein band from cells infected with a virus defective in the gene U_L13 is not *per se* an indication that the protein is phosphorylated by U_L13 PK as has been reported recently by Coulter *et al.* (44). Since in the absence of U_L13 a subset of late proteins is reduced in amount, the mere decrease in phosphoprotein accumulation may reflect reduction in the total amount of protein rather than reduction in its phosphorylation.

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