

# Replication of Herpes Simplex Virus 1 Depends on the $\gamma_134.5$ Functions That Facilitate Virus Response to Interferon and Egress in the Different Stages of Productive Infection

Xianghong Jing, Melissa Cervený, Kui Yang, and Bin He\*

*Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, Illinois 60612*

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**The ability of the  $\gamma_134.5$  protein to suppress the PKR response plays a crucial role in herpes simplex virus pathogenesis. In this process, the  $\gamma_134.5$  protein associates with protein phosphatase 1 to form a large complex that dephosphorylates eIF-2 $\alpha$  and thereby prevents translation shutoff mediated by PKR. Accordingly,  $\gamma_134.5$  null mutants are virulent in PKR-knockout mice but not in wild-type mice. However,  $\gamma_134.5$  deletion mutants, with an extragenic compensatory mutation, inhibit PKR activity but remain avirulent, suggesting that the  $\gamma_134.5$  protein has additional functions. Here, we show that a substitution of the  $\gamma_134.5$  gene with the NS1 gene from influenza A virus renders viral resistance to interferon involving PKR. The virus replicates as efficiently as wild-type virus in SK-N-SH and CV-1 cells. However, in mouse 3T6 cells, the virus expressing the NS1 protein grows at an intermediate level between the wild-type virus and the  $\gamma_134.5$  deletion mutant. This decrease in growth, compared to that of the wild-type virus, is due not to an inhibition of viral protein synthesis but rather to a block in virus release or egress. Virus particles are predominantly present in the nucleus and cytoplasm. Notably, deletions in the amino terminus of the  $\gamma_134.5$  protein lead to a significant decrease in virus growth in mouse 3T6 cells, which is independent of eIF-2 $\alpha$  dephosphorylation. In correlation, a series of deletions in the amino-terminal domain impair nuclear as well as cytoplasmic egress. These results indicate that efficient viral replication depends on the  $\gamma_134.5$  functions required to prevent the PKR response and to facilitate virus egress in the different stages during virus infection.**

Herpes simplex viruses (HSV) are human pathogens responsible for a variety of diseases, including localized mucocutaneous infection, encephalitis, and disseminated disease (49). Following primary infection, HSV establishes a latent infection or lytic infection in which viruses undergo transcription, replication, assembly, and egress. While many viral factors are involved in this complex process, the  $\gamma_134.5$  protein has been demonstrated to be a critical determinant of virus infection (18). Several lines of evidence indicate that the  $\gamma_134.5$  protein contributes to HSV virulence in vivo (18, 34, 35, 46, 48). HSV type 1 (HSV-1) mutants that fail to express the  $\gamma_134.5$  protein are incapable of multiplying and causing encephalitis in experimental animal models (18, 35, 48). Similar phenotypes have been observed for HSV-2 mutants lacking the  $\gamma_134.5$  gene (34, 38).

The precise roles of the  $\gamma_134.5$  protein in HSV infection are not fully understood. In HSV-infected cells, the double-stranded RNA-dependent protein kinase (PKR) is activated to phosphorylate the  $\alpha$  subunit of translation initiation factor 2 (eIF-2 $\alpha$ ) (17, 19). This leads to the translation arrest and subsequent inhibition of viral replication (19). As a way to evade the host response, the  $\gamma_134.5$  protein recruits cellular protein phosphatase 1 (PP1), forming a high-molecular-weight complex that dephosphorylates eIF-2 $\alpha$  (28, 29). Studies indicate that dephosphorylation of eIF-2 $\alpha$  facilitated by the  $\gamma_134.5$

protein is linked to viral resistance to alpha/beta interferon (14, 31). Consistent with these findings, the  $\gamma_134.5$  null mutant is virulent in PKR-knockout mice but not in wild-type mice (18, 32, 48). Paradoxically, a  $\gamma_134.5$  null mutant with a secondary mutation in the U<sub>S</sub>11 promoter region inhibits PKR activity but nevertheless remains avirulent (11, 39, 40). The virus is cleared a few days after ocular infection in experimental mice (47). Moreover, a  $\gamma_134.5$  null mutant with an additional mutation in the other regions of the viral genome partially restores virulence (10).

The  $\gamma_134.5$  gene is located in the inverted repeats of the HSV genome flanking the unique long sequence and is present in two copies per genome (1, 21, 22). In HSV-1, the  $\gamma_134.5$  gene encodes a protein of 263 amino acids consisting of an amino-terminal domain, a linker region of three-amino-acid repeats (Ala-Thr-Pro), and a carboxyl-terminal domain (21). The triplet repeats are a constant feature of the  $\gamma_134.5$  protein in HSV-1, but the number of repeats varies among different strains (6, 21). The number of triplet repeats in the  $\gamma_134.5$  protein appears to affect the ability of HSV to invade the central nervous system from the peripheral tissue (6, 37). However, the triplet repeats are not present in the  $\gamma_134.5$  protein of HSV-2 (38). The carboxyl terminus of the  $\gamma_134.5$  protein consists of a PP1-binding domain and an effector domain, both of which are essential to antagonize the antiviral activity of PKR (12, 15, 28). This portion of the protein is homologous to the corresponding domains of the growth arrest and DNA damage response protein GADD34 and a virulence factor, NL/I14L, of the African swine fever virus (25, 33, 50, 51). Currently, the biological function of the amino-terminal domain of the  $\gamma_134.5$

\* Corresponding author. Mailing address: Department of Microbiology and Immunology (M/C 790), College of Medicine, University of Illinois at Chicago, 835 S. Wolcott Ave., Chicago, IL 60612. Phone: (312) 996-2391. Fax: (312) 996-6415. E-mail: tshuo@uic.edu.

protein is unknown. Published data suggest that mutations in this region affect neurovirulence, but this domain itself is not sufficient to confer virulence (2, 18).

Previous studies indicated that the  $\gamma_134.5$  protein of HSV-1(F) accumulates both in the nucleus and in the cytoplasm during virus infection (1). In agreement with this observation, the  $\gamma_134.5$  protein is found in both the nucleus and the cytoplasm when expressed alone in mammalian cells (13, 36). Deletion analysis showed that the  $\gamma_134.5$  protein bears nuclear import and export signals that direct shuttling of the  $\gamma_134.5$  protein between the cytoplasm, nucleus, and nucleolus (13). A proposed model is that this dynamic process is required for the different activities performed by the  $\gamma_134.5$  protein during viral infection (13).

Accumulating evidence suggests that the  $\gamma_134.5$  protein is a multifunctional protein. In mouse 3T6 cells (8), a  $\gamma_134.5$  deletion mutant derived from the HSV-1 (17) strain is defective in egress, although the way by which the  $\gamma_134.5$  protein is involved is unknown. Notably, the growth of the  $\gamma_134.5$  null mutant is severely impaired in resting 3T6 cells but less so in actively dividing cells (7). Like its cellular homologue GADD34, the carboxyl-terminal domain of the  $\gamma_134.5$  protein complexes with PCNA (proliferating cell nuclear antigen), a nuclear protein involved in DNA replication and cell cycle regulation (9). In addition, the  $\gamma_134.5$  protein inhibits autophagy (42). Recent experiments showed that the triplet repeats of the  $\gamma_134.5$  protein are implicated in glycoprotein processing in infected cells (6, 37). The  $\gamma_134.5$  protein also blocks the surface expression of major histocompatibility complex class II molecules in HSV-infected cells, which is thought to inhibit the functions of CD4<sup>+</sup> T cells (45). In HSV-infected cells, eIF-2 $\alpha$  dephosphorylation mediated by the  $\gamma_134.5$  protein is coupled with viral resistance to interferon but is not sufficient for efficient viral replication (16). The observations that the  $\gamma_134.5$  protein has different activities are intriguing, but contributions of these activities to HSV infection are not yet clear.

The present study was undertaken to dissect the functions of the  $\gamma_134.5$  protein during HSV infection. Here, we report that a critical function of the  $\gamma_134.5$  protein is to block translation shutoff mediated by PKR. In addition, the  $\gamma_134.5$  protein facilitates virus egress or release late in infection. Evidence is presented that the amino terminus of the  $\gamma_134.5$  protein contributes to virus egress that is independent of the function required to prevent the PKR response. These results indicate that efficient HSV infection depends on different functions of the  $\gamma_134.5$  protein during productive infection.

#### MATERIALS AND METHODS

**Cells and viruses.** Vero, 143tk<sup>-</sup>, SK-N-SH, CV-1, and mouse embryo fibroblast (MEF) 3T6 cell lines were obtained from the American Type Culture Collection and propagated in Dulbecco's modified Eagle's medium supplemented with 5% (Vero) or 10% (143tk<sup>-</sup>, SK-N-SH, CV-1, and MEF 3T6) fetal bovine serum.

HSV-1(F) is a prototype HSV-1 strain used in these studies (24). In recombinant virus R3616, a 1-kb fragment from the coding region of the  $\gamma_134.5$  gene was deleted (18). In recombinant virus H9813, codons encoding Val<sup>193</sup> and Phe<sup>195</sup> of the  $\gamma_134.5$  gene were replaced with those encoding Glu and Leu, respectively (15). In recombinant viruses R4002, R931, R908, and R909, the sequences of the  $\gamma_134.5$  gene encoding amino acids 1 to 30, 30 to 72, 72 to 106, and 106 to 146 were deleted, respectively (20).

To construct recombinant virus JL0253R, the plasmid pJL0203 was transformed into an *Escherichia coli* RR1 strain that harbored a wild-type HSV-

bacterial artificial chromosome (BAC) by electroporation (30). HSV-BAC is derived from HSV-1(F) with an insertion of miniF in the *tk* locus (a gift from Brian Horsburgh and Frank Tafaro). After a 2-h incubation at 30°C in Luria-Bertani (LB) broth, the bacteria were plated on zeocin-chloramphenicol (CHL) (25 and 20  $\mu$ g/ml, respectively) plates and incubated overnight at 43°C for integration. Several clones were picked up and diluted serially in LB broth, plated on CHL-5% sucrose LB plates, and incubated at 30°C overnight. The sucrose-CHL-resistant clones were screened by PCR. The primers used for this purpose were OligBH0018 (CCACCCGGCAGCTCTCTGTCTC) and OligBH0020 (TATAGCGGGCTCCTGCCATCGTC), which are specific to the  $\gamma_134.5$  gene at nucleotides -25 and +886, respectively. Additional primers used were OligMC0203 (TCAAGCTTTCAGGTAGATTGCTTTCT) and OligMC0204 (TTCTCATTACTGCTTCTCCAAGCGA), which are specific to the NS1 gene at nucleotides +45 and +652, respectively. The positive clones were used to prepare HSV-BAC DNA with the QIAGEN plasmid purification kit. Viral DNA was transfected into Vero cells by using Lipofectamine reagent (Invitrogen). Virus was harvested 3 to 4 days after transfection and amplified on Vero cells. To restore the thymidine kinase (*tk*) gene, recombinant viral DNA was transfected along with plasmid pRB4867 containing the *tk* gene into Vero cells (15). The recombinant progeny was selected and purified on 143tk<sup>-</sup> cells overlaid with HAT medium (0.1 mM sodium hypoxanthine, 0.4  $\mu$ M aminopterin, 16  $\mu$ M thymidine). Preparation of viral stock and titration of infectivity were carried out on Vero cells.

**Plasmids.** Plasmid pCAGGS-PR8 NS1 SAM contains the EcoRI-XhoI fragment encoding the NS1 protein of influenza A (PR8) virus (43). Plasmid pRB143 contains a BamHI S fragment of HSV-1(F) in the BamHI site of pBR322. To construct plasmid pKY0102, a BamHI S fragment from pRB143 was cloned into the BamHI site of pBluescript SK. To construct pKY0103, the BstEII-BspEI fragment in the  $\gamma_134.5$  gene was replaced with a polylinker (oligonucleotide GTAACCACTAAGT and its complement, CCGGAAGTACTG) containing a ScaI site. Plasmid pKY0104 was constructed by ligating the BamHI fragment of pKY0103 into the BamHI site of pKO5y. To construct pKY0140, the BglIII-DraIII/Klenow fragment from pCDNA3 (Invitrogen) was cloned into the ScaI/Klenow site pKY0104. As a result, a cytomegalovirus promoter was inserted into pKY0140. To construct pJL0203, the EcoRI-XhoI fragment encoding the NS1 protein was isolated from pCAGGS-PR8 NS1 SAM and cloned into the EcoRI-XhoI sites of pKY0140.

**Southern blot analysis.** Vero cells were infected with viruses at 10 PFU per cell. At 18 h after infection, cells were harvested and resuspended in ice-cold Tris-EDTA buffer (pH 7.8) containing NP-40 (0.5%) and RNase A (50  $\mu$ g/ml). The cytoplasmic fraction was collected and treated with proteinase K (0.5 mg/ml) for 30 min at 37°C. Viral DNAs were prepared and subjected to restriction digestions, electrophoretic separation in agarose gels, transfer to nitrocellulose membranes, and hybridization with the <sup>32</sup>P-labeled DNA fragments as described previously (20). Autoradiographic images were obtained by exposure to Kodak X-ray film.

**Virus growth assay.** Monolayers of SK-N-SH, CV-1, or MEF 3T6 cells were infected with viruses at either 0.01 or 10 PFU per cell. After adsorption for 2 h, the monolayers were overlaid with Dulbecco's modified Eagle's medium and incubated at 37°C. At 24 or 48 h postinfection, samples were harvested, and viruses, released by three cycles of freezing and thawing, were titrated on Vero cells.

**Immunoblotting.** Virus-infected cells were washed, harvested, and solubilized in disruption buffer containing 50 mM Tris-HCl (pH 7.0), 5% 2-mercaptoethanol, 2% sodium dodecyl sulfate, and 2.75% sucrose. Samples were then sonicated, boiled, subjected to electrophoresis on denaturing 12% polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and reacted with a selected primary antibody. The membranes were rinsed in phosphate-buffered saline and reacted with donkey anti-rabbit immunoglobulin conjugated to horseradish peroxidase. Protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech Inc.). The primary antibodies used include anti- $\gamma_134.5$  antibody, anti-HSV-1 antibody (Dako Corporation), anti-eIF2 $\alpha$  antibody, anti-eIF2 $\alpha$  ser51p antibody (Cell Signaling Technology), anti-glycoprotein C (gC) antibody, anti-gD antibody (a gift from Gary Cohen and Roselyn Eisenberg), and anti-NS1 antibody (4).

**Interferon assay.** Monolayers of Vero cells grown to 80% confluency were either untreated or pretreated with human leukocyte alpha interferon (1,000 U/ml; Sigma) for 20 h. Cells were then infected with viruses at 0.05 PFU per cell and incubated at 37°C. At 48 h after infection, cells were harvested, and virus yields were determined on Vero cells (14).

**Electron microscopy analysis.** Monolayers of MEF 3T6 cells were infected with viruses at 0.5 PFU per cell in 35-mm dishes. At 24 h postinfection, samples were first fixed in 4% glutaraldehyde with 100 mM phosphate buffer (pH 6.8 to

7.2), fixed in 1% osmium tetroxide in phosphate buffer, dehydrated in a series of 50, 70, 85, 95, and 100% ethanol, and embedded in LX112 resin (Ladd Research Industries). Samples were removed from the petri dishes and remounted on aluminum stubs. The ultrathin sections were cut with a Leica Ultracut UCT, placed on 200 mesh copper grids, and stained with uranyl acetate and lead citrate. Grids were viewed with a Joel 1220 transmission electron microscope at 80 kV. Images were taken at various magnifications with a digital charge-coupled device (CCD) camera (Software digital micrograph; Gatan Inc.).

## RESULTS

**Construction of a recombinant virus in which the  $\gamma_134.5$  gene is replaced with the NS1 gene of influenza A virus.** Studies demonstrated that the  $\gamma_134.5$  protein of HSV-1 is essential to promote viral virulence in vivo (18, 35, 48). Although the protein antagonizes the antiviral effect of interferon mediated by PKR (14, 19, 29, 32), its precise roles in HSV infection remain unresolved. This is partly attributed to the fact that the  $\gamma_134.5$  protein appears to have multiple functions. In order to dissect the functions of the  $\gamma_134.5$  protein, we sought to develop a model system. By design, we constructed a recombinant virus, JL0253R, in which the  $\gamma_134.5$  gene is replaced by the NS1 gene of influenza A virus (Fig. 1). The objective was to assess to what extent the NS1 protein complements the  $\gamma_134.5$  protein in the context of HSV infection. The NS1 protein was chosen because it is an extensively characterized viral protein that blocks interferon response by preventing the activation of PKR (5, 27, 44).

As illustrated in Fig. 1A, the BstEII-BspEI fragment containing the  $\gamma_134.5$  gene was replaced with the cDNA fragment of the NS1 gene from influenza A virus in the recombinant virus JL0253R. This was done by homologous recombination using the BAC system as described in Materials and Methods. The BAC plasmid inserted in the *tk* gene was removed by cotransfection of viral DNA and a plasmid containing the *tk* gene into Vero cells. The recombinant progeny JL0253R was then selected on 143tk<sup>-</sup> cells overlaid with medium containing hypoxanthine-aminopterin-thymidine. To verify the virus construct, Southern blot analysis was carried out after BamHI, BstEII, and DraIII digestion of viral DNAs (Fig. 1B). As expected, HSV-1(F), R3616, and JL0253R yielded a 3-kb BamHI Q fragment containing the *tk* gene (Fig. 1B, lanes 1 to 3). In addition, JL0253R produced a 720-bp EcoRI-XhoI fragment containing the NS1 gene (lane 3), and HSV-1(F) yielded a 526-bp BstEII-DraIII fragment representing the  $\gamma_134.5$  gene (lane 1). To examine protein expression, Western blot analysis was performed by using anti-NS1 and anti- $\gamma_134.5$  antibodies, respectively. The results in Fig. 1C show that in virus-infected cells, HSV-1(F) expressed the  $\gamma_134.5$  protein, whereas the recombinant virus JL0253R produced the NS1 protein (lanes 2 and 4). The  $\gamma_134.5$  protein was not detected in cells infected with either JL0253R (lane 4) or R3616, which lacks the  $\gamma_134.5$  gene (lane 3).

**Substitution of the  $\gamma_134.5$  gene with the NS1 gene does not affect HSV response to alpha interferon.** Since the  $\gamma_134.5$  protein is involved in HSV resistance to interferon (14), we examined whether a substitution of the  $\gamma_134.5$  gene with the NS1 gene had any effect in this process. Specifically, monolayers of Vero cells were untreated or pretreated with alpha interferon (1,000 U/ml) to induce the antiviral state. Cells were then infected with the indicated viruses, and virus yields were de-

termined 48 h after infection. As seen in Fig. 2, in the absence of interferon, HSV-1(F) replicated to a titer of  $8.7 \times 10^8$  PFU/ml and R3616 reached a titer of  $2.6 \times 10^7$  PFU/ml. Similarly, JL0253R replicated to a titer of  $1.6 \times 10^8$  PFU/ml. When cells were pretreated with interferon, replication of HSV-1(F) decreased slightly (fourfold), with a titer at  $2.4 \times 10^8$  PFU/ml. Due to a deletion of the  $\gamma_134.5$  gene, replication of R3616 decreased dramatically to a titer of  $2.4 \times 10^5$  PFU/ml, exhibiting an interferon-sensitive phenotype. Under this condition, JL0253R still replicated efficiently, reaching a titer of  $9.3 \times 10^7$  PFU/ml. Thus, like wild-type HSV-1(F), the recombinant virus JL0253R is capable of blocking the antiviral action of alpha interferon. We conclude from this experiment that the NS1 protein functions to confer viral resistance to interferon when expressed in the context of the HSV genome.

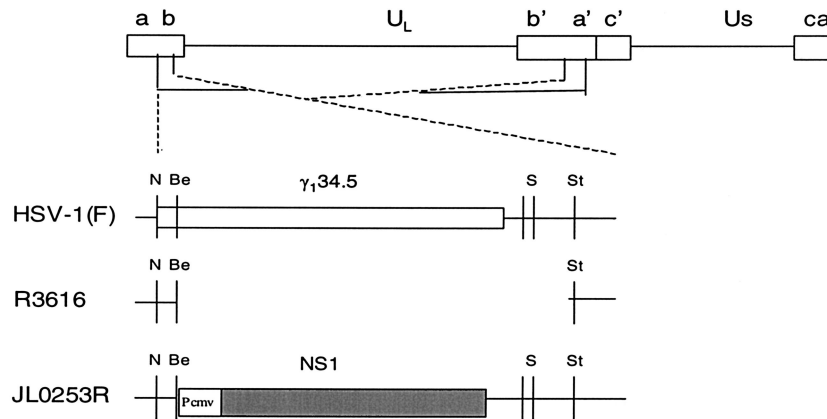
**Viruses lacking the  $\gamma_134.5$  gene exhibit differential growth defects in mammalian cell lines.** Based on the above analysis, we evaluated the growth properties of JL0253R in human neuroblastoma SK-N-SH cells and African monkey kidney CV-1 cells. These cell lines are restrictive to the  $\gamma_134.5$  deletion mutant due to the shutoff of protein synthesis triggered by viral DNA synthesis (19). In this series of experiments, monolayers of cells were infected with HSV-1(F), R3616, or JL0253 at 0.01 PFU, and virus yields were measured. The results in Fig. 3A show that in SK-N-SH cells, HSV-1(F) replicated to a titer of  $2.2 \times 10^6$  PFU/ml 24 h after infection. This virus maintained an efficient growth, reaching a titer of  $3.8 \times 10^7$  PFU/ml 48 h after infection. As expected, R3616 replicated poorly, with a titer of  $3.7 \times 10^3$  PFU/ml at 24 h and  $4 \times 10^4$  PFU/ml at 48 h. The decrease in virus growth correlated with the inability of R3616 to prevent the shutoff of protein synthesis (17). Over the same growth period, JL0253R replicated as efficiently as did wild-type HSV-1(F), with titers reaching  $2.6 \times 10^6$  PFU/ml at 24 h and  $3.8 \times 10^7$  PFU/ml at 48 h. Virtually identical growth patterns were also seen for these viruses in CV-1 cells (Fig. 3B). Further analysis showed similar phenotypes in mouse fibroblast 10T1/2 cells infected with HSV-1(F), R3616, or JL0253R (data not shown). Therefore, the growth property of the recombinant virus JL0253R is indistinguishable from that of wild-type HSV-1 (F) in these cell lines.

To further examine the requirement of the  $\gamma_134.5$  protein in HSV infection, we also measured virus growth patterns in MEF 3T6 cells. Monolayers of cells were infected with HSV-1(F), R3616, or JL0253R at 0.01 PFU per cell, and virus yields were then determined. Figure 3C shows that wild-type HSV-1(F) replicated to a high titer of  $1.9 \times 10^7$  PFU/ml at 24 h postinfection. It continued to maintain efficient replication at 48 h after infection, with a titer of  $4.7 \times 10^7$  PFU/ml. R3616 replicated poorly, with a titer of  $7 \times 10^1$  PFU/ml at 24 h postinfection, which then increased to a titer of  $3.2 \times 10^3$  PFU/ml at 48 h postinfection. Surprisingly, JL0253R replicated to a titer of only  $2.6 \times 10^3$  PFU/ml at 24 h, which then increased to  $8.5 \times 10^4$  PFU/ml at 48 h after infection. The growth of this virus was approximately 500-fold lower than that of HSV-1(F) but was 26- to 37-fold greater than that of R3616. The modest increase for JL0253R was consistently observed in several independent experiments. These results indicated that the NS1 protein only partially complements the functions of the  $\gamma_134.5$  protein required for viral growth in MEF 3T6 cells.

Based on the unique growth phenotype of JL0253R in MEF



## A. Schematic diagram of virus constructs



## B. Southern blot analysis

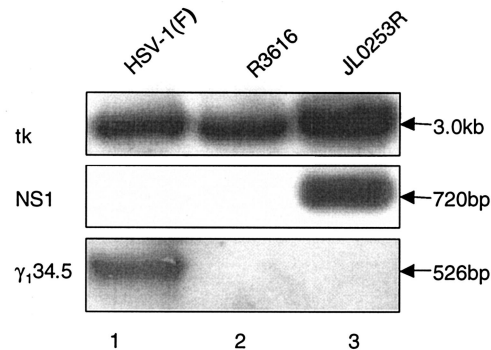
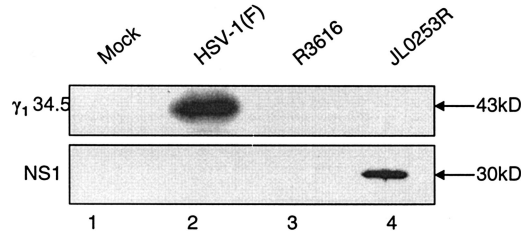
C. Immunoblot with anti- $\gamma_1$  34.5 and NS1 antibodies

FIG. 1. (A) Schematic representation of the genome structure of HSV-1 and its derivatives. The two covalently linked components of HSV-1 DNA, L and S, each consist of unique sequences,  $U_L$  and  $U_S$ , respectively, flanked by inverted repeats (41). The reiterated sequences flanking  $U_L$ , designated as  $ab$  and  $b'a'$ , are each 9 kbp in size, whereas the repeats flanking  $U_S$ , designated  $a'c'$  and  $ca$ , are 6.3 kbp in size. The location of the  $\gamma_1$ 34.5 gene is shown in the expanded portions of the inverted repeat sequences  $b$  and  $b'$ . Since the  $b$  sequence is repeated in an inverted orientation, there are two copies of the  $\gamma_1$ 34.5 gene per genome. HSV-1(F) is the prototype strain used in our laboratory (24). In R3616, the coding region between BstEII-StuI sites of the  $\gamma_1$ 34.5 gene is deleted (21). In recombinant virus JL0253R, the  $\gamma_1$ 34.5 gene is replaced with the influenza A virus NS1 gene (43), which is driven by a cytomegalovirus promoter. Restriction site designations are as follows: N, NcoI; Be, BstEII; S, SacI; and St, StuI. (B) Autoradiographic images of viral DNAs. Vero cells were infected with the indicated viruses at 10 PFU per cell. At 18 h postinfection, cells were harvested, and viral DNA was prepared and then digested with either BamHI, EcoRI and XhoI, or BstEII and DraIII. Samples were electrophoretically separated on 0.8% agarose gels and transferred to a nitrocellulose membrane. The *tk* gene was detected by hybridization to a  $^{32}$ P-labeled BamHI Q fragment of HSV-1. Similarly, the NS1 gene was probed with a  $^{32}$ P-labeled NS1 fragment spanning nucleotides 45 to 652, and the  $\gamma_1$ 34.5 gene was probed with a  $^{32}$ P-labeled  $\gamma_1$ 34.5 fragment spanning nucleotides 345 to 579. (C) Expression of the  $\gamma_1$ 34.5 protein and the NS1 protein. Vero cells were either mock infected or infected with the indicated viruses at 10 PFU per cell. At 18 h postinfection, the cells were harvested and subjected to electrophoresis, transferred to a nitrocellulose membrane, and reacted with either anti-NS1 antibody or anti- $\gamma_1$ 34.5 antibody.

3T6 cells, we tested whether the virus grew similarly at a high multiplicity of infection. For this purpose, monolayers of MEF 3T6 cells were infected with viruses at 10 PFU per cell. At 24 h after infection, virus titers were determined. As shown in Fig.

3D, HSV-1(F) replicated to a titer of  $1.1 \times 10^7$  PFU/ml, whereas R3616 replicated to a titer of  $1.2 \times 10^5$  PFU/ml. Under this condition, replication of R3616 was 100-fold less than that of HSV-1 (F), which may result from a block that

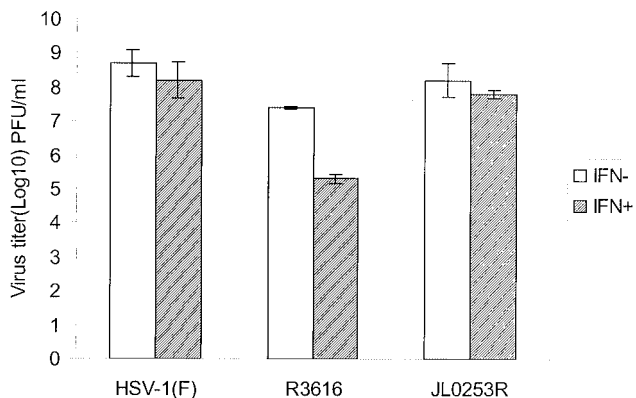


FIG. 2. Viral response to alpha interferon. Monolayers of Vero cells were either untreated or pretreated with human leukocyte alpha interferon (1,000 U/ml; Sigma) for 20 h. Cells were then infected with viruses at 0.05 PFU per cell and incubated at 37°C. At 48 h postinfection, cells were harvested, and virus yields were determined on Vero cells (14). Data represent the average from three independent experiments, with the standard deviation indicated.

inhibited viral replication. Unlike R3616, JL0253R reached a titer of  $6.3 \times 10^6$  PFU/ml, which is close to that of HSV-1(F), with a virus yield only 1.7-fold lower than that for HSV-1(F). These results suggest that once infection is initiated, JL0253R

is able to complete its replication cycle within an infected cell, albeit at a slightly lower level than that of HSV-1(F).

The different growth patterns between JL0253R and R3616 suggest that JL0253R, but not R3616, is able to overcome the PKR response in 3T6 cells. To address this issue, viral protein synthesis in 3T6 cells was examined by Western blot analysis using anti-HSV antibodies. As indicated in Fig. 4A, a high level of viral protein was detected in cells infected with HSV-1(F) or JL0253R. A slightly lower level of viral protein synthesis in JL0253R correlated with the 1.7-fold decrease in viral growth at a high multiplicity of infection (Fig. 3D). In sharp contrast, little or no viral protein was detected in 3T6 cells either mock infected or infected with R3616. Immunoblot analysis indicated that levels of eIF-2 $\alpha$  were comparable in mock-infected and virus-infected cells. However, a significant amount of phosphorylated eIF-2 $\alpha$  was seen only in cells infected with R3616. Obviously, HSV-1(F) as well as JL0253R prevented the translation shutoff mediated by PKR in 3T6 cells, whereas R3616 did not. Collectively, these data suggest that the marked decrease in virus growth associated with R3616 resulted from the translation block mediated by PKR. However, the growth defect associated with JL0253R, derived from a defect(s) after viral protein translation, could be virus release or egress.

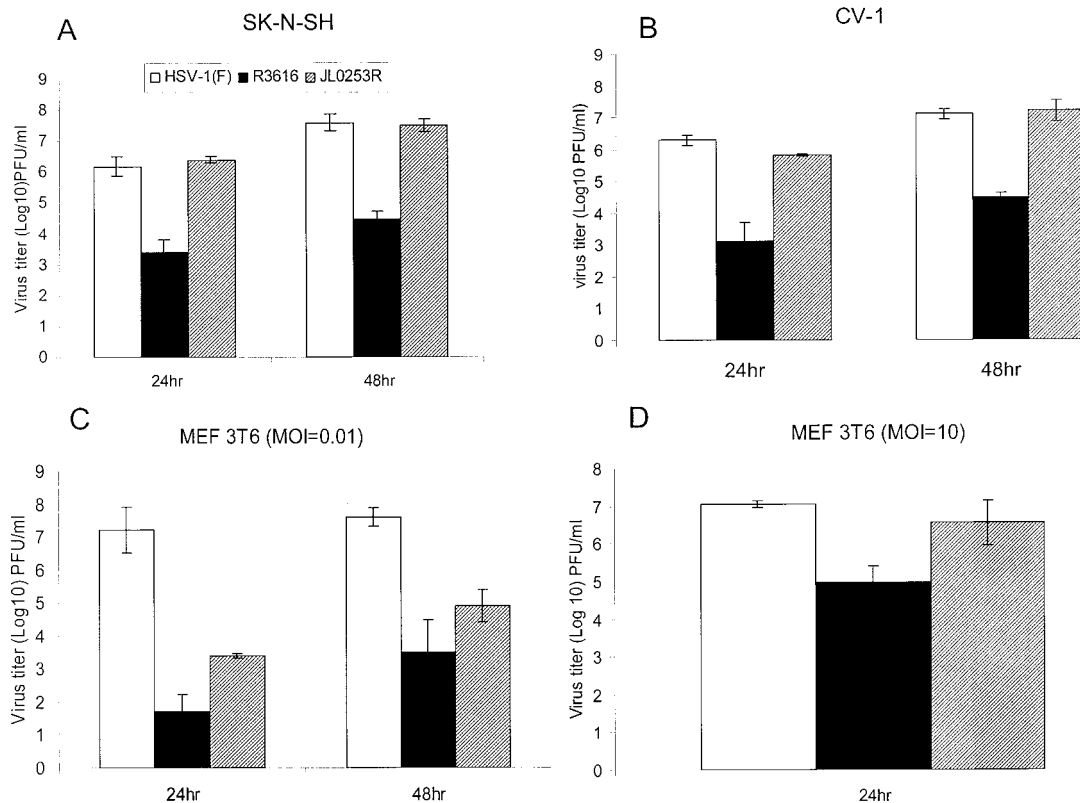


FIG. 3. Growth properties of wild-type HSV-1(F), the  $\gamma_1$ 34.5 deletion mutant, and the recombinant virus expressing the NS1 protein. Confluent monolayers of SK-N-SH (A), CV-1 (B), and MEF 3T6 (C) cells were infected with HSV-1(F), R3616, or JL0253R at 0.01 PFU per cell and incubated at 37°C. Viruses were harvested at 24 and 48 h postinfection. Samples were freeze-thawed three times and titrated on Vero cells at 37°C. As a parallel experiment, confluent monolayers of MEF 3T6 cells were also infected at 10 PFU per cell (D). Viruses were harvested at 24 h postinfection and titrated as described above. The data represent an average from three independent experiments, and the error bars indicate standard deviations. MOI, multiplicity of infection.

## A. Immunoblot with anti-HSV antibody

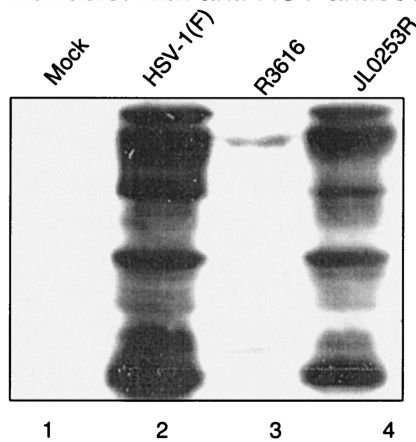
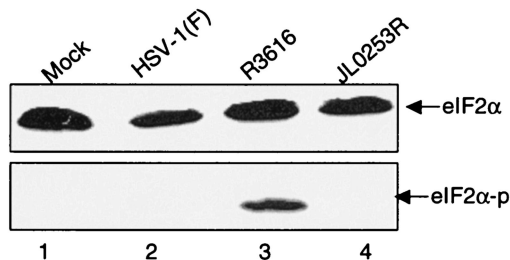
B. Phosphorylation state of eIF2 $\alpha$ 

FIG. 4. (A) Synthesis of viral proteins in virus-infected MEF 3T6 cells. Confluent monolayers of MEF 3T6 cells were either mock infected or infected with the indicated viruses at 10 PFU per cell. At 16 h postinfection, cells were harvested, solubilized, subjected to polyacrylamide gel analysis, transferred to a nitrocellulose sheet, and reacted with polyclonal antibodies against whole HSV-1 antigens (Dako Corporation). (B) Phosphorylation state of eIF-2 $\alpha$ . The same membrane described above (A) was stripped and probed with antibodies against eIF-2 $\alpha$  and phosphorylated eIF-2 $\alpha$  (Cell Signaling Technology). The positions of eIF-2 $\alpha$  and phosphorylated eIF-2 $\alpha$  are shown on the right.

**Virus release is decreased in MEF 3T6 cells infected with viruses lacking the  $\gamma_1$ 34.5 gene.** To analyze the growth defect associated with JL0253R, we determined yields of viruses that remained associated with cells and that were released into the medium. In this experiment, monolayers of MEF 3T6 cells were infected with HSV-1(F), R3616, or JL0253R at 0.5 PFU per cell. At 24 h after infection, viruses associated with the cells or in the medium were collected and measured for infectivity on Vero cells. The data in Table 1 show that for HSV-1(F)-infected cells, the total virus yield was  $8.93 \times 10^7$  PFU/ml.

Among these infectious virus particles, 63% were found in the cell body and 37% were present in the medium. As expected, for cells infected with R3616, the overall virus yield was low, with a titer of  $1.73 \times 10^4$  PFU/ml. There was approximately a 1,000-fold drop in virus production compared to that of HSV-1(F). Moreover, a larger fraction (80%) was associated with the cells, and a smaller fraction (20%) was detected in the medium. Virus particles released into the medium were reduced by 17% in relation to HSV-1(F). Interestingly, in cells infected with JL0253R, the total virus yield was  $8.25 \times 10^6$  PFU/ml, which was close to that for HSV-1(F). However, most of the virus particles (90%) were associated with cells, and a very small portion (10%) was released into the medium. There was a 27% decrease in virus release compared to HSV-1(F). Therefore, despite the quantitative difference, virus release was less efficient in cells infected with JL0253R or R3616. However, the overall virus production for JL0253R was about 400-fold greater than that for R3616. This result is concordant with the ability of JL0253R to overcome eIF-2 $\alpha$  phosphorylation mediated by PKR.

**Virus particles are predominantly present within MEF 3T6 cells infected with viruses lacking the  $\gamma_1$ 34.5 gene.** As JL0253R is less efficient in virion release than HSV-1(F), we further examined the distribution of virus particles within the infected 3T6 cells. Cells were infected with HSV-1(F), R3616, or JL0253R and fixed 24 h after infection. Samples were prepared for thin sections and examined by electron microscopic analysis. As presented in Fig. 5, in cells infected with HSV-1(F), a large number of enveloped virions were observed both in the cytoplasmic vesicles and in the extracellular space (Fig. 5A). The significant number of virus particles seen on the cell surface suggests efficient viral production and maturation. In cells infected with R3616, there was a drastic decrease in overall virus particle production. In addition, virus particles were predominantly seen in the nucleus (Fig. 5B). In some cases, the virus particles were localized to areas close to the nuclear membrane. Very few virus particles were found in the cytoplasm or in the extracellular space. This phenotype is similar to that observed for the  $\gamma_1$ 34.5 deletion mutant derived from HSV-1(17<sup>+</sup>) (8). Importantly, in cells infected with JL0253R, there were a large number of virus particles being produced. Nevertheless, these virus particles were mainly confined to the nucleus (Fig. 5C). Some particles were in close proximity to the nuclear membrane, whereas others formed an aggregate or cluster. To quantitate the distribution of virus particles, 15 to 20 cells were examined for each virus. Locations and numbers of virus particles were enumerated, and the data are summarized in Table 2. In HSV-1(F)-infected cells, 23% of virus

TABLE 1. Yields of secreted and cell-associated infectious virions<sup>a</sup>

Virus	No. of virion particles (%)		
	Cell-free particles	Cell-associated particles	Total
HSV-1(F)	$(3.20 \pm 1.1) \times 10^7$ (37)	$(5.73 \pm 2.8) \times 10^7$ (63)	$(8.93 \pm 3.7) \times 10^7$ (100)
R3616	$(3.67 \pm 2.0) \times 10^3$ (20)	$(1.37 \pm 0.4) \times 10^4$ (80)	$(1.73 \pm 0.6) \times 10^4$ (100)
JL0253R	$(7.83 \pm 1.5) \times 10^5$ (10)	$(7.47 \pm 2.3) \times 10^6$ (90)	$(8.25 \pm 2.2) \times 10^6$ (100)

<sup>a</sup> Confluent monolayers of MEF 3T6 cells were infected with HSV-1(F), R3616, and JL0253R at 0.5 PFU per cell and incubated at 37°C. At 24 h postinfection, cell-associated viruses and viruses in the supernatant were collected separately and titrated on Vero cells. The data represent an average from three independent experiments, with standard deviations indicated.



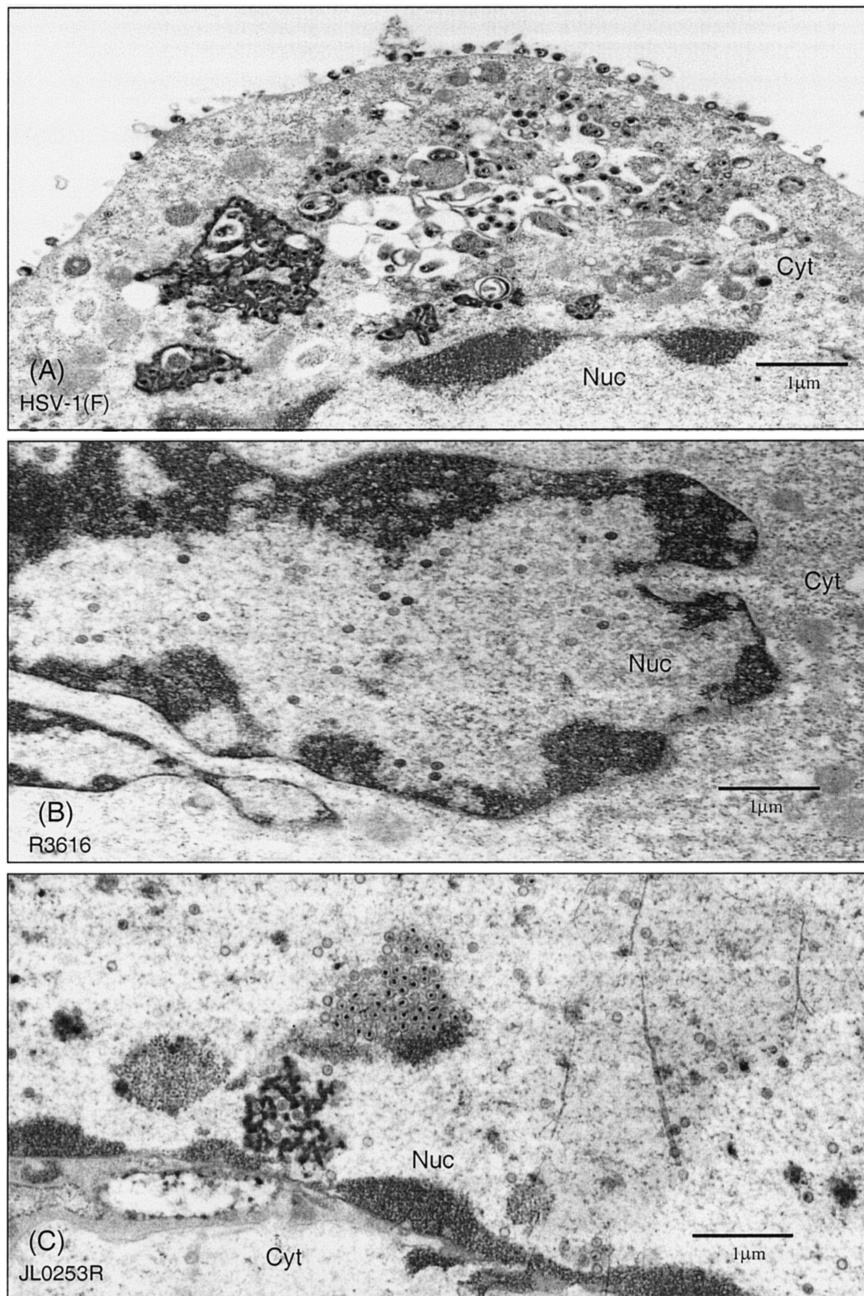


FIG. 5. Cellular distribution of virus particles in MEF 3T6 cells. Confluent monolayers of MEF 3T6 cells were infected with HSV-1(F), R3616, or JL0253R at 0.5 PFU per cell. At 24 h postinfection, cells were first fixed in 4% glutaraldehyde in 100 mM phosphate buffer (pH 6.8 to 7.2) and then fixed in 1% osmium tetroxide. Cells were dehydrated in ethanol, embedded in LX112 resin, and stained with uranyl acetate and lead citrate. Thin sections were prepared and viewed with a Joel 1220 transmission electron microscope at 80 kV. Images were captured with a Gatan digital CCD camera. (A) HSV-1(F)-infected MEF 3T6 cells. (B) R3616-infected MEF 3T6 cells. (C) JL0253R-infected MEF 3T6 cells. Scale bars are shown in each panel. Abbreviations: Nuc, nuclear region; Cyt, cytoplasm.

particles were in the nucleus, 30% were in the perinuclear region or cytoplasm, and 47% were in the extracellular space. In R3616-infected cells, 48% of virus particles were in the nucleus, 40% were in the perinuclear region or cytoplasm, and 12% were in the extracellular space. Thus, in the absence of the  $\gamma_134.5$  gene, a significant fraction of virus particles were trapped in the nucleus or cytoplasm. Similarly, in cells infected

with JL0253R, a large fraction of virus particles were retained in the nucleus or cytoplasm, with 62% of particles in the nucleus, 26% in the perinuclear region or cytoplasm, and only 13% in the extracellular space. Therefore, substitution of the  $\gamma_134.5$  protein with the NS1 protein did not promote virus release or egress, suggesting that an additional function(s) of the  $\gamma_134.5$  protein is required for this process in MEF 3T6 cells.

TABLE 2. Distribution of virions in MEF 3T6 cells

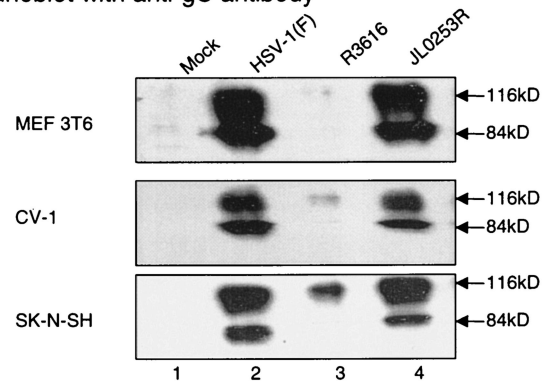
Virus	Avg no. (%) of particles/cell in <sup>a</sup> :		
	Nucleus	Perinuclear region/cytoplasm	Extracellular space
HSV-1(F)	58 (23)	5/67 (30)	113 (47)
R3616	29 (48)	17/7 (40)	7 (12)
JL0253R	62 (61)	3/24 (26)	13 (13)
R4002	87 (55)	2/32 (22)	37 (23)
R931	10 (9)	78/12 (83)	8 (8)
R908	11 (10)	80/8 (85)	5 (5)
R909	38 (24)	3/47 (32)	69 (44)

<sup>a</sup> Virus particles present in the nucleus, perinuclear region, and cytoplasm and on the cell surface were counted in electron micrographs of at least 15 to 20 randomly sampled MEF 3T6 cells infected with the indicated viruses. The numbers represent the average number of virus particles per cellular compartment, and the numbers in the parentheses denote the percentage of virus particles in the different sections of a cell.

**Glycoproteins are processed in cells infected with wild-type virus or virus expressing the NS1 protein of influenza A virus.** Recent studies showed a connection between the  $\gamma_134.5$  protein and processing of gC and gD (6, 37). This process is thought to influence virion maturation and egress (6). To characterize whether the substitution of the  $\gamma_134.5$  gene with the NS1 gene affected glycoprotein processing, monolayers of CV-1, SK-N-SH, and MEF 3T6 cells were mock infected or infected with HSV-1(F), R3616, or JL0253R. At 16 h postinfection, lysates of cells were processed for Western blot analysis by using anti-gC antibody or anti-gD antibody. Figure 6A shows that, like HSV-1(F), JL0253R virus yielded both immature (84 kDa) and mature (116 kDa) gC in all infected cells, which is indicative of efficient glycoprotein processing. Similarly, gD was also efficiently processed in virus-infected cells, with the 52- and 55-kDa bands representing immature and mature forms, respectively. However, R3616 expressed little gC or gD, which resulted from a block in viral protein synthesis due to the deletion of the  $\gamma_134.5$  gene. These data indicate that the defect in virion maturation or egress in 3T6 cells infected with JL0253R is not linked to the processing of viral gC and gD.

**Deletions in the amino-terminal domain of the  $\gamma_134.5$  protein reduce viral growth that is independent of eIF-2 $\alpha$  dephosphorylation in MEF 3T6 cells.** Previous studies demonstrated that the carboxyl terminus of the  $\gamma_134.5$  protein is essential to prevent the shutoff of protein synthesis mediated by PKR (19, 28), but the function of the amino-terminal domain is unknown. To address whether the amino terminus is required for virus growth in MEF 3T6 cells, we analyzed the growth properties of a panel of  $\gamma_134.5$  mutants. These mutants have either a series of deletions spanning the region from amino acids 1 to 146 or site-specific mutations in the carboxyl terminus of the  $\gamma_134.5$  protein (15, 20). Cells were infected with viruses at 0.01 PFU per cell, and virus yields were determined at 24 h postinfection. The data in Fig. 7 show that wild-type HSV-1(F) grew to a titer of  $1.1 \times 10^7$  PFU/ml, whereas the  $\gamma_134.5$  deletion mutant R3616 reached a titer of only  $7.7 \times 10^1$  PFU/ml. A deletion of the entire  $\gamma_134.5$  gene severely hindered viral replication, with a  $10^5$ -fold decrease in virus growth. Mutant H9813, which has Val<sup>193</sup>Glu and Phe<sup>195</sup>Leu substitutions in the PP1-interacting motif of the  $\gamma_134.5$  protein, grew poorly,

### A. Immunoblot with anti-gC antibody



### B. Immunoblot with anti-gD antibody

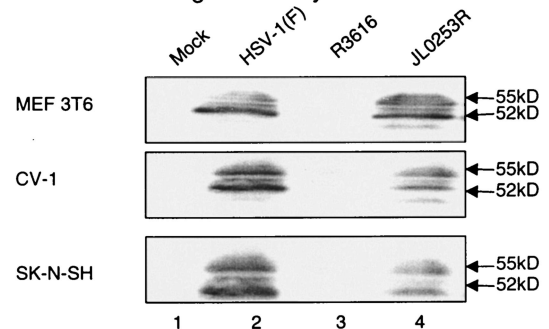
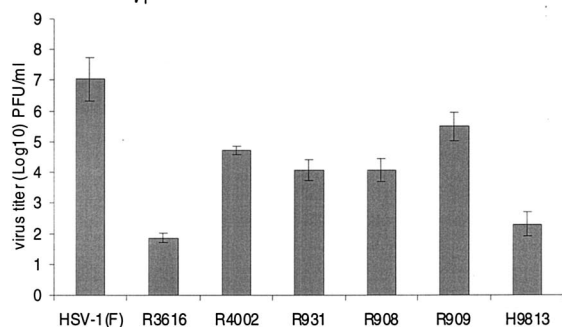


FIG. 6. Processing of gC and gD in virus-infected cells. (A) Confluent monolayers of MEF 3T6, CV-1, and SK-N-SH cells were either mock infected or infected with the indicated viruses at 10 PFU per cell. At 16 h postinfection, cells were harvested, solubilized, subjected to polyacrylamide gel analysis, transferred to a nitrocellulose membrane, and reacted with anti-gC antibody. The positions of mature (116 kDa) and premature (84 kDa) forms are indicated on the right. (B) The same membrane described above (A) was stripped and incubated with anti-gD antibody. The positions of mature (55 kDa) and premature (52 kDa) forms are indicated on the right.

with a titer reaching only  $2.6 \times 10^2$  PFU/ml, which is similar to that for R3616. Hence, a defect that disrupts the ability of the  $\gamma_134.5$  protein to block the antiviral action of interferon involving PKR results in a block in viral replication. Importantly, mutants R4002 (with a deletion of the region of the  $\gamma_134.5$  protein containing amino acids 1 to 30 [ $\Delta 1-30$ aa]), R931 ( $\Delta 30-72$ aa), and R908 ( $\Delta 72-106$ aa) grew more efficiently than R3616, with titers reaching  $1.5 \times 10^4$  to  $5.5 \times 10^4$  PFU/ml. However, these mutants also displayed a growth defect compared to HSV-1(F). There was an approximately 1,000-fold decrease in virus titer for these mutants. In addition, mutant R909 ( $\Delta 106-146$ aa) exhibited a moderate decrease (25-fold) in virus growth. These results suggest that the region containing amino acids 1 to 146 is crucial for efficient viral growth in MEF 3T6 cells.

To explore the basis for the phenotype described above, we analyzed viral protein synthesis in 3T6 cells. Monolayers of cells were either mock infected or infected with the indicated viruses, and cell lysates were processed for immunoblot analysis by using anti-HSV antibodies. The data in Fig. 7B show that viral proteins were produced in cells infected with HSV-1(F) but not in cells infected with R3616 or H9813, which



A. Growth of  $\gamma_1$ 34.5 deletion mutants in MEF 3T6 cells

## B. Viral protein accumulation in MEF 3T6 cells

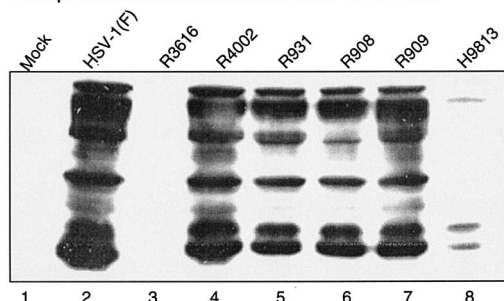
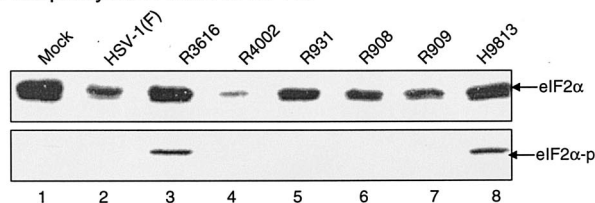
C. Phosphorylation state of eIF-2 $\alpha$ 

FIG. 7. (A) Growth of  $\gamma_1$ 34.5 deletion mutants in MEF 3T6 cells. Confluent monolayers of MEF 3T6 cells were infected with viruses at 0.01 PFU per cell and incubated at 37°C. Twenty-four hours postinfection, viruses were harvested, freeze-thawed three times, and titrated on Vero cells at 37°C. The data represent an average from three independent experiments, and the error bars indicate standard deviations. HSV-1(F) is a wild-type virus, whereas R3616 lacks the  $\gamma_1$ 34.5 gene (18, 24). H9813 has Val<sup>193</sup>Glu and Phe<sup>195</sup>Leu substitutions in the PP1-binding motif of the  $\gamma_1$ 34.5 protein (15). R4002 ( $\Delta$ 1-30aa), R931 ( $\Delta$ 30-72aa), R908 ( $\Delta$ 72-106aa), and R909 ( $\Delta$ 106-146aa) have a series of deletions in the amino terminus consisting of amino acids 1 to 146 (20). (B) Synthesis of viral polypeptides in virus-infected MEF 3T6 cells. Confluent monolayers of MEF 3T6 cells were either mock infected or infected with the indicated viruses at 10 PFU per cell. At 16 h postinfection, cells were harvested and processed for immunoblot analysis with antibodies against whole HSV-1 antigens (Dako Corporation). (C) Phosphorylation state of eIF-2 $\alpha$ . Confluent monolayers of MEF 3T6 cells were either mock infected or infected with the indicated viruses at 10 PFU per cell. At 16 h postinfection, cells were harvested and processed for immunoblot analysis with antibodies against eIF-2 $\alpha$  and phosphorylated eIF-2 $\alpha$  (Cell Signaling Technology). The positions of eIF-2 $\alpha$  and phosphorylated eIF-2 $\alpha$  are shown on the right.

correlated well with viral growth properties shown in Fig. 7A. Notably, in cells infected with R4002, R931, R908, or R909, viral proteins were produced at a level comparable to that seen for HSV-1(F). Further analysis of the phosphorylation state of

eIF-2 $\alpha$  indicated that eIF-2 $\alpha$  was phosphorylated in cells either mock infected or infected with R3616 or H9813. A low level of phosphorylated eIF-2 $\alpha$  in mock-infected cells represents background. Thus, failure to produce viral proteins in cells infected with R3616 or H9813 resulted from the antiviral action of PKR. Importantly, eIF-2 $\alpha$  remained nonphosphorylated in cells infected with HSV-1(F), R4002, R931, R908, or R909. This correlated well with efficient synthesis of viral proteins in cells infected with these viruses. As R4002, R931, R908, and R909 were able to inhibit the activity of PKR, the growth defects associated with these viruses in MEF 3T6 cells are due to a step after viral protein production (Fig. 7A). These phenotypes paralleled those seen in cells infected with JL0253R, in which the  $\gamma_1$ 34.5 gene was substituted with the NS1 gene.

**Deletions in the amino terminus of the  $\gamma_1$ 34.5 protein impair nuclear as well as cytoplasmic egress in MEF 3T6 cells.**

To investigate the role of the amino-terminal domain of the  $\gamma_1$ 34.5 protein, we further analyzed the localization of virus particles in cells infected with HSV-1(F), R3616 ( $\Delta\gamma_1$ 34.5), R4002 ( $\Delta$ 1-30aa), R931 ( $\Delta$ 30-72aa), R908 ( $\Delta$ 72-106aa), or R909 ( $\Delta$ 106-146aa). MEF 3T6 cells infected with viruses were processed for thin sections and examined by electron microscopic analysis 24 h after infection. As expected, in cells infected with HSV-1(F), virus particles were evident not only in the cytoplasm but also in the extracellular space. In cells infected with R3616, there was a drastic decrease in overall virus particle production. In addition, virus particles were predominantly seen in the nucleus (data not shown). Notably, in cells infected with R4002, virus particles were confined mainly to the nucleus (Fig. 8A). Although a significant number of virus particles were present in infected cells, very few were found in the cytoplasmic vesicles or in the extracellular space. This mutant displayed a defect in nucleocapsid transit from the nucleus to the cytoplasm. However, the most striking observation was that in cells infected with R931 or R908, a majority of the virus particles were trapped in the cytoplasm (Fig. 8B and C). A large number of these particles were in areas close to or associated with the outer nuclear membrane. Virus particles were rarely seen in the nucleus or on the cell surface (Fig. 8B and C). It seems that R931 and R908 were capable of budding into the cytoplasm from the nucleus but incapable of reaching the cell surface. In cells infected with R909, the distribution pattern of virus particles was indistinguishable from that for HSV-1(F) (Fig. 8D), but with a slight decrease in virus particle number. To quantitate the observed differences, the subcellular distribution of virions was counted in 15 to 20 cells for each virus. As summarized in Table 2, in the absence of the  $\gamma_1$ 34.5 gene, a significant fraction (48%) of virus particles were trapped in the nucleus. A similar phenotype was seen with R4002 (55%), indicating a block or delay in virus budding from the nucleus to the cytoplasm. In contrast, in cells infected with R931 or R908, more than 80% of virus particles accumulated in the cytoplasm, whereas less than 10% were on the cell surface. This finding indicates that these mutants have a defect in egress from the cytoplasm to the cell surface. These results correlated with those seen in the virus growth assay for the mutants described above (Fig. 7A). The distribution of R909 seems similar to that of HSV-1(F). Given that this mutant exhibited a moderate growth defect (Fig. 7A), it is likely that the small difference in egress may not be detectable under this

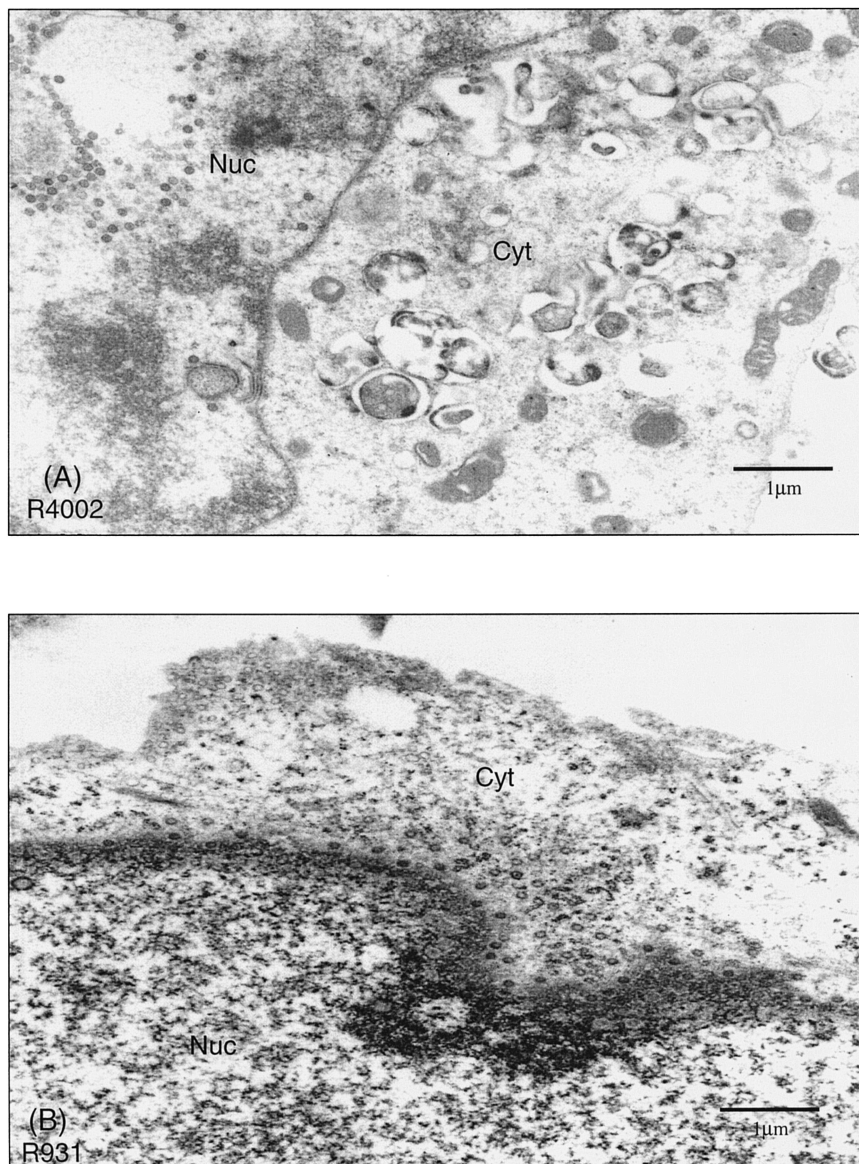


FIG. 8. Intracellular distribution of virions in cells infected with  $\gamma_{134.5}$  mutants with deletions in the amino-terminal domain. Confluent monolayers of MEF 3T6 cells in 35-mm dishes were infected with the indicated viruses at 0.5 PFU per cell. At 24 h postinfection, cells were harvested and processed for electron microscopic analysis as described in Materials and Methods. Digital images were taken at various magnifications with a Gatan digital CCD camera. R4002-infected MEF 3T6 cells (A), R931-infected MEF 3T6 cells (B), R908-infected MEF 3T6 cells (C), and R909-infected MEF 3T6 cells (D) are shown. Scale bars are shown in each picture. Nuc, nuclear region; Cyt, cytoplasm.

experimental condition. Collectively, these data suggest that the amino-terminal domain of the  $\gamma_{134.5}$  protein acts to facilitate virus egress at two steps. While the region containing amino acids 1 to 30 is required for nuclear egress, the region spanning amino acids 30 to 106 is crucial for cytoplasmic egress.

#### DISCUSSION

Several studies have shown that the  $\gamma_{134.5}$  protein is essential to promote virulence in experimental animal models (18, 34, 35, 46, 48). It functions to inhibit the PKR response in HSV infection (14, 17, 19, 28, 29). Importantly,  $\gamma_{134.5}$  null mutants are virulent in PKR-knockout mice but not in wild-type mice

(18, 32). However,  $\gamma_{134.5}$  null mutants, with an extragenic mutation, are capable of blocking PKR activity but nonetheless remain avirulent or partially virulent in vivo (10, 40). One hypothesis to reconcile these findings is that the  $\gamma_{134.5}$  protein has additional functions required for virus infection or virulence. In the present study, we report that efficient virus replication requires the  $\gamma_{134.5}$  functions that promote viral protein synthesis and virus release or egress in the different stages of the virus life cycle.

The cell line-dependent virus growth distinguishes the functions of the  $\gamma_{134.5}$  protein in virus infection. The recombinant virus expressing the NS1 protein was resistant to the antiviral



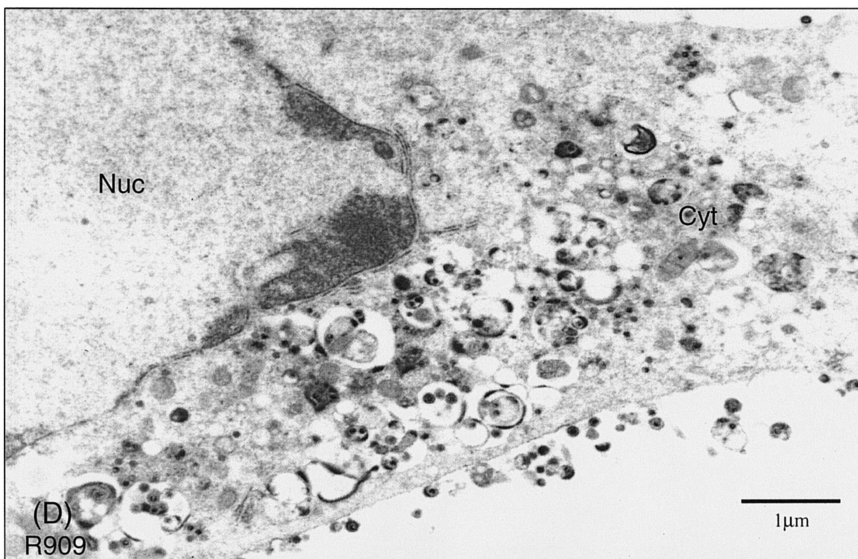
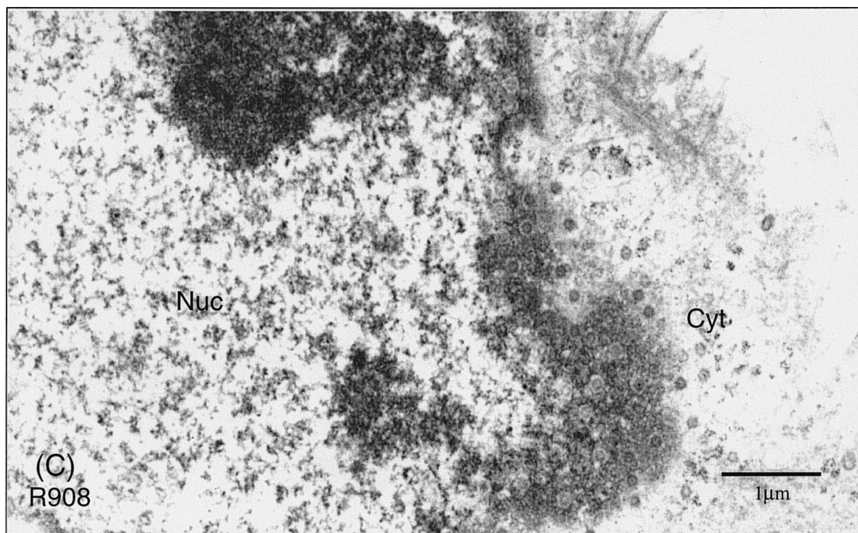


FIG. 8—Continued.

action of interferon. The virus was replication competent in both SK-N-SH and CV-1 cells, which are nonpermissive to the  $\gamma_134.5$  deletion mutants because of the PKR response (19). Thus, the  $\gamma_134.5$  protein and the NS1 protein are functionally interchangeable in antagonizing interferon response involving PKR. Surprisingly, in MEF 3T6 cells, the virus expressing the NS1 protein displayed a completely different phenotype. At a low multiplicity of infection, its growth was intermediate between the wild-type virus and the  $\gamma_134.5$  deletion mutant, which would seemingly result from a defect in viral DNA replication or spread from infected cells. However, as the virus expressing the NS1 protein grew efficiently at a high multiplicity of infection where all cells are infected initially, it is unlikely that viral DNA replication is inhibited. Two lines of evidence support this argument. First, viral polypeptides were produced at comparable levels in MEF 3T6 cells infected with either

wild-type virus or virus expressing the NS1 protein (Fig. 4A). Second, gC (a  $\gamma_2$  gene), whose expression depends strictly on viral DNA replication, was fully expressed in cells infected with the virus expressing the NS1 protein (Fig. 6A). These results are consistent with previous findings that a deletion of the  $\gamma_134.5$  gene from HSV-1 has no effect on viral DNA replication. The fact that the virus expressing the NS1 protein blocked eIF-2 $\alpha$  phosphorylation indicates that the growth defect seen for the virus stems from a block after viral protein synthesis.

It is of interest that the  $\gamma_134.5$  deletion mutant exhibited a severe defect in SK-N-SH, CV-1, and MEF 3T6 cells. The impaired growth in SK-N-SH and CV-1 cells is attributable to the shutoff of protein translation induced by virus infection (19), but the growth defect in MEF 3T6 cells has been suggested to result from a depletion of cellular factors involved in virus replication (7). Brown and colleagues and Harland et al.



reported that the  $\gamma_134.5$  protein interacts with PCNA, a cellular protein required for DNA replication and cell cycle control (9, 26). The interaction between the two proteins was postulated to release cells from growth arrest and facilitate viral replication in HSV-infected MEF 3T6 cells (9). While these observations are interesting, the PKR response in MEF 3T6 cells has not been examined. In this regard, it is notable that viral proteins were barely detectable in MEF 3T6 cells infected with the  $\gamma_134.5$  deletion mutant (Fig. 4A and 6A). Moreover, eIF-2 $\alpha$  is phosphorylated in MEF 3T6 cells infected with the  $\gamma_134.5$  deletion mutant only. These results suggest that the antiviral activity mediated by PKR is operational in MEF 3T6 cells. This conclusion is further supported by the evidence that amino acid substitutions in the PP1-binding domain of the  $\gamma_134.5$  protein caused a severe defect in viral growth, which paralleled viral translation shutoff and eIF-2 $\alpha$  phosphorylation (Fig. 7). Given that viral DNA replication triggers the shutoff of protein synthesis approximately 6 h after infection (19), the ability of the  $\gamma_134.5$  protein to counteract the PKR response seems to be essential during the early phase of virus infection. Thus, the restricted growth pattern of the  $\gamma_134.5$  deletion mutant in MEF 3T6 cells resulted partly from a block in viral protein synthesis mediated by PKR.

Previous studies indicated that a  $\gamma_134.5$  deletion mutant derived from the HSV-1(17<sup>+</sup>) strain is defective in egress (8). However, whether this block in egress is secondary to a defect in synthesis of other viral late proteins has not been resolved. In MEF 3T6 cells infected with the  $\gamma_134.5$  deletion mutant, there is not only a dramatic decrease in virus production but also a decrease in virus release. Thus, it is difficult to assess the specific involvement of egress in productive infection. We have resolved this problem and extended the previous finding by constructing a recombinant virus in which the  $\gamma_134.5$  gene was substituted with the NS1 gene of influenza A virus. As noted above, the substitution of  $\gamma_134.5$  with NS1 restored the function required to antagonize the PKR response but partially rescued virus growth in MEF 3T6 cells. The virus expressing the NS1 protein yielded more virus particles than the  $\gamma_134.5$  deletion mutant in MEF 3T6 cells, but only a smaller fraction was released (Table 1). These results are strengthened by electron microscopic analysis of virus-infected 3T6 cells, where more virus particles are trapped in the nucleus or cytoplasm in cells infected with the  $\gamma_134.5$  deletion mutant or the recombinant virus expressing the NS1 protein than in wild-type HSV-1(F). It is apparent that inhibition of the PKR response early in infection is not sufficient for efficient productive infection. An additional function(s) of the  $\gamma_134.5$  protein is required to promote virus release or egress in MEF 3T6 cells.

Clinical HSV-1 isolates involved in a block in glycoprotein processing and a limited virion release have been described previously (6, 23). During HSV infection, glycoproteins are integrated into the envelope within the endoplasmic reticulum and processed from the high-mannose precursor form to the sialylated mature form as the virus is released. A comparison of the  $\gamma_134.5$  proteins from clinical HSV-1 isolates suggested that a large plaque-producing variant is efficient in virus release that correlates with efficient processing of glycoproteins (6, 37). Unexpectedly, the recombinant virus expressing the NS1 protein is competent in the processing of glycoproteins (gC and gD), yet the virus displayed decreased virus release or egress.

The basis for this is unclear, and additional experiments are needed to resolve this issue.

The present study shows that the amino-terminal domain of the  $\gamma_134.5$  protein is required for efficient virus replication and egress. Previous studies indicated that deletions in the amino terminus of the  $\gamma_134.5$  protein do not affect HSV response to interferon involving PKR (14). Similarly, in MEF 3T6 cells infected with the amino-terminal deletion mutants, viral protein synthesis was not significantly different from that of wild-type virus, and eIF-2 $\alpha$  was not phosphorylated. However, deletions in the amino terminus of the  $\gamma_134.5$  protein resulted in a decrease in virus growth in MEF 3T6 cells. Relevant to these observations are two interesting phenotypes. First, a deletion of the region containing amino acids 1 to 30 from the  $\gamma_134.5$  protein led to an increased accumulation of capsid in the nucleus. It is notable that this portion of the protein contains a nucleolar import signal, which determines the nucleocytoplasmic shuttling of the  $\gamma_134.5$  protein (13). As the  $\gamma_134.5$  protein is a virion component (26), it is possible that this *cis* element is required to direct virus egress from the nucleus to the cytoplasm. Second, a deletion of the region encoding either amino acids 30 to 72 or 72 to 106 from the  $\gamma_134.5$  protein inhibited cytoplasmic egress. Thus, it is conceivable that this region may represent a functional domain required for cytoplasmic transport of virions to the extracellular space. Work is in progress to test these hypotheses.

Lastly, it should be pointed out that  $\gamma_134.5$  mutants exhibit deficient virus growth or egress in mouse 3T6 cells but not in other cells, for example, SK-N-SH and Vero cells. We speculate that the mouse 3T6 cell line may lack a cellular factor that is critical for egress of  $\gamma_134.5$  mutants. Alternatively, this cell line may express an inhibitor that may act to block virus egress during HSV infection. It remains to be established whether  $\gamma_134.5$  mutants are defective in virus egress in human cell lines. Nevertheless, our experimental results appear to correlate with the pathogenesis of HSV infection. Previous studies suggested that mutations in the amino-terminal domain of the  $\gamma_134.5$  protein affect neurovirulence in a mouse model, but this domain is not sufficient to confer virulence (2, 3, 18). Consistent with these observations, the  $\gamma_134.5$  null mutants, with secondary mutations in other regions of the viral genome, block the translation shutoff mediated by PKR. However, these mutants remain highly attenuated *in vivo* (10, 40). Collectively, this study and others suggest that the  $\gamma_134.5$  protein has an additional function(s) required for viral virulence. The fact that  $\gamma_134.5$  mutants are defective in virus egress suggests that efficient virus egress mediated by the  $\gamma_134.5$  protein is crucial for the pathogenesis of HSV infection.

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