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Suppression of the Phenotype of $\gamma_1$34.5$^-$ Herpes Simplex Virus 1: Failure of Activated RNA-Dependent Protein Kinase To Shut Off Protein Synthesis Is Associated with a Deletion in the Domain of the α47 Gene

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Earlier studies have shown that infection of human cells by herpes simplex virus 1 (HSV-1) results in the activation of RNA-dependent protein kinase (PKR) but that the α subunit of eIF-2 is not phosphorylated and that protein synthesis is unaffected. In the absence of the viral $\gamma_1$34.5 gene, eIF-2α is phosphorylated and protein synthesis is prematurely shut off (J. Chou, J. J. Chen, M. Gross, and B. Roizman, Proc. Natl. Acad. Sci. USA 92:10516–10520, 1995). A second recent paper reported the selection of second-site suppressor mutants characterized by near-wild-type protein synthesis in cells infected with $\gamma_1$34.5$^-$ mutants (I. Mohr and Y. Gluzman, EMBO J. 15:4759–4766, 1996). Here, we report the properties of the spontaneous HSV-1 suppressor mutant Sup-1, which is characterized by spontaneous deletion of 503 bp encompassing the domain of the α47 gene and junction with the inverted repeats flanking the unique short (U_S) sequence of the HSV-1 DNA resulting in the juxtaposition of the α47 promoter to the coding domain of the U_S11 gene. This mutant does not exhibit the shutoff of protein synthesis characteristic of the $\gamma_1$34.5$^-$ virus. Specifically, Sup-1 in SK-N-SH human neuroblastoma cells (i) did not exhibit the function of the α47 gene characterized by a reduction in the transport of peptides across the endoplasmic reticulum of permealized cells consistent with the absence of α47 gene sequences, (ii) accumulated U_S11 protein at levels analogous to those of the wild-type parent but the protein was made at earlier times after infection, as would be expected from a change in the promoter, and (iii) activated PKR like that of the parent, $\gamma_1$34.5$^-$ virus, but (iv) did not cause premature shutoff of protein synthesis and therefore was similar to the wild-type parent virus rather than the $\gamma_1$34.5$^-$ virus from which it was derived. We conclude that the mechanism by which Sup-1 blocks the shutoff of protein synthesis associated with phosphorylation of eIF-2α by the activated PKR is not readily explainable by a secondary mutation characterized by a deletion.

The $\gamma_1$34.5 gene of herpes simplex virus 1 (HSV-1) encodes a protein consisting of a 159-amino-acid amino-terminal domain, the amino acids AlaThrPro repeated 5 to 10 times depending on the virus strain, and a 74-amino-acid carboxyl-terminal domain (1, 5, 6). The gene maps in the inverted-repeat sequence *ab* and *b′a′* flanking the unique long (U_L) sequence, between the terminal *a* sequence and the gene encoding the infected cell protein 0 (ICP0), and encodes at least two functions (1–7, 31). One function enables the virus to multiply in the central nervous systems of experimental animal systems. Mutations in both the amino-terminal and carboxyl-terminal domains confer the attenuated phenotype (2, 31). The second function blocks the phosphorylation of the α subunit of the eIF-2 translation initiation factor and, in consequence, precludes the total shutoff of protein synthesis induced after the onset of viral DNA synthesis (4, 6, 7). This function is not affected by mutations in the amino-terminal 159 amino acids but is abolished by deletions in the carboxyl-terminal domain (8). Of particular interest is the observation that the carboxyl-terminal domain of ICP34.5 is homologous to the carboxyl terminus of a murine, Chinese hamster, and human protein known as GADD 34 (8, 9, 16, 18). This protein belongs to a set of proteins induced under conditions which favor growth arrest after serum deprivation, DNA damage, or differentiation (33). The homology is in fact meaningful, inasmuch as in the context of the viral genome, the carboxyl terminus of murine GADD 34 (MyD116) can replace the carboxyl terminus of $\gamma_1$34.5 in blocking the premature shutoff of protein synthesis (11).

The mechanism by which $\gamma_1$34.5 precludes the shutoff of protein synthesis is not known. Recent studies (2) have shown that in HeLa cells infected with wild-type virus, the RNA-dependent protein kinase (PKR) is activated, although protein synthesis is unaffected. Precipitates obtained with polyclonal rabbit anti-human PKR contain, in addition, to the kinase, a phosphorylated protein with an apparent M_r of approximately 200,000. In contrast, precipitates obtained from lysates of HeLa cells infected with mutants lacking either the entire $\gamma_1$34.5 gene or the domain encoding the carboxyl terminus of the $\gamma_1$34.5 protein contain a phosphorylated protein with an apparent M_r of 90,000 in addition to the activated PKR and the phosphoprotein with an M_r of 200,000.

Mohr and Gluzman (20) recently reported that serial passage of a $\gamma_1$34.5$^-$ virus on a human neuroblastoma cell line resulted in the selection of a series of suppressor mutants capable of growing in these cells. They reported that these mutants contain variable size deletions at the junction between the right terminus of the unique short (U_S) sequence and the inverted repeat *ca* flanking U_S. They also reported that trans-

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fer of this rearranged region, which affects the domain of the U_S11-α47 genes, rescued the premature termination of the protein synthesis phenotype of a $\gamma_1 34.5^-$ virus.

In this article, we report on the phenotypic properties of a wild-type virus, a $\gamma_1 34.5$ deletion mutant, and one suppressor mutant described by Mohr and Gluzman (20). Relevant to this report are the following. U_S11 is a virion protein localized in the tegument, i.e., between the capsid and the envelope. This protein belongs to the γ_2 group in that its synthesis requires the onset of viral DNA synthesis (reviewed in reference 23). This protein localizes in nucleoli and associates with ribosomes both upon entry into cells during infections and after de novo synthesis late in infection (24–27). Its function is not known; in infected cells, it binds RNA in a sequence- and conformation-specific manner and precludes the accumulation of a truncated mRNA derived from the 5′ terminus of the U_L34 gene (25). α47 is one of a set of α genes whose expression does not require de novo synthesis of proteins after infection (reviewed in reference 23). The gene is not essential for viral replication in cells in culture (17). Recent studies (10, 12, 13, 21, 32) have shown that the infected-cell protein 47 (ICP47), the product of the α47 gene, binds the transport-associated proteins TAP1 and TAP2 and prevents the translocation of antigenic peptides from the cytoplasm across the endoplasmic reticulum for presentation by major histocompatibility complex (MHC) class I molecules to $CD8^+$ cells.

In this report, we show that the Sup-1 mutant is capable of sustained protein synthesis, but at a reduced level. Both the wild type and the spontaneous Sup-1 mutant activate PKR, but the amounts of the phosphorylated p90 precipitated from lysates of either the Sup-1 or the wild-type strain are much reduced relative to those present in precipitates of cells infected with the corresponding $\gamma_1 34.5^-$ virus. We also report that the amounts and electrophoretic properties of the U_S11 protein in cells infected with the Sup-1 mutant are identical to those found in wild-type-infected cells. However, the U_L11 protein accumulates earlier in infection, which is in agreement with the juxtaposition of the α47 promoter next to the coding domain of the U_S11 gene. Moreover, as could be expected from the genotype of the Sup-1 mutant, the function associated with the α47 protein is not detectable in lysates of cells infected with this virus.

MATERIALS AND METHODS

Cells and viruses. The Vero, HeLa, and human neuroblastoma SK-N-SH cell lines used were obtained from the American Type Culture Collection. Human embryonic lung cells were the kind gift of R. Spaete, Aviron Inc. Mountainview, Calif. The cells were propagated in Dulbecco's modified Eagle's medium supplemented with 5% (Vero and HeLa cells) or 10% (SK-N-SH cells) fetal bovine serum. HSV-1(F) is the wild-type prototype strain used in the University of Chicago Laboratory. Recombinant virus R3631 has deletions in the α47 gene (17). The parental viruses used in this study were HSV-1(Patton) and HSV-1(F). The construction of the $\gamma_1 34.5^-$ mutant of HSV-1(Patton), which was designated SPBg5e, and the suppressor mutant Sup-1 was described by Mohr and Gluzman (20). A schematic representation of these viruses is shown in Fig. 1.

[^{35}S]methionine labeling of infected-cell extracts. The procedures used for labeling of infected-cell proteins with [^{35}S]methionine has been described previously (7). Briefly, replicate 25-cm^2 cultures of SK-N-SH cells were either mock infected or exposed to the viruses for 2 h at 37°C. The inocula were then removed, and the cells were incubated at 37°C in medium 199V consisting of mixture 199 (Sigma Chemical Co., St. Louis, Mo.) supplemented with 1% calf serum. At 14 h after infection, the cells were overlaid with 1 ml of medium lacking methionine but supplemented with 50 μCi of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; 1 Ci = 37 GBq; Amersham, Arlington Heights, Ill.) for 1 h. The cells were then harvested, solubilized, subjected to electrophoresis in denaturing polyacrylamide gels, transferred to a nitrocellulose sheet, and subjected to autoradiography as previously described (7, 8).

Immunoblotting of viral proteins with antibodies. Proteins in cell lysates were electrophoretically separated in polyacrylamide gels cross-linked with *N,N′*-diallyltartardiamide, transferred electrically onto nitrocellulose paper, and reacted with mouse monoclonal antibodies to U_S11 or polyclonal antibody to a glutathione *S*-transferase–U_L38 chimeric protein. The preparation and properties of the antibodies have been described elsewhere (25, 30).

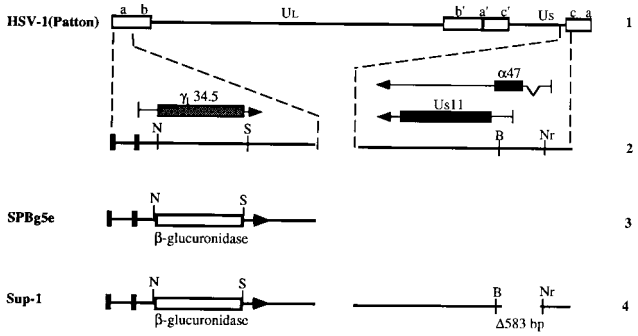

FIG. 1. Schematic representation of the genome structure and sequence arrangements of HSV-1(Patton) and of related mutants. Line 1, the two covalently linked components of HSV-1 DNA, L and S, each consisting of unique sequences (U_L and U_S) flanked by inverted repeats (28, 29). The reiterated sequences flanking U_L (designated *ab* and *b′a′*) are each 9 kb in size, whereas the repeats flanking U_S are 6.3 kb in size (29). Line 2 (left side), enlarged portions of the inverted-repeat sequences *ab* containing the $\gamma_1 34.5$ gene. The filled boxes represent the 20-bp direct repeat sequence (DR1) flanking the *a* sequence (19). The thin line and filled bar represent the transcribed and coding domains of the $\gamma_1 34.5$ gene, respectively, and the vertical line indicates the promoter region. Line 2, right side, expansion of the region of the S component and of terminal repeats containing U_S11 and α47 genes. The thin lines and filled bars represent transcribed and coding regions of the U_S11 and α47 genes, respectively, and the vertical lines indicate the promoter regions. Arrows indicate the directions of transcription. Line 3, enlarged portions of the inverted-repeat sequence *ab* of recombinant virus SPBg5e. The open bar represents the coding sequences of β-glucuronidase. Arrow indicates the direction of transcription. In this virus, a 0.8-kbp *Nco*I-*Sac*I fragment containing the entire coding sequences of the $\gamma_1 34.5$ gene of wild-type HSV-1(Patton) was replaced with a 2.1-kbp fragment harboring the β-glucuronidase gene (20). Although this substitution is not shown diagrammatically, it was made in both the *ab* and the *b′a′* domains of the recombinant genome (20). Line 4, designations are as described above. Recombinant virus Sup-1 was derived from mutant virus SPBg5e (20). In addition to the deletion generated in the coding region of the $\gamma_1 34.5$ gene, this virus had a spontaneous deletion of 583 bp between the *Bst*EII and *Nru*I sites in the junction of the S component and terminal repeats *ca*. The 583-bp deletion disrupted the α47 coding sequences and was presumed to affect the *cis*-acting elements of the U_S11 promoter (20). The uppercase letters indicate restriction sites. N, *Nco*I; S, *Sac*I; *Bst*EII; and Nr, *Nru*I.

Preparation of S10 fractions. Replicate cultures of HeLa cells were either mock infected or infected with HSV-1(Patton)SPBg5e or Sup-1 virus. At 6 h after infection, the cells were harvested, and S10 fractions, which included all cytoplasmic materials, were prepared as described elsewhere (22).

Peptide translocation assays to measure the function of α47. The synthetic peptide TYNRTRALI (single-letter amino acid notation) was synthesized, and its identity was confirmed by sequencing. Approximately 20 μg of the peptide was labeled by the chloramine T method, with 2 mCi of $Na^{125}I$ and a radioiodination kit from ICN Pharmaceuticals Inc., Irvine, Calif. Free iodine was separated from bound iodine by ion-exchange chromatography on AG 1-X8 resin from Bio-Rad. The labeled peptide, at a concentration of 10 μg/ml, was frozen in dry ice and stored at −80°C.

Peptide translocation assays were done as follows. Approximately 3×10^6 embryonic lung fibroblasts were used in each assay by a modified procedure (12, 21). Cells were detached with a mixture of trypsin and EDTA, rinsed three times with Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, and washed once in transport buffer (130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM HEPES). The cells were then resuspended in 80 μl of transport buffer containing 2 U of streptolysin O (SLO; Murex Inc.) per ml at 4°C and then transferred to a water bath at 37°C for 10 min. SLO was omitted from control samples. SLO is a bacterial toxin used in this instance to permeabilize the cells in order to allow the entry of exogenously added labeled peptides. This procedure resulted in >70% permeabilization. The labeled peptide (100 ng/sample) was added to each tube on ice, together with ATP (Sigma) at a final concentration of 5 mM. The reaction mixtures were then incubated at 37°C for 10 min. Cells were transferred on ice and finally lysed by the addition of 100 μl of lysis buffer (100 mM Tris-HCl, 10 mM $MgCl_2$, 1% Nonidet P-40) for 10 min on ice, with occasional vortexing. Nuclei were spun down by centrifugation for 3 min at 14,000 rpm in a microcentrifuge, and the supernatant fluids were added to 100 μl of packed concanavalin A-Sepharose beads (Pharmacia), preequilibrated in a mixture of equal volumes of transport and lysis buffers. After overnight incubation at 4°C, the beads were washed four

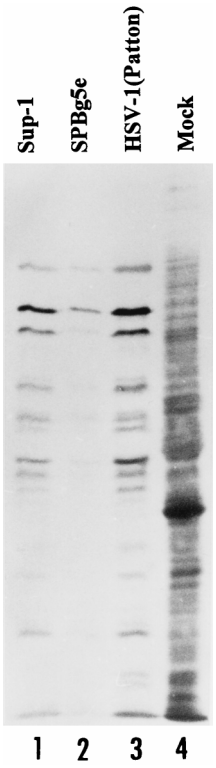

FIG. 2. Autoradiographic images of SK-N-SH cells mock infected or infected with wild-type HSV-1(Patton), SPBg5e, or Sup-1 at 20 PFU per cell in 199V medium (mixture 199 with 1% calf serum) and incubated at 37°C. At 14 h after infection, the cells were overlaid with 1 ml of medium 199V lacking methionine but supplemented with 50 μCi of [^{35}S]methionine (specific activity, >1,000 Ci/mmol; 1 Ci = 37 GBq), incubated for 1 h, harvested, solubilized in disruption buffer containing sodium dodecyl sulfate, subjected to electrophoresis in a 12% polyacrylamide gel cross-linked with *N,N′*-diallyltartardiamide, electrically transferred to a nitrocellulose sheet, and subjected to autoradiography as previously described (2).

times in the same buffer, the bound peptides were eluted by competition with 0.1 M methyl-α-mannoside (Sigma), and the radioactivity was quantitated.

PKR assays. PKR assays were done as described previously (2). Briefly, S10 fractions, which were prepared from replicate cultures of HeLa cells either mock infected or infected with viruses, were incubated with [γ-^{35}P]ATP (100 μCi per sample) for 20 min at 30°C. The fractions were then mixed with antibody to PKR (Santa Cruz Biotechnology). Proteins were precipitated with protein A-agarose beads; washed with phosphate-buffered saline buffer containing 1% Nonidet P40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate; solubilized in disruption buffer; electrophoretically separated in a 12% denaturing polyacrylamide gel; transferred to a nitrocellulose sheet; and exposed to X-ray film.

RESULTS

Protein synthesis is turned off in cells infected with HSV-1(Patton) $\gamma_1 34.5^-$ (designated SPBg5e) but is sustained, at a reduced rate, in suppressor mutant Sup-1. The procedure was the same as that described earlier (6). Replicate cultures of SK-N-SH cells were mock infected or infected with 20 PFU of HSV-1(Patton), SPBg5e, or Sup-1 and incubated in medium containing [^{35}S]methionine for one h at 37°C after infection. The cells were harvested, solubilized, subjected to electrophoresis in denaturing polyacrylamide gels, and analyzed by autoradiography. As shown in Fig. 2, incorporation of labeled methionine into polypeptides was barely detected in cells infected with the $\gamma_1 34.5^-$ virus SPBg5e. The level of incorporation of labeled methionine in cells infected with Sup-1 virus was significantly higher than that in cells infected with SPBg5e. In this and other experiments (data not shown), incorporation of labeled methionine into cells infected with Sup-1 was lower than that observed for wild-type-virus-infected cells.

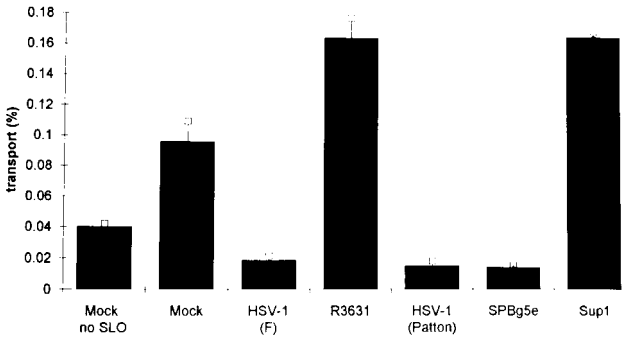

FIG. 3. Results of functional assays for the presence of α47 in the HSV-1 mutants. Transport is measured as percentages of input radioactivity (means of duplicate samples ± standard deviations).

The effects of the deletion in the U_S11-α47 gene domain on the accumulation and or function of these proteins. The suppressor mutants derived by Mohr and Gluzman (20) were reported to contain deletions at or near the junction between U_S and specifically between the genes encoding U_S11 and α47 and the inverted-repeat sequence *ca*. To establish whether these gene products were present, two series of experiments were done.

The purpose of the first series of experiments was to probe the wild-type and mutant viruses for the presence of functions associated with the α47 gene product. Earlier studies (12, 21, 32) have shown that ICP47 binds to the TAP1-TAP2 complex and inhibits the translocation of antigenic peptides into the endoplasmic reticulum for major histocompatibility complex class I-mediated presentation to cytotoxic $CD8^+$ T cells (12, 21, 32). The assay for the translocation of peptides used in this study is described in Materials and Methods and is based on the uptake and glycosylation by permeabilized cells of an ^{125}I-labeled peptide which contains an N-linked glycosylation site (NRT). The addition of the N-linked glycan takes place in the endoplasmic reticulum (12). Glycosylated peptides are then specifically bound to and recovered from Sepharose beads conjugated with the lectin concanavalin A. In this series of experiments, replicate human embryonic lung fibroblast cultures were infected with 10 PFU of HSV-1(Patton), SPBg5e, or Sup-1 per cell. At 9 h after infection, the cells were harvested and processed as described in Materials and Methods. The results of this assay are shown in Fig. 3 as the average percentages of input radioactivity that is glycosylated for duplicate samples ± 1 standard deviation. The results may be summarized as follows.

(i) Exposure of mock-infected cells without permeabilization (SLO [Fig. 3, first bar]) followed by addition of peptides and immediate lysis measured entry of labeled peptides without permeabilization, nonspecific attachment of peptides to proteins which may adhere to the lectin, etc.

(ii) Bars 2, 3, 4, 5, 6, and 7 (Fig. 3) show the percentages of glycosylated peptides recovered from permeabilized mock-infected or virus-infected cells. Cells that were mock infected or infected with HSV-1(F) and R3631 served as controls. The data show that the permeabilized cells infected with HSV-1(Patton) glycosylated half as many peptide as mock-infected, nonpermeabilized cells and at least four times less than the amount of peptides glycosylated by mock-infected, permeabilized cells. In contrast, permeabilized cells infected with Sup-1 glycosylated nearly seven times the amount of peptide glyco-

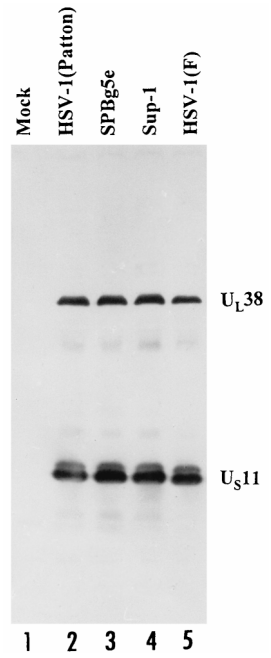

FIG. 4. Photograph of immunoblots of lysates of Vero cells mock infected or infected with parent or mutant viruses, electrophoretically separated on a 15% denaturing polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with anti-U_S11 monoclonal and anti-U_L38 polyclonal antibodies. The cells were harvested 15 h after infection with 10 PFU/cell. The positions of bands representing U_S11 and U_L38 proteins are as indicated on the right.

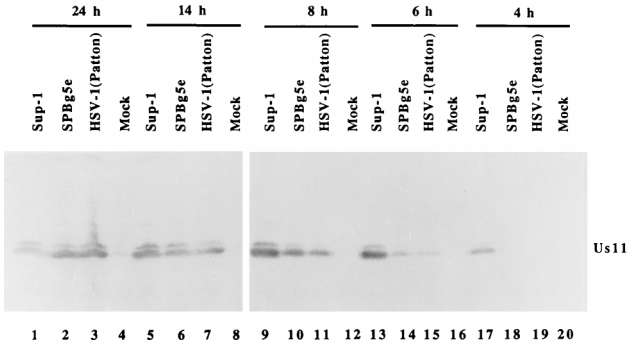

FIG. 5. Photograph of immunoblots of lysates of infected Vero cells harvested at different times after infection with 10 PFU of parent or mutant viruses per cell, electrophoretically separated on a 15% denaturing polyacrylamide gel, electrically transferred to a nitrocellulose sheet, and reacted with anti-U_S11 monoclonal antibody.

sylated by wild-type and $\gamma_1 34.5^-$ viruses. This high level of peptide glycosylation is similar to that observed in permeabilized cells infected with the recombinant R3631 that lacks the α47 gene. We infer from these results that the function of ICP47 was impaired in the Sup-1 mutant.

The objective of the second series of experiments was to characterize the expression of the U_S11 gene in cells infected with the mutant and wild type. In the initial experiments, 4×10^6 cells were mock infected or infected with 10 PFU of HSV-1(Patton), SPBg5e, or Sup-1 per cell. At 15 h after infection, the cells were harvested, solubilized, electrophoretically separated on denaturing gels, electrically transferred to a nitrocellulose sheet, and reacted with polyclonal antibody to U_L38 encoding the capsid protein VP19C and monoclonal antibody to U_S11 (25). As shown in Fig. 4, all of the viruses tested made equivalent amounts of both U_L38 and U_S11. We infer from these data that the deletion does not extend into the coding domain of the U_S11 gene and has no effect on the overall accumulation of U_S11 protein.

Next, we determined the kinetics of expression of the U_S11 protein. In this series of experiments, replicate cultures of Vero cells were mock infected or infected with 10 PFU of the wild-type or mutant viruses. At 4, 6, 8, 14, or 24 h after infection, the cells were harvested and processed as described above and reacted with the anti-U_S11 antibody. The results shown in Fig. 5 indicate that U_S11 expression was detected in cells infected with Sup-1 but not in those infected with HSV-1(Patton) or SPBg5e at 4 h after infection (lanes 17 to 20). At 6 and 8 h after infection, the level of U_S11 was highest in Sup-1-infected cells and lowest in cells infected with the wild-type parent virus (Fig. 5, lanes 9 to 16). The amounts of U_S protein reacting with the U_S11 antibody were approximately the same at 14 h after infection or slightly diminished in cells infected with the Sup-1 mutant at 24 h after infection. These results are consistent with the hypothesis that the deletion in the Sup-1 gene brought the α promoter of the α47 gene into the immediate proximity of the U_S11 coding sequences and altered the kinetics of expression of the U_S11 gene.

Phosphorylation of PKR in cells infected with wild-type and mutant viruses. Earlier studies (2) have shown that antibody to PKR precipitated an activated PKR and one additional phosphorylated protein with an apparent M_r of 200,000 from lysates of cells infected with wild-type virus. In contrast, the precipitate from uninfected cell lysates contained no activated PKR, whereas the precipitate from cells infected with the $\gamma_1 34.5^-$ virus R3616 contained the activated PKR (M_r, 68,000), the phosphoprotein with an M_r of 200,000, and an additional phosphorylated protein with an M_r of 90,000. The objective of the present studies was to determine the state of the PKR and of the associated proteins with M_rs of 200,000 and 90,000 in cells infected with the HSV-1(Patton) strain and derivative mutants.

Figure 5 shows one of several series of experiments that were done as described in Materials and Methods. In all studies, the antibody to PKR brought down from lysates of cells infected with the wild type, the $\gamma_1 34.5^-$ mutant, and the Sup-1 viruses phosphorylated proteins with apparent M_rs of 68,000 and approximately 200,000, respectively. In the autoradiographic image shown in Fig. 6, p200 formed two bands, although in most experiments only a single band was resolved. These two phosphoproteins were not phosphorylated in precipitates of uninfected cells. In all of these experiments, p90 was heavily phosphorylated only in extracts of cells infected with $\gamma_1 34.5^-$ viruses. In some but not all experiments, the antibody to PKR also precipitated trace amounts of p90 from lysates of cells infected with Sup-1 virus or with wild-type virus. It should be stressed that the failure of PKR, p90, and p200 to be phosphorylated in lysates of mock-infected cells is highly reproducible, as is the phosphorylation of p90 in $\gamma_1 34.5^-$-infected cells. The phosphorylation of PKR and of p200 in infected cells was highly reproducible, but the relative amounts of each varied from one experiment to the next.

We conclude from these studies that the phenotype of Sup-1 approximates that of wild-type virus with respect to sustained protein synthesis, effective block of phosphorylation of eIF-2α by activated PKR, and absence of phosphorylated p90 in anti-PKR immune complexes.

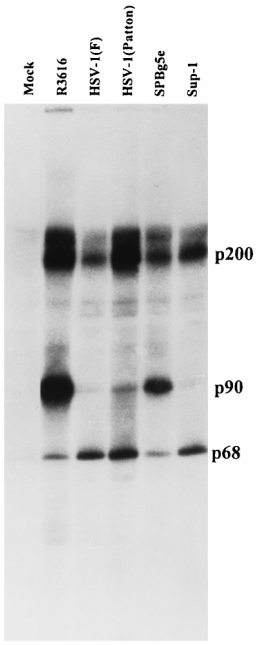

FIG. 6. Autoradiographic images of electrophoretically separated immune precipitate of PKR S10 fractions (22) prepared from replicate cultures of HeLa cells either mock infected or infected for 6 h with wild-type HSV-1(Patton) or SPBg5e or Sup-1 mutant were incubated with [γ-^{35}P]ATP (100 μCi per sample) for 20 min at 30°C. Fractions were then mixed with antibody to PKR (Santa Cruz Biotechnology). The proteins were precipitated with protein A-agarose beads, washed with phosphate-buffered saline buffer, solubilized in disruption buffer, electrophoretically separated in a denaturing polyacrylamide gel, transferred to a nitrocellulose sheet, and exposed to X-ray film. The molecular weights of p68 (PKR), p90, and p200 were calculated from the electrophoretic mobilities of molecular weight markers.

DISCUSSION

Earlier studies have shown that PKR is activated in cells infected with wild-type virus but that the α subunit of the translation initiation factor eIF-2 is not phosphorylated. In cells infected with genetically engineered $\gamma_1$34.5$^-$ viruses, a protein with an apparent M_r of 90,000 (p90) is phosphorylated, the eIF-2α is phosphorylated, and protein synthesis is shut off (2). The shutoff of protein synthesis and, consequently, the phosphorylation of eIF-2α are induced after the onset of viral DNA synthesis. These studies raised the possibility that there exists an alternate pathway to prevent the shutoff of protein synthesis by activated PKR. This issue is of particular interest, since the $\gamma_1$34.5 gene is not highly conserved among herpesviruses and, therefore, herpesviruses lacking this gene must have evolved alternative pathways to block the activation of PKR.

Mohr and Gluzman (20) reported that serial passage of a $\gamma_1$34.5$^-$ virus in a human neuroblastoma cell line yielded a series of spontaneous mutants capable of sustained viral protein synthesis and enhanced multiplication in these cells. The suppressor mutation was mapped to a sequence encompassing a variable size deletion at the junction between the right terminus of the U_S and the inverted repeat *ca* flanking that sequence. In recent studies, we (1a) genetically engineered a virus similar to the Sup-1 mutant with respect to genotype and phenotype without serial passage under highly selective conditions in human cells. The rationale for the studies described in this report were based on observations that further analyses of suppressor mutations may shed light on alternative pathways whereby HSV-1 may block phosphorylation of eIF-2α.

This article reports two sets of experiments. First, we attempted to determine the status of the genes flanking the sequences spontaneously deleted from the Sup-1 mutant. Our results show that the function attributed to α47 is not expressed by the Sup-1 mutant. We also showed that all of the viruses tested make U_S11 in amounts comparable to those of cells infected with the wild-type parent and that the electrophoretic properties of the protein made in Sup-1-infected cells could not be differentiated from those made by wild-type and parent viruses. However, the U_S11 protein was made at an earlier time after infection, which is consistent with the juxtaposition of the promoter of the α47 gene next to the coding sequence of the U_S11 gene. These findings are consistent with the report that the sequences deleted from the Sup-1 genome map upstream of the coding sequences of U_S11 but include those of the α47 gene (20). In addition, we showed that the phenotype of the Sup-1 mutant in human SK-N-SH cells is clearly differentiated from those of the $\gamma_1$34.5$^-$ parent.

The second set of experiments concerned the status of the PKR and of the associated proteins. Our studies showed that the PKR is activated in cells infected with every one of the viruses tested in this study. p90 was highly phosphorylated in cells infected with $\gamma_1$34.5$^-$ and was phosphorylated at most in trace amounts only in cells infected with Sup-1 or wild type virus. The phenotype of the Sup-1 virus was therefore similar to that of wild-type virus with respect to sustained protein synthesis, the effective block of phosphorylation of the α subunit of the translation initiation factor eIF-2, and the absence of phosphorylated p90 in anti-PKR immune complexes.

Our initial expectations regarding the mechanism of suppression by Sup-1 were twofold. The first was that Sup-1 contains a mutation which disables the ability of the virus to induce activation of PKR. Based on studies done earlier (14, 15), it could be argued, for example, that a large fraction of the viral genome is transcribed symmetrically, yielding RNA capable of annealing into double-stranded RNA, that a specific subset symmetric RNAs arising from the domain of the U_S11-α47 is particularly competent in activating PKR, and that the spontaneous deletion ablates the formation of this RNA. This does not appear to be the case for two reasons. First, in earlier studies, Roller and Roizman (20) reported that U_S11 binds in a sequence- and conformation-specific fashion an RNA sequence identical to the sequence antisense to that of the promoter domain of the U_S11 gene. All efforts to detect the existence of that RNA proved to be fruitless. Second, and perhaps more appropriate, the data presented in this report show that the mutation in the Sup-1 virus does not prevent the activation of PKR.

The second mechanism by which the mutation in Sup-1 could block total shutoff of protein synthesis would be through a compensatory mutation in another gene. For example, if the product of gene X were to interact with $\gamma_1$34.5 to block the pathway leading to the phosphorylation of eIF-2α, a mutation in protein X may partially compensate for the absence of $\gamma_1$34.5 gene product. This does not appear to be the case here, since deletions in the U_S11-α47 domain is the prevailing genotype of the Sup-1 mutants.

The third formal hypothesis which could be advanced to explain the phenotype of the Sup-1 mutant is that a product of the deletion interacts with the activated PKR and blocks the phosphorylation of eIF-2α. In this instance, the only product apparent from our studies is the conversion of the U_S11 gene from a γ_2 protein to a protein accumulating very early in infection as a consequence of the juxtaposition of the α47 promoter with the coding domain of the U_S11 gene. This juxtaposition resulted in the synthesis of U_L11 protein early in infection and the appearance of a novel mRNA containing 5′

noncoding domains of the α47 gene linked to the coding domain of the U_S11 gene. The hypothesis that U_S11 made early in infection blocks activated PKR from phosphorylating eIF-2α since the kinetics of shutoff of protein synthesis are similar to those of U_S11 protein synthesis in wild-type-virus-infected cells (Fig. 5). The possibility that the altered α47-U_S11 mRNA takes on a secondary structure which binds to the activated PKR and renders it nonfunctional cannot be refuted, although Mohr and Gluzman reported that the deletions in their U_S11-α47 domain were highly variable in size.

The results were not concordant with these expectations. One possible alternative, that a viral function which maps in the domain of the α47 gene and which enhances rather than prevents the eventual phosphorylation of eIF-2α independent of PKR, remains to be explored.

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