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Differential Response of Human Cells to Deletions and Stop Codons in the $\gamma_1$34.5 Gene of Herpes Simplex Virus

JOANY CHOU, ALICE P. W. POON, JENNIFER JOHNSON, AND BERNARD ROIZMAN*

The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, Chicago Illinois 60637

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Earlier studies have shown that herpes simplex virus mutants lacking the $\gamma_1$34.5 gene are totally avirulent on intracerebral inoculation of the virus into mice and induce premature shutoff of protein synthesis in human neuroblastoma (SK-N-SH) cells but not in Vero cells. We report the following. (i) Whereas deletion mutant R3616, lacking 1,000 bp of the $\gamma_1$34.5 gene, caused premature shutoff of protein synthesis in both SK-N-SH and human foreskin fibroblasts (HFF), mutants R4009 and R930 (mutant F), carrying stop codons in all six frames, 27 and 210 codons from the initiation codon of the $\gamma_1$34.5 genes, respectively, induced shutoff of protein synthesis in SK-N-SH cells but not in HFF. The differences in behavior between the R3616 deletion and R4009 stop codon mutants cannot be attributed to differences in the rate of induction of premature shutoff of protein synthesis and the multiplicity of infection. HFF do not produce detectable truncated $\gamma_1$34.5 protein or truncated mRNA. (ii) Some clonal lines of SK-N-SH cells carrying a $\gamma_1$34.5 gene driven by a metallothionein promoter express the $\gamma_1$34.5 gene constitutively and do not require induction by cadmium to complement the $\gamma_1$34.5$^-$ virus. One clonal cell line complements the $\gamma_1$34.5$^-$ virus only after induction by cadmium. These results are consistent with previous conclusions that the phenotype of premature shutoff of protein synthesis is associated with absence of the $\gamma_1$34.5 protein and indicate that the amounts of $\gamma_1$34.5 protein necessary to complement the $\gamma_1$34.5$^-$ viruses are small. We conclude that human cells differ in the manner in which they respond to the presence of stop codons. Shutoff of protein synthesis in HFF infected with the stop codon mutants could have been precluded by small amounts of $\gamma_1$34.5 protein produced by splicing out of an intron containing the stop codon, downstream initiation of translation, or tRNA suppression of the stop codon.

Several years ago, a report from this laboratory described a most unusual herpes simplex virus 1 (HSV-1) open reading frame (ORF). This ORF, designated $\gamma_1$34.5, is located in the *ab* and *b'a'* inverted repeat sequences flanking the long (L) component of the HSV-1 DNA and is therefore present in two copies per genome (5). Its TATAA-less promoter was contained in the terminal *a* sequence. The nucleotide sequence of the HSV-1 strain F [HSV-1(F)] $\gamma_1$34.5 gene predicted a protein of 263 amino acids consisting of a 159-amino-acid amino-terminal domain, 10 repeats of the amino acids Ala, Thr, and Pro, and a 74-amino-acid carboxyl-terminal domain. Because the number of triplet repeats varied from strain to strain, its role was most likely that of a linker or swivel region of the protein (6). A genetically engineered mutant lacking 1,000 bp of the coding domain replicated in Vero cells to the same level as the wild-type parent HSV-1(F) and the recombinant virus in which the deleted sequences were restored [HSV-1(F)R]. The deletion mutant did not multiply in the central nervous systems of mice given the virus by the intracerebral route, although limited multiplication was seen in mice inoculated by other routes (4). The phenotypic properties of another mutant, R4009, which carries a stop codon in all six frames inserted between codons 27 and 28 of the $\gamma_1$34.5 gene, were identical to those of the R3616 deletion mutant (4).

A remarkable property of the R3616 and R4009 mutants emerged in the course of studies of their replication in human neuroblastoma cell line SK-N-SH. Whereas the phenotype of Vero cells infected with these mutants could not be differentiated from that of those infected with the wild-type or repaired virus, in SK-N-SH cells infected with the R3616 and R4009 mutants, there was a premature, total shutoff of protein synthesis (7). The shutoff of protein synthesis was triggered by the onset of viral DNA synthesis inasmuch as in infected cells treated with phosphonoacetate, a specific inhibitor of viral DNA synthesis, the premature shutoff of protein synthesis did not ensue. Premature shutoff of protein synthesis was therefore a cell type-specific stress response induced by the onset of viral DNA synthesis in the absence of the $\gamma_1$34.5 gene product (7).

The predicted amino acid sequence of the $\gamma_1$34.5 gene is partially homologous to two eukaryotic genes, MyD116 and GADD34, which are members of a diverse family known as the growth arrest and DNA damage (GADD) genes. The various members of this family of genes are expressed in cells whose growth is arrested as a consequence of differentiation or whose DNA is damaged. The function of these genes appears to be to cause growth arrest and protect cells from programmed cell death (10). Thus, MyD116, the first of the homologs described, is induced by interleukin 6 in murine myeloid leukemia cells. The MyD116 protein is predicted to consist of 657 amino acids comprising a long amino-terminal domain, a sequence of 38 amino acids repeated 4.5 times, and a short carboxyl-terminal domain (14). The size and sequence arrangement of the predicted product of GADD34, a hamster gene, are similar to those of the MyD116 protein. A stretch of 64 amino acids of the carboxyl-terminal domain of the $\gamma_1$34.5 protein shows a high degree of homology to the corresponding domains of the MyD116 and GADD34 proteins. Analyses of a series of in-frame deletions and insertions in the $\gamma_1$34.5 gene have shown that the carboxyl terminus of the $\gamma_1$34.5 gene is necessary to preclude premature shutoff of protein synthesis and thus suggest a relationship between the functions of the GADD and $\gamma_1$34.5 genes (8).

It has also been reported that the strand antisense to the

* Corresponding author. Mailing address: The Marjorie B. Kovler Viral Oncology Laboratories, The University of Chicago, 910 E. 58th St., Chicago, IL 60637. Phone: (312) 702-1898. Fax: (312) 702-1631.

$\gamma_1$34.5 gene is transcribed in cells in which the major regulatory protein, infected cell protein 4 is not expressed or is inactive. Work done in this laboratory recently showed that an ORF designated ORF-P that is contained in the transcribed domain is expressed. The ORF is antisense but overlaps the coding domain of the $\gamma_1$34.5 gene, and therefore all mutations and deletions introduced into the $\gamma_1$34.5 gene would also appear as deletions in ORF-P (13).

This report concerns two observations relevant to the function of the $\gamma_1$34.5 gene. First, we report that $\gamma_1$34.5 deletion mutant R3616, but not mutant R4009, carrying the stop codon after codon 27, induces premature shutoff of protein synthesis in human foreskin fibroblasts (HFF). The discordance between SK-N-SH and HFF cultures was also noted in another mutant, mutant F (8), carrying a stop codon at codon 210 of the $\gamma_1$34.5 gene. These studies indicate that interaction of cellular machinery with stop codons may be cell type dependent.

Second, we report that SK-N-SH clonal cell lines carrying the $\gamma_1$34.5 gene fused to a mouse metallothionein promoter and expressing the gene after cadmium induction complemented the R3616 mutant in precluding premature shutoff of protein synthesis. The results of these experiments support the conclusion that premature shutoff of protein synthesis is due to absence of the $\gamma_1$34.5 gene product in HSV-infected cells.

MATERIALS AND METHODS

Cells. The Vero and human neuroblastoma SK-N-SH cell lines used were obtained from the American Type Culture Collection. The HFF cell strain used was obtained from George Kemble (Aviron Inc., Burlingame, Calif.). The cells were propagated in Dulbecco's modified Eagle's medium supplemented with 5% (Vero cells) or 10% (SK-N-SH cells and HFF) fetal bovine serum.

Viruses. HSV-1(F) is the prototype HSV-1 strain used in this laboratory (9). The construction and phenotype of all other $\gamma_1$34.5 gene mutants have been described elsewhere (4, 7, 8). They were as follows (Fig. 1). R3616 contains a 1-kbp deletion in the coding domain of the $\gamma_1$34.5 gene. In recombinant HSV-1(F)R, the $\gamma_1$34.5 sequences missing from R3616 had been restored. The phenotype and genotype of this virus are those of the wild-type parent, HSV-1(F). In R4009, a stop codon was inserted at amino acid 28 of the coding sequence. R4003 (mutant A) contains an insertion of 17 codons in frame with the first codon of the authentic $\gamma_1$34.5 genes. In R4002 (mutant B), 28 codons of the $\gamma_1$34.5 gene were deleted and replaced with the initiation codon of the gene specifying glycoprotein H. In R931 (mutant C), 39 codons were deleted in frame after codon 31. In R908 (mutant D), 41 codons were deleted in frame after codon 72. In R909 (mutant E), 39 codons were deleted after codon 107. In R939 (mutant F), a sequence encoding a stop codon in all six ORFs was inserted at codon 11 after the last three amino acid repeats. All of the mutations introduced were present in both copies of the $\gamma_1$34.5 gene.

Plasmids. Plasmid pMK, containing the metallothionein gene promoter fused to the HSV-1 thymidine kinase gene (*tk*), was the generous gift of R. Palmitter. The *Eco*RI-*Bgl*II fragment of pMK, containing only the promoter sequence, was joined to the $\gamma_1$34.5 leader sequence at the *Bam*HI site 100 bp upstream of the ATG initiation codon, near the transcription initiation site (5). The chimeric fragment was then subcloned into vector pSV2-neo between *Eco*RI and *Bam*HI restriction sites as shown in Fig. 1C.

[^{35}S]methionine labeling of infected-cell extracts. The procedure used for labeling of infected-cell proteins with [^{35}S]methionine has already been described (7). Briefly, replicate cultures were either mock infected or exposed to the viruses described in Results for 1 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 12 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).

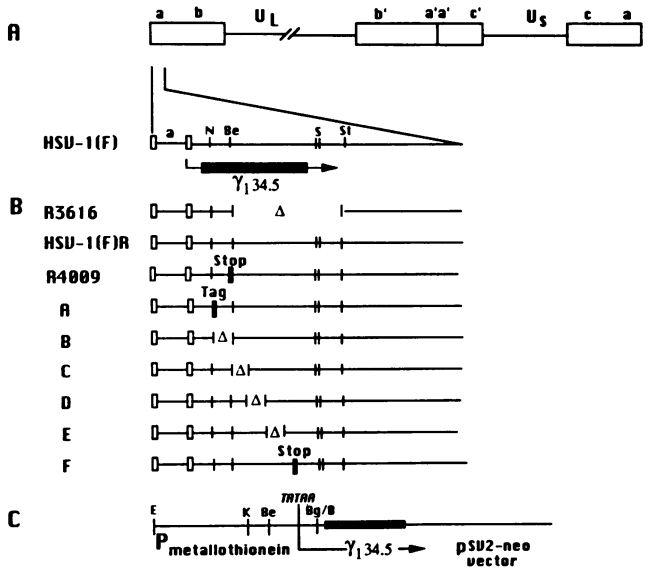

FIG. 1. (A) Schematic diagram of the HSV-1 genome and the location of the $\gamma_1$34.5 gene and its coding region. $\gamma_1$34.5 is present in two copies of the viral genome: in the junction between the L and S components (not shown) and at the terminus of the L component, as shown in the diagram (5). *ab* and *b'a'* represent the inverted repeats flanking the unique sequences of the L component (U_L), whereas *a'c'* and *ca* represent the inverted repeats flanking the S component (U_S) (11, 19, 20). The $\gamma_1$34.5 promoter is contained within the terminal 500-bp *a* repeat (5). The vertical rectangles represent 20-bp direct-repeat (DR1) sequences of the *a* sequence (16). The solid bar and the arrow indicate the coding region and the direction of transcription of $\gamma_1$34.5. (B) Schematic representation of the deletions and stop codons within the coding sequence of the $\gamma_1$34.5 gene. The construction and properties of these mutants have been described previously (4, 7, 8). The triangle indicates the deletion domain. Stop indicates the six-frame termination codons inserted at the *Bst*EII (R4009 virus) and *Dra*III (R939 virus) sites. Tag represents a 17-amino-acid codon insertion at the methionine translation initiation codon. (C) Plasmid construct of the pMet-$\gamma_1$34.5 gene. The $\gamma_1$34.5 sequence was fused to the promoter of the mouse metallothionein gene in a pSV2-neo vector. TATA indicates the proximal location of transcription initiation. The solid bar indicates the coding sequence of the $\gamma_1$34.5 gene. N, Be, S, St, E, K, Bg, and B are abbreviations for the restriction enzyme cleavage sites *Nco*I, *Bst*EII, *Sac*I, *Stu*I, *Eco*RI, *Kpn*I, *Bgl*II, and *Bam*HI, respectively.

RNA extraction and Northern (RNA) blotting. RNAs were extracted from cells that were either mock infected or infected with wild-type virus HSV-1(F) or mutant R4009 at 13 h after infection by procedures using guanidinium thiocyanate (18). Poly(A)$^+$ RNAs which represent approximately 5% of total RNAs were purified with a kit (PolyATract mRNA isolation

system; Promega, Madison, Wis.). Approximately 7 to 8 μg of poly(A)$^+$ RNA was then electrophoretically separated on a 1.5% agarose-formaldehyde gel, transferred to a nitrocellulose sheet, probed with an antisense $\gamma_1$34.5 RNA probe synthesized in vitro with an SP6-directed RNA transcription kit (Promega), and subjected to autoradiography.

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 rabbit polyclonal antibody R4 made against the Ala–Thr–Pro-10 repeats of the $\gamma_1$34.5 protein (1).

RESULTS

The ability of $\gamma_1$34.5 deletion mutant R3616 and stop codon mutant R4009 to induce premature shutoff of protein synthesis is cell line specific. Previously we have shown that all protein synthesis, both cellular and viral, is prematurely shut off in human neuroblastoma cells (SK-N-SH) infected with HSV-1(F) mutant R3616, in which 1 kb of the coding domains of both copies of the $\gamma_1$34.5 gene has been deleted, or with the R4009 mutant, in which the $\gamma_1$34.5 protein has been truncated by a stop codon insertion at amino acid 28 (7). A remarkable observation which we report here is that in HFF cells, shutoff of protein synthesis was observed after infection with deletion mutant R3616 but not after infection with stop codon mutant R4009. In this series of experiments, SK-N-SH, Vero, and HFF cells were infected at equal multiplicities, as described in Materials and Methods, with HSV-1(F), R3616, R4009, and HSV-1(F)R. The infected cells were labeled for 1 h with [^{35}S]methionine at 12 h after infection and then harvested and processed as described in Materials and Methods. As shown in Fig. 2, protein synthesis was detected in Vero cells infected with all of the strains tested, in HFF infected with all of the strains except deletion mutant R3616, and in SK-N-SH cells infected with the wild-type and repaired viruses but not in cells infected with either the R4009 or R3616 mutant virus.

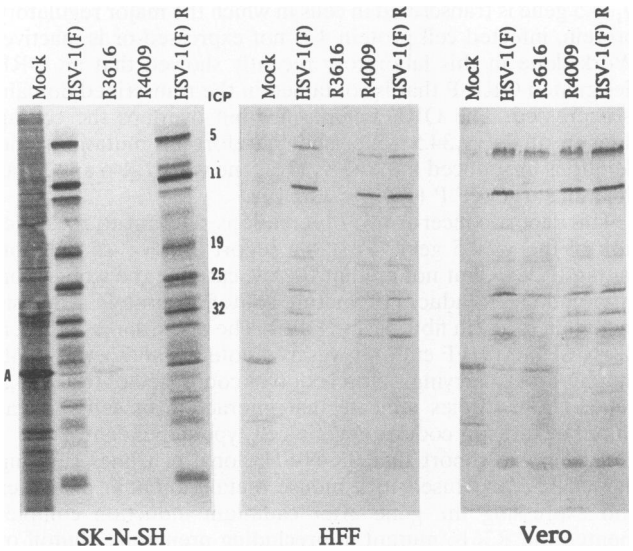

FIG. 2. Autoradiographic image of electrophoretically separated, [^{35}S]methionine-labeled proteins from lysates of cells infected with the wild-type virus or mutant viruses. The cells were infected with 10 PFU per cell and labeled at 12 h after infection for 1 h. The procedures for infection, [^{35}S]methionine labeling, preparation of cell lysates, electrophoresis in *N,N'*-diallyltartardiamide–polyacrylamide gels, electrical transfer of proteins to nitrocellulose sheets, and autoradiography have been previously described (7). Infected-cell protein (ICP) numbers were assigned in accordance with Honess and Roizman (12) and Morse et al. (17). Mock, mock infection; A, actin.

The temporal patterns of premature shutoff of protein synthesis by deletion mutant R3616 and stop codon mutant R4009 in neuroblastoma cells are virtually identical. To determine whether the discordance observed in HFF could be a reflection of the temporal pattern of shutoff of protein synthesis, we selected SK-N-SH cells in which shutoff of protein synthesis follows infection with either virus. In this series of experiments, we examined the temporal pattern of premature shutoff of protein synthesis in neuroblastoma cells either mock infected or infected with wild-type virus HSV-1(F), deletion virus R3616, and stop codon virus R4009 at 2-h intervals from 5 to 20 h after infection. As shown in Fig. 3, premature shutoff of protein synthesis in cells infected with deletion mutant R3616 or stop codon mutant R4009 was at least partial at 5 h after infection and complete by 11 h after infection. In this series of experiments, no significant difference between the phenotypes of the deletion and stop codon mutants was apparent.

The discordance between the stop codon and the deletion mutants in HFF is not due to differences in multiplicity of infection. In a series of experiments, replicate HFF cultures were exposed to R3616 at 15 to 250 PFU per cell or to R4009 at 20 to 1,000 PFU per cell. The cultures were processed as described above. The cells were labeled for 1 h at 12 h after infection and then harvested and processed as described in Materials and Methods. The results shown in Fig. 4 indicate that the effect of R4009 on viral protein synthesis was independent of the multiplicity of infection. We conclude that the differences in the premature shutoff of protein synthesis seen in the infected HFF cultures described above are intrinsic properties of the cells; i.e., the results described above are due solely to the fact that SK-N-SH cells are affected by the presence of the stop codon in the $\gamma_1$34.5 genes of R4009 whereas HFF are not.

Protein synthesis activities in cells infected with $\gamma_1$34.5 partial deletion viruses and C terminus stop codon mutant virus F. Elsewhere we reported that the carboxyl terminus domain of the $\gamma_1$34.5 protein is required to preclude premature shutoff of protein synthesis in SK-N-SH cells (8). In that study, we tested a series of mutants generated by in-frame deletions or insertion of a stop codon 11 codons after the Ala–Thr–Pro-10 repeats (Fig. 1). In light of the results described above, it was of interest to determine the phenotype of these mutants in HFF cultures. In this series of experiments, replicate SK-N-SH or HFF cultures were infected with 10 PFU of wild-type or mutant virus per cell. At 12 h after infection, the cells were labeled for 1 h with [^{35}S]methionine and then harvested and processed as described in Materials and Methods. The results (Fig. 5) were as follows. (i) As previously reported, there was no premature shutoff of protein synthesis in Vero cells infected with wild-type and mutant viruses. Similarly, there was no premature shutoff of protein synthesis in SK-N-SH cells infected with the wild-type virus or mutant A, B, C, D, or E. Premature shutoff of protein synthesis was observed in cells infected with mutant R3616 or F. (ii) In HFF cultures, premature shutoff of protein synthesis was observed only following infection of the cells with deletion mutant R3616.

Once again, HFF and SK-N-SH cultures yielded discordant results. In this instance again, SK-N-SH cells were affected by the presence of the stop codon in the domain encoding the

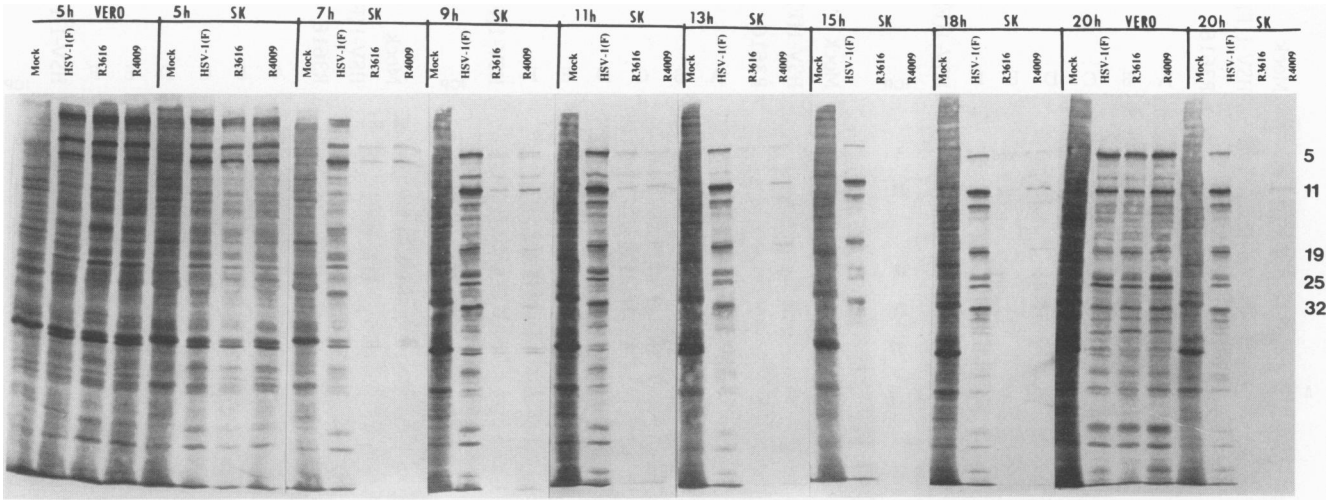

FIG. 3. Temporal pattern of labeling of proteins in Vero and SK-N-SH cells either mock infected or infected with the wild-type virus or mutant R3616 or R4009. The cells were labeled with [^{35}S]methionine for 1 h at 5, 7, 9, 11, 13, 15, 18, and 20 h after infection. The major protein bands formed by lysates of infected cells are identified by the infected-cell protein numbers on the right.

carboxyl-terminal 80 amino acids of the $\gamma_1$34.5 gene, whereas HFF were not.

Deletion and stop codon mutants do not express detectable quantities of $\gamma_1$34.5 proteins or of truncated mRNA. In the first of two series of experiments, HFF, SK-N-SH, and Vero cells were infected with HSV-1(F), R3616, or R4009 virus. At 20 h after infection, the cells were harvested and assayed for the presence of $\gamma_1$34.5 protein as described in Materials and Methods. The results shown in Fig. 6 indicate that cells infected with R3616 or R4009 do not make detectable amounts of protein reactive with the rabbit polyclonal antibody to the Ala–Thr–Pro-10 sequence characterized in a preceding publication. We have previously shown that this antibody reacts with a variety of truncated $\gamma_1$34.5 proteins.

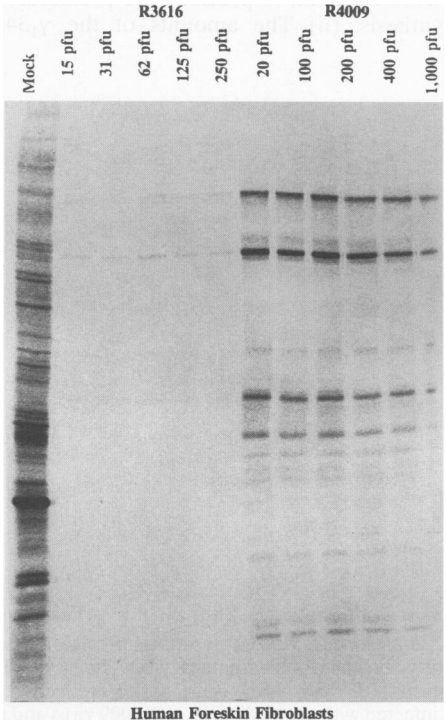

FIG. 4. Autoradiographic image of [^{35}S]methionine-labeled proteins in HFF cells either mock infected or infected with virus R3616 or R4009 at increasing multiplicities of infection. The number of PFU of virus per cell in each infection is shown.

In the second series of experiments, RNA was extracted from HFF, SK-N-SH, or Vero cells infected with the same viruses. The RNA was electrophoretically processed and hybridized to a $\gamma_1$34.5 DNA probe as described in Materials and Methods. Figure 7 shows that cells infected with the deletion mutant or the stop codon mutants accumulated two species of RNA reactive with a $\gamma_1$34.5-specific probe. One RNA (A band), with a predicted length of 5 kb, consists of an RNA which contains both $\gamma_1$34.5 and α0 sequences. The second RNA (B band) is 1.3 kb long and contains $\gamma_1$34.5 sequences only. Both RNAs have been previously described (5).

The results of these experiments revealed no detectable quantities of either a truncated RNA that could express a truncated form of $\gamma_1$34.5 protein or the presence of a truncated $\gamma_1$34.5 protein that can contribute to the partial protein synthesis in R4009 stop codon mutant virus-infected HFF cells.

The SK-N-SH cell line, which is capable of expressing the $\gamma_1$34.5 protein, complements the $\gamma_1$34.5$^-$ phenotype of the R3616 deletion mutant. In the first of two series of experiments, SK-N-SH cells were transfected with pRB3210 and plated in the presence of G418 (400 μg/ml of medium). The resulting G418-resistant clones were subcloned and tested for the presence of $\gamma_1$34.5 protein (Fig. 8) and the ability of the $\gamma_1$34.5 gene, by cadmium sulfate (50 μM) induction, to restore protein synthesis in cells infected with the R3616 virus (Fig. 9). Three clonal cell lines carrying the $\gamma_1$34.5 gene and a line carrying only the G418 resistance marker were selected for these studies. As shown in Fig. 8, clonal lines Pmet-$\gamma_1$34.5-11 and Pmet-$\gamma_1$34.5-1 expressed the $\gamma_1$34.5 protein at relatively high levels after cadmium induction and at lower levels in the absence of cadmium. The Pmet-$\gamma_1$34.5-2 cell line expressed barely detectable levels of the $\gamma_1$34.5 protein in the presence of cadmium, and expressed no detectable $\gamma_1$34.5 protein in uninduced cells.

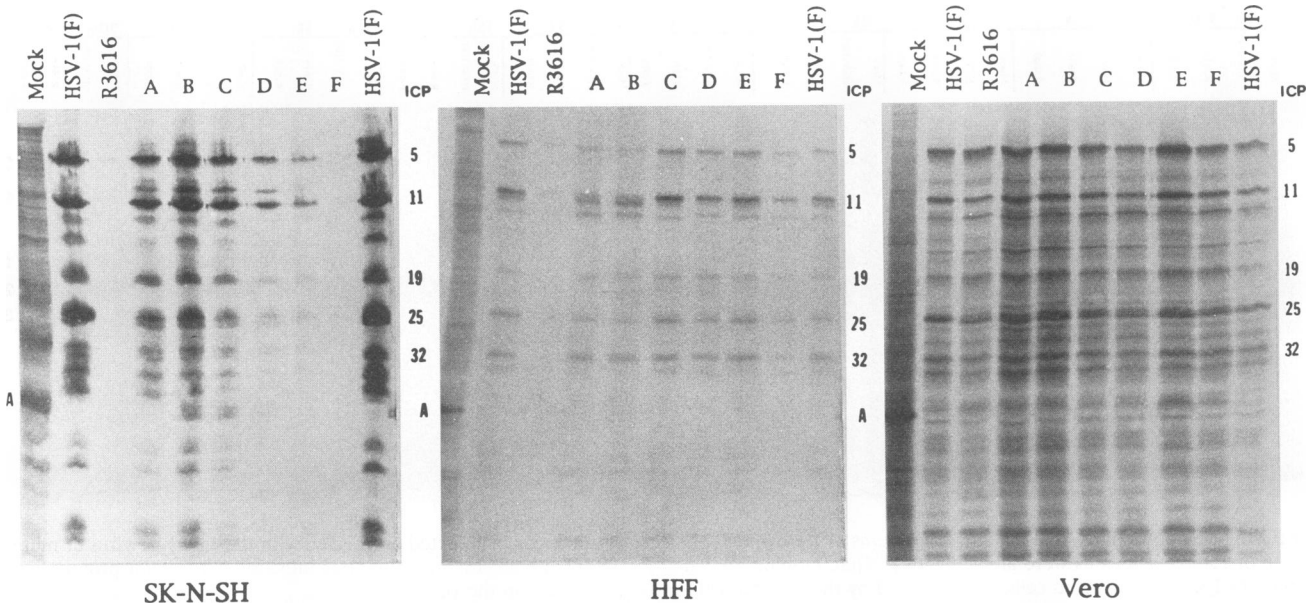

FIG. 5. Autoradiographic image of [^{35}S]methionine-labeled proteins in SK-N-SH cells, HFF, and Vero cells either mock infected or infected with wild-type virus HSV-1(F) or deletion mutant R3616, A, B, C, D, E, or F. ICP, infected-cell protein; A, actin.

In the second series of experiments, parent and clonal lines were infected with the wild type and the deletion mutant in the presence or absence of cadmium. The key findings, shown in Fig. 9, were that protein synthesis was shut off by the R3616 virus in the parent cell line and in cells carrying the G418 resistance marker. Protein synthesis was present at nearly the wild-type level when the R3616 virus infected the Pmet-$\gamma_1$34.5-11 and Pmet-$\gamma_1$34.5-1 clonal cell lines in the absence of cadmium and was enhanced in both cell lines when cadmium was added to the medium. The significant finding is that in the Pmet-$\gamma_1$34.5-2 clonal cell line, which did not express the $\gamma_1$34.5 protein at a detectable level in the absence of cadmium (Fig. 8), protein synthesis was shut off by the R3616 virus to the same level as in control cell lines. This premature shutoff of protein synthesis in the Pmet-$\gamma_1$34.5-2 clonal cell line infected with R3616 was precluded by addition of cadmium to the medium.

We conclude the following. (i) Induction of the activity which precluded the shutoff of protein synthesis was tied to the induction of the metallothionein promoter by cadmium. Consistent with these results, the $\gamma_1$34.5 protein in expressor cell lines complemented the deletion in the $\gamma_1$34.5 gene of the R3616 virus in infection to preclude the premature shutoff of protein synthesis. (ii) The amounts of the $\gamma_1$34.5 protein

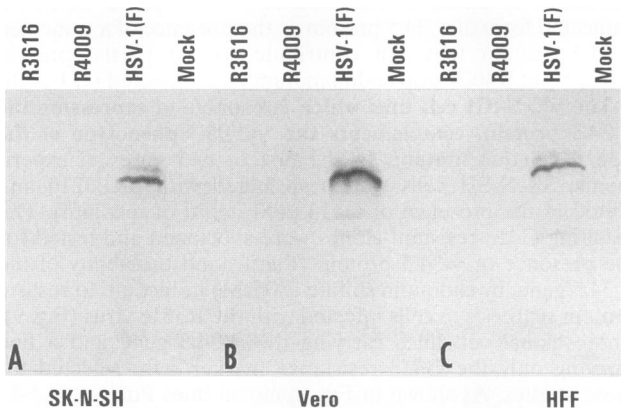

FIG. 6. Photographic image of $\gamma_1$34.5 proteins in infected-cell extracts, visualized by staining with polyclonal antibody R4. SK-N-SH cells, HFF, and Vero cells were either mock infected or infected with R3616, R4009, or wild-type virus HSV-1(F) for 24 h. The procedures used for harvesting of extracts, electrophoresis, transfer, and immunoblotting are described in Materials and Methods.

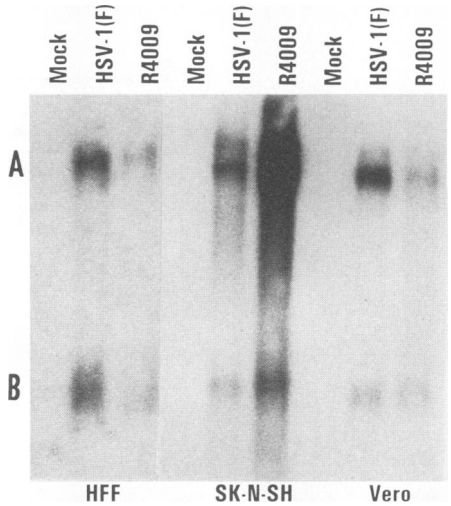

FIG. 7. $\gamma_1$34.5-specific RNA transcripts in cells infected with the wild-type virus or the R4009 mutant virus. Poly(A)$^+$ RNAs were extracted from HFF, SK-N-SH cells, and Vero cells either mock infected or infected with the HSV-1(F) or R4009 virus and probed with an antisense RNA probe specific for $\gamma_1$34.5. A, 5-kb transcript initiated from the *a* sequence promoter of $\gamma_1$34.5 and 3′ coterminate with that of α0 transcripts; B, 1.3-kb transcript of the $\gamma_1$34.5 coding sequence only. Both transcripts have been reported previously (5).

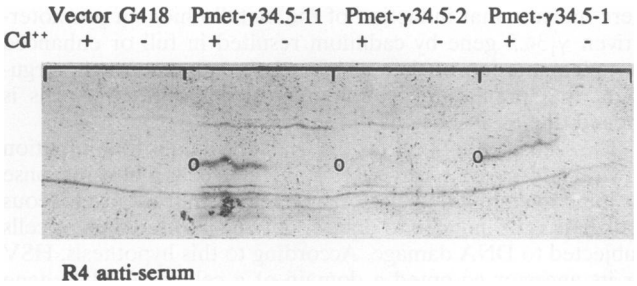

FIG. 8. Photographic image of $\gamma_1$34.5 proteins expressed by clonal lines of SK-N-SH cells. Vector G418 is the designation of a control cell line selected in the presence of G418. Pmet-$\gamma_1$34.5-11, Pmet-$\gamma_1$34.5-2, and Pmet-$\gamma_1$34.5-1 were selected from SK-N-SH cultures transfected with the plasmids shown in Fig. 1C and plated in medium containing G418. The clonal cell lines were untreated (−) or exposed to 50 μM cadmium sulfate (+) during infection. The procedures used for preparation of cell lysates, electrophoresis in *N,N'*-diallyltartardiamide–polyacrylamide gels, electrical transfer of proteins to nitrocellulose sheets, and reaction with antibody to the $\gamma_1$34.5 protein with rabbit polyclonal antibody R4 were as previously described. O, position of $\gamma_1$34.5 protein.

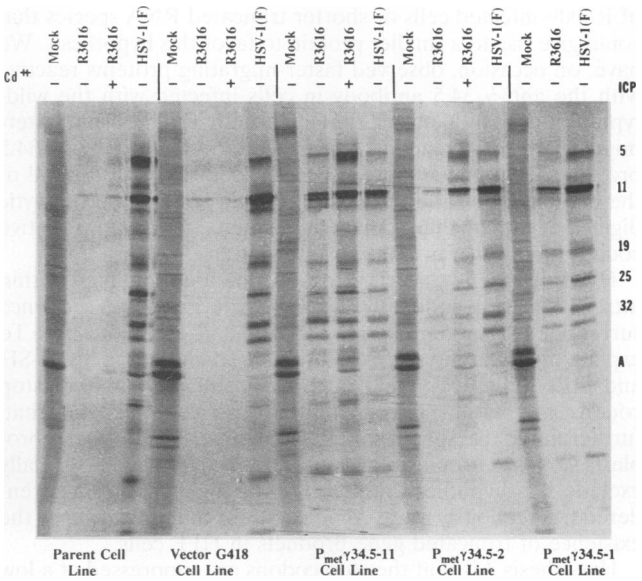

FIG. 9. Autoradiographic images of electrophoresis-separated, [^{35}S]methionine-labeled proteins in SK-N-SH clonal cells infected with viruses. The various SK-N-SH clonal cell lines shown in Fig. 8 were infected with virus HSV-1(F) or R3616 and were either untreated (−) or treated (+) with 50 μM cadmium sulfate during infection prior to labeling with [^{35}S]methionine. Mock, mock infection. ICP, infected-cell protein. A, actin.

required to preclude shutoff of protein synthesis were low. For example, the amounts of the $\gamma_1$34.5 protein expressed in the uninduced Pmet-$\gamma_1$34.5-11 and Pmet-$\gamma_1$34.5-1 clonal cell lines appear to be sufficient to preclude premature shutoff of protein synthesis whereas the amounts expressed in the Pmet-$\gamma_1$34.5-2 clonal line were not sufficient. In the presence of cadmium, the small amount made in the Pmet-$\gamma_1$34.5-2 cell line was sufficient to complement the $\gamma_1$34.5$^-$ virus.

DISCUSSION

The salient features of this report are as follows. (i) Cultures of two human cell lines, SK-N-SH neuroblastoma cells and HFF, responded identically to infection with $\gamma_1$34.5 deletion mutant R3616 but in very different ways to infection with stop codon mutants R4009 and F. Specifically, SK-N-SH cells and HFF infected with $\gamma_1$34.5 deletion mutant R3616 exhibited the same phenotype, i.e., premature, total shutoff of protein synthesis. Whereas SK-N-SH cells infected with stop codon mutants R4009 and R939 (mutant F) exhibited the same phenotype as that observed after infection with deletion mutant R3616, HFF cells infected with the stop codon mutants exhibited a wild-type phenotype; i.e., protein synthesis was not prematurely shut off. (ii) The $\gamma_1$34.5 protein expressed in SK-N-SH cells complemented the R3616 deletion mutant and precluded premature shutoff of protein synthesis. The amounts of $\gamma_1$34.5 protein required for this function appeared to be small.

Relevant to the results presented in this report are the following findings. (i) In our studies, R3616 and R4009 behaved identically in both Vero and SK-N-SH cells in culture. Furthermore, as shown in this report, the temporal patterns of premature shutoff of protein synthesis in SK-N-SH cells by the two viruses were virtually identical. Both R3616 and R4009 are highly attenuated in mice infected by the intracerebral route (4, 21). Specifically, the [R4009 PFU/LD_{50}]/[HSV-1(F) PFU/LD_{50}] ratio, where LD_{50} is the 50% lethal dose, was $>10^5$ (4). In contrast to our results, Bolovan et al. (3) reported that an HSV-1 strain 17 mutant, 1771, in which a stop codon was inserted at the same position as in the R4009 mutant, i.e., after codon 27 of the $\gamma_1$34.5 gene, exhibited a (1771 PFU/LD_{50})/(HSV-1 strain 17 PFU/LD_{50}) ratio of 25 with the same route of inoculation, i.e., several orders of magnitude different from the results reported in our studies. Their data are difficult to interpret, particularly in light of the studies by McKie et al. (15), who inserted a stop codon after codon 9 of both copies of the $\gamma_1$34.5 gene (mutant termA) and reported that the (termA PFU/LD_{50})/(HSV-1 strain 17 PFU/LD_{50}) ratio was $>10^5$, i.e., similar to that reported by Chou et al. (4). Preliminary studies indicate that R939 (mutant F) is also highly attenuated following intracerebral inoculation (12a).

(ii) The experiments reported here indicate that the $\gamma_1$34.5 gene product is essential for full viral gene expression in HFF. This conclusion is based on the observation that the $\gamma_1$34.5 gene deletion mutant caused premature shutoff of protein synthesis. It follows that the failure of the stop codon mutants to induce premature shutoff of protein synthesis indicates that the requirement for the $\gamma_1$34.5 protein was satisfied in some fashion. The disparity between the replicative cycles of the R3616 and R4009 viruses is not due to some gene dosage effect, inasmuch as we have shown that the phenotype of the mutants in HFF cells is independent of the multiplicity of infection.

The formal possibilities which could account for the results observed are the following. Hypothesis 1 is that protein synthesis is initiated downstream from the stop codons. Formally, we cannot exclude this hypothesis. However, the $\gamma_1$34.5 gene contains a single methionine codon. Although alternative (non-methionine) initiations abound, to explain the observation that recombinant R939 (mutant F), with the stop codon downstream from the triplet repeat at codon 205, also fails to induce premature shutoff of protein synthesis in HFF cells, it is necessary to postulate either a family of truncated RNAs and corresponding shorter $\gamma_1$34.5 proteins or that the initiator codon is 3′ to the stop codon in R939 (mutant F), which has a coding capacity of only 60 amino acids. As shown in Fig. 6 and 7, we have not detected a smaller, truncated protein in lysates

of R4009-infected cells or shorter truncated RNA species that could give rise to a smaller protein to favor this hypothesis. We have, on occasion, observed faster-migrating proteins reactive with the anti-$\gamma_1$34.5 antibody in cells infected with the wild-type virus but not in cells infected with R4009. The faster-migrating bands reacting with the antibody to the $\gamma_1$34.5 protein shown in Fig. 6 were not reproducibly found in all of the immunoblots, and those shown in Fig. 6 reflect proteolytic digestion rather than polypeptides obtained by alternative codon usage for translation of the protein.

Hypothesis 2 is that the stop codons are contained within introns. The stop codon in the R4009 virus is within a sequence surrounded by potential splice donor and acceptor sites. To explain the differential growth of the R939 mutant in SK-N-SH and HFF cells, we would have to postulate that both stop codons are within introns and are spliced out and that, furthermore, the splicing of RNA is human cell type (fibroblasts versus neuroblastoma cells) specific. We cannot formally exclude this hypothesis, but testing of this hypothesis is rendered tremendously more complex by our inability to verify the existence of truncated gene products in HFF cells.

Hypothesis 3 is that the stop codons are suppressed at a low level. Stop codon suppression by a tRNA suppressor is a well-documented event, and suppression can vary from 1 to 20% in some cells. This hypothesis differs from the preceding two in that it does not require the existence of truncated RNA or proteins, and furthermore, such events may well be tissue specific. Consonant with this hypothesis are two observations. First, stop codon mutant R4009 and deletion mutant R3616 were equally avirulent in mice inoculated intracerebrally and little or no virus could be detected in central nervous system tissues in the days following inoculation. However, both of these viruses were detected in vaginal tissues and the stop codon mutant was present at a titer greater than that of the deletion mutant after vaginal inoculation (21). These observations suggest that both mutants have a reduced capacity to multiply in the central nervous system, a conclusion that is consistent with the results of studies on the human neuroblastoma cell line. However, both viruses have a residual capacity to multiply in tissues peripheral to the central nervous system, and moreover, the stop codon mutant is better able to multiply; these findings are also consistent with the behavior of the two viruses in HFF. The second relevant observation stems from results presented in Fig. 8 and 9 showing that cadmium induction of the $\gamma_1$34.5 gene in SK-N-SH cells constitutively expressing small amounts of the $\gamma_1$34.5 protein was not necessary to complement the $\gamma_1$34.5$^-$ mutant. The cell line which required induction to complement the $\gamma_1$34.5$^-$ mutant produced no detectable $\gamma_1$34.5 prior to induction and barely detectable amounts afterward. These observations suggest that trace amounts of $\gamma_1$34.5 proteins are sufficient to preclude the shutoff of protein synthesis induced by a deletion virus in the absence of the $\gamma_1$34.5 protein.

Lastly, two series of experiments indicate that both the in vivo attenuated phenotype and the premature shutoff of protein synthesis in vitro are associated with mutations in the $\gamma_1$34.5 gene and both reflect defects introduced into that gene and not into ORF-P, which is located exactly antisense to the $\gamma_1$34.5 gene. First, McKie et al. (15) took note of the existence of the transcript reported by Bohenzky et al. (2) and by Yeh and Schaffer (22) and constructed its $\gamma_1$34.5 gene stop codon mutant in such a manner as to preclude mutagenesis of ORF-P. The observation that the mutant produced by McKie et al. (15) is nearly identical to R3616 and R4009 in the murine model indicates that the properties associated with neurovirulence are indeed due to the $\gamma_1$34.5 gene. The studies presented here indicate that induction of the metallothionein promoter-driven $\gamma_1$34.5 gene by cadmium resulted in full or enhanced complementation of the $\gamma_1$34.5$^-$ virus and support the argument that premature shutoff of protein synthesis in cells is indeed due to absence of the $\gamma_1$34.5 gene.

The scenario that best fits the available data is that initiation of viral DNA synthesis triggers in infected cells a host response to infection similar to those triggered in murine myelogeous leukemia cells induced to differentiate by interleukin 6 or cells subjected to DNA damage. According to this hypothesis, HSV or its ancestor co-opted a domain of a cellular GADD gene and tacked it on at the carboxyl terminus of a forbear of the $\gamma_1$34.5 gene to preclude cells from shutting off all protein synthesis and thus interfere with viral replication.

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