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A Five-Amino-Acid Deletion of the Eastern Equine Encephalitis Virus Capsid Protein Attenuates Replication in Mammalian Systems but Not in Mosquito Cells[▽]

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Eastern equine encephalitis virus (EEEV) is a human and veterinary pathogen that causes sporadic cases of fatal neurological disease. We previously demonstrated that the capsid protein of EEEV is a potent inhibitor of host cell gene expression and that this function maps to the amino terminus of the protein. We now identify amino acids 55 to 75, within the N terminus of the capsid, as critical for the inhibition of host cell gene expression. An analysis of stable EEEV replicons expressing mutant capsid proteins corroborated these mapping data. When deletions of 5 to 20 amino acids within this region of the capsid were introduced into infectious EEEV, the mutants exhibited delayed replication in Vero cells. However, the replication of the 5-amino-acid deletion mutant in C710 mosquito cells was not affected, suggesting that virus replication and assembly were affected in a cell-specific manner. Both 5- and 20-amino-acid deletion mutant viruses exhibited increased sensitivity to interferon (IFN) in cell culture and impaired replication and complete attenuation in mice. In summary, we have identified a region within the capsid protein of EEEV that contributes to the inhibition of host gene expression and to the protection of EEEV from the antiviral effects of IFNs. This region is also critical for EEEV pathogenesis.

Eastern equine encephalitis virus (EEEV), a member of the family *Togaviridae*, genus *Alphavirus*, is responsible for sporadic cases of human and equine disease in the Americas. In the United States, Georgia, Louisiana, Massachusetts, New Jersey, and Florida have recorded the largest numbers of eastern equine encephalitis cases. The increase in the number of cases during recent years has underscored the importance of EEEV as a public health threat in the Americas. In addition, due to its potential use as a biological weapon and the lack of an approved vaccine or antiviral for humans, EEEV has been included in the category B priority-pathogen list by the National Institutes of Health (NIH) (11, 29).

EEEV possesses a single-stranded, positive-sense RNA genome of approximately 11.7 kb. The genome encodes four viral nonstructural proteins (nsP1 to nsP4) that are important for viral RNA synthesis and polyprotein processing. The three major structural proteins, the capsid, and the two envelope proteins, E1 and E2, are involved in virus assembly and in receptor recognition, virus attachment, and membrane fusion during viral entry (47). Based on studies with the alphaviruses Sindbis and Semliki Forest, it is believed that the capsid protein is important for the encapsidation of the viral RNA, nucleocapsid assembly, and interaction with the E1 and E2 viral glycoproteins during virus budding (13, 26, 37, 39). The capsid also possesses serine protease activity that allows the protein to release itself into the cytoplasm from the nascent structural polyprotein chain during translation (14, 52). Upon the release of the capsid, the protease active site is occupied by the C terminus of the mature protein, rendering the protease inactive (14). In addition to these functions, we recently implicated the capsid protein of EEEV as a possible virulence determinant (4). The expression of the capsid in mammalian cells strongly inhibits host cell gene expression, and this inhibition correlates with a reduction of host cell RNA levels and the phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF-2α). The capsid protease activity does not appear to be necessary for the inhibition of host gene expression, since the shutoff function maps to the N terminus while the protease domain lies at the C terminus. Additionally, mutation of the serine catalytic site does not affect the inhibitory effect of the capsid on host cell gene expression (4, 25). This newly identified function of the EEEV capsid is analogous to the function of the Sindbis virus and Semliki Forest virus nsP2 proteins (9, 22), which also suppress host cell gene expression. How the capsid and the nsP2 proteins inhibit gene expression remains unclear. In this study, we sought to more precisely define regions within the N terminus of the EEEV capsid important for the inhibition of gene expression. Mapping analyses identified a cluster of 20 amino acids that are necessary for this function. The deletion of amino acids within this region of the capsid generated viruses with wild-type levels of replication in mosquito cells. In contrast, compared to the wild type, these mutants exhibited increased sensitivity to interferon (IFN), delayed replication in mammalian cells, and impaired replication and virulence in mice.

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MATERIALS AND METHODS

Plasmids and viruses. The cloning of the EEEV capsid gene into the pCAGGS mammalian expression vector has been described previously (4). The deletion of sequences within the capsid gene corresponding to amino acids 55 to 75, 55 to 59, 60 to 64, 65 to 69, and 70 to 75 was done by using overlapping PCRs. A hemagglutinin (HA) tag was introduced at the N termini of the capsid mutant

proteins to confirm the expression and subcellular localization of the proteins. To confirm the importance of amino acids 55 to 75 of the capsid in the inhibition of host gene expression and the cellular localization of the capsid, this region and the N terminus of the capsid were fused to the C terminus of the green fluorescent protein (GFP) by overlapping PCRs, and the expression of the constructs was confirmed using antibodies against GFP (Clontech, Mountain View, CA). Maps and primer sequences are available upon request.

For the virus work, the EEEV strain FL93-939 was used to produce an infectious cDNA clone as described previously (2). Strain FL93-939 from a 1993 Florida pool of *Culiseta melanura* mosquitoes was isolated in Vero cells and passaged once in newborn mouse brains to produce RNA before the generation of the full-length cDNA. Deletions within the capsid gene were introduced into the EEEV infectious clone by site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The DNA sequences were linearized using an appropriate restriction site and employed to generate RNA by in vitro transcription. Then RNAs were introduced into BHK cells by electroporation to generate infectious viruses as described previously (5). Virus titers in the stocks were determined by plaque assays. Tenfold dilutions of virus were adsorbed to the monolayers for 1 h at 37°C. A 3-ml overlay consisting of minimal essential medium with 0.4% agarose was added, and the cells were incubated at 37°C for 48 h. Agar plugs were removed, and the cells were stained with 0.25% crystal violet in 20% methanol. The sensitivity of the assay corresponded to a detection limit of 2 $\log_{10}$ PFU/ml.

The complete sequences of the mutant viruses were obtained to confirm the mutations in the capsid gene and exclude the possibility that any other mutation could have arisen during the cloning process.

Reporter assays and STAT-1 expression. 293T cells were transfected with 1 μg of a plasmid encoding the firefly luciferase protein or GFP in the presence or absence of a plasmid encoding the EEEV full-length capsid or a deletion version. Twenty-four hours after transfection, the cells were lysed and luciferase activity was measured according to the protocol of the luciferase assay system manufacturer (Promega). GFP expression was monitored using a fluorescence microscope. In another set of experiments, 293T cells were transfected with 1 μg of the empty plasmid or a plasmid encoding the full-length capsid, the N terminus of the capsid, the C terminus, or one of the capsid deletion mutant proteins. One day after transfection, the cells were treated with 1,000 IU of IFN-β (PBL Biomedical Laboratories). Twelve hours after treatment, protein was extracted from the cells by using the PARIS kit according to the protocol of the manufacturer (Ambion, Austin, TX). Cell lysates were separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (10% PAGE) under reducing conditions. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane, blocked for 1 h in 5% nonfat dry milk dissolved in wash buffer (phosphate-buffered saline [PBS] containing 0.2% Tween and 1% bovine serum albumen) for 1 h, and then incubated with a 0.2-μg/ml concentration of mouse antibody raised against GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Abcam, Cambridge, MA) and a 0.25-μg/ml concentration of mouse antibody raised against STAT-1 (BD Transduction Labs, San Jose, CA). After being rinsed three times with wash buffer, the membrane was incubated overnight at 4°C with a 0.5-μg/ml concentration of allophycocyanin-conjugated goat antibodies raised against mouse immunoglobulin G (Molecular Probes, Eugene, OR). After being rinsed with wash buffer, the membrane was scanned on a Storm phosphorimager (Amersham, Piscataway, NJ) in the red fluorescence mode.

EEEV capsid expression, purification, and antibody production. The EEEV capsid protein was expressed in *Escherichia coli* for the production of a rabbit capsid antibody. The EEEV capsid gene was cloned into the pET15b vector (Novagen, Madison, WI), and the plasmid was used to transform BL21-CodonPlus (DE3)-RIPL cells (Stratagene). For the large-scale expression of the EEEV capsid, cells in a 2-liter volume were grown in 2XYT medium (MP Biomedicals, Solon, OH) at 37°C with agitation until an A_{600} of 0.7 was reached, at which time the cells were induced with 0.1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (Roche, Indianapolis, IN). Following induction, cells were incubated at 37°C with agitation for 4 h to allow for maximal protein expression. The cells were pelleted at 5,000 rpm for 10 min, and the capsid protein was extracted and purified using the His-Bind resin and the Ni-nitrilotriacetic acid buffer kit (Novagen). Protein purity was assessed by SDS-PAGE with silver staining, and the purified protein was used to immunize rabbits for antibody production. To eliminate nonspecific reactivity, the rabbit capsid antibody was preabsorbed onto a monolayer of Vero cells overnight at 4°C. The next day, the antibody was collected and centrifuged to eliminate cell debris.

Subcellular localization of capsid in EEEV-infected cells. Vero cells were seeded onto 12-mm glass coverslips and infected with EEEV at multiplicities of infection (MOI) of 0.1 and 10. Eight or 24 h after infection, the cells were fixed and decontaminated with 10% buffered formalin for 2 days. The coverslips were then washed with PBS containing 50 mM ammonium chloride and 10 mM glycine. After permeabilization with 0.075% saponin and 0.5% bovine serum albumin in PBS (blocking buffer), the cells were incubated in a humidified chamber with 7 μg of rabbit anticapsid antibody/ml for 1 h at room temperature. After being rinsed three times with blocking buffer, the samples were incubated with a 2-μg/ml concentration of Alexa 488-conjugated donkey anti-mouse antibody (Molecular Probes, Eugene, OR) in blocking buffer containing 0.1 μg of Hoechst 33342 (Molecular Probes, Eugene, OR)/ml. The coverslips were washed with blocking buffer and mounted onto glass slides by using Prolong antifade reagent (Molecular Probes, Eugene, OR). Images were acquired on a Leica SP5 confocal microscope using a 63× oil immersion lens objective and a 2× digital zoom. After acquisition, individual channels were pseudocolored and merged using NIH Image.

Construction of EEEV replicons. The schematic representations of the EEEV replicons generated are summarized below (see Fig. 3A). Maps and sequences are available upon request. To produce cDNA templates for RNA synthesis, plasmids were linearized using the NotI site located downstream of the poly(A) tail. In vitro transcription was performed using the mMessage mMachine T7 kit according to the protocol of the manufacturer (Ambion). Five-microgram samples of in vitro-transcribed RNAs were introduced into equal numbers of BHK cells by electroporation as described previously (5), and 22 h after electroporation, 2×10^5 cells were lysed in reporter lysis buffer and lysates were assayed for luciferase activity by following the protocol of the luciferase assay manufacturer (Promega, Madison, WI). To establish a cell line stably expressing the capsid mutant replicon, in vitro-transcribed RNA was introduced into BHK cells by electroporation and, 1 day after electroporation, the medium was replaced with Dulbecco's minimal essential medium–10% fetal bovine serum containing 12.5 μg of puromycin. Single puromycin-resistant cells were isolated, the population was expanded, and RNA was extracted from the cells for PCR and sequencing purposes. Cells were lysed in reporter lysis buffer and lysates were assayed for luciferase activity by following the protocol of the luciferase assay manufacturer (Promega).

Virus replication curve. Vero and C710 cells were seeded into 12-well plates and infected with parental and capsid mutant viruses at a high or low MOI (10 or 0.1). Supernatants were collected at 0, 4, 6, 8, 12, and 24 h after infection, and virus titers were determined by plaque assays using Vero cells in 12-well plates as described above.

Detection of phosphorylated eIF-2α. Vero cells were infected with parental and capsid mutant EEEVs at an MOI of 0.1. Twelve and 24 h after infection, cell lysates were separated by SDS–10% PAGE under reducing conditions. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane, blocked for 1 h in 5% nonfat dry milk dissolved in Tris-buffered saline, and then probed with antibodies against phosphorylated eIF-2α (phosphorylated at serine 52; Cell Signaling Technology, Danvers, MA), eIF-2α (Cell Signaling), or α-tubulin (Sigma, St. Louis, MO). Secondary antibodies conjugated to horseradish peroxidase and the chemiluminescence detection system from Perkin-Elmer (Wellesley, MA) were used to visualize the proteins.

IFN sensitivity assay. Vero cells were infected with parental and capsid mutant EEEVs (MOI = 2) and incubated for 1 h at 37°C. After incubation, cells were washed twice with PBS to remove unbound virus, and then the cells were mock treated or treated with 1,000 IU of IFN-β (PBL Biomedical Laboratories). Eight and 12 h after infection, supernatants were collected to determine virus titers by plaque assays.

Infection of mice. Six-week-old NIH Swiss mice ($n = 6$) were infected subcutaneously with 1,000 PFU of virus, serial blood samples from three animals were collected 24, 48, and 72 h after infection, and virus titers were determined by plaque assays as described above. All animals were weighed and monitored daily for signs of illness and mortality.

Statistical analysis. For virus replication levels and viremia titers, statistical comparisons were performed using a one-way analysis of variance with Tukey's multiple-comparison test as included in the Prism program (GraphPad, San Diego, CA). Values of P of ≤ 0.05 were considered significant.

RESULTS

Amino acids 55 to 75 within the capsid are necessary for the inhibition of gene expression. We previously demonstrated that the EEEV capsid inhibits host cell gene expression. Untagged and HA-tagged capsid proteins are equally able to inhibit protein synthesis (data not shown), and the inhibition correlates with a decrease in mRNA levels and with the phos-

phorylation of eIF-2α (4). To identify the residues important for the inhibition of gene expression, we first deleted a 21-amino-acid (QRAPNPPAGPPAKRKKPAPKP) positively charged region within the N-terminal half of the capsid protein, amino acids 55 to 75, yielding the *d*55-75 capsid. This region was chosen because it is highly conserved in EEEV and Venezuelan equine encephalitis virus (VEEV), which are both known to use the capsid for the inhibition of host gene expression (25). Also, it was previously suggested that this region is important for protein-protein interaction (30), and it possesses a putative nuclear localization signal (NLS) identified by using the Psort II and VirusPloc programs (data not shown) (15, 44, 45). 293T cells were transfected with a firefly luciferase or GFP plasmid in the presence or absence of a plasmid encoding a full-length capsid or the *d*55-75 capsid mutant protein (Fig. 1A). Luciferase activity and GFP expression were reduced only in the presence of the full-length-capsid plasmid but not in the presence of the plasmid encoding the *d*55-75 capsid mutant protein, suggesting that the region of amino acids 55 to 75 is necessary for the inhibition of host gene expression (Fig. 1B and C). Next, we introduced a series of 5-amino-acid deletions within the region of amino acids 55 to 75 of the capsid and assayed the mutant proteins for their abilities to inhibit the expression of reporter genes in cotransfected cells. A reduction in the expression of GFP or luciferase activity in cotransfected cells was observed only in the presence of the full-length capsid and the *d*70-75 capsid mutant protein, suggesting that the region spanning amino acids 55 to 69 of the capsid is important for the inhibition of gene expression (Fig. 1A to C). Western blotting confirmed the expression of each of the capsid constructs and indicated that the *d*55-75, *d*55-59, *d*60-64, and *d*65-69 capsid mutant proteins were expressed at much higher levels than either the full-length capsid or the *d*70-75 capsid mutant construct, possibly reflecting the abilities of the latter two constructs to inhibit gene expression (Fig. 1D).

We previously demonstrated that the capsid inhibits the expression of endogenous IFN-inducible genes (4). Thus, to determine whether the capsid mutant proteins affected the expression of endogenous STAT-1, the expression of which is induced by IFN-β, 293T cells were transfected with empty plasmids or plasmids expressing the full-length capsid, the capsid N terminus, the capsid C terminus, or the capsid deletion mutant proteins. For this experiment, we evaluated only the *d*55-75 and *d*65-69 capsid mutant proteins because the evidence from the experiments described above suggested that the *d*55-59 and *d*60-64 deletion proteins would behave similarly to the *d*55-75 and *d*65-69 deletion proteins. One day posttransfection, cells were treated with IFN-β and levels of endogenous STAT-1 were subsequently analyzed by Western blotting. Note that in these experiments, a pool of cells remained untransfected and, therefore, should have remained unaffected by capsid expression. A 60% decrease in STAT-1 expression was observed only in cells transfected with a plasmid expressing the full-length capsid, the capsid N terminus, or the *d*70-75 capsid deletion mutant protein, while those mutant proteins previously unable to inhibit the expression of a reporter construct in cotransfected cells (Fig. 1) did not inhibit STAT-1 expression (Fig. 2A and B).

Amino acids 55 to 75 are sufficient for the partial inhibition of gene expression. To determine if the expression of amino acids 55 to 75 was sufficient to inhibit gene expression, we fused this region as well as the N terminus of the capsid to GFP and assayed the abilities of the constructs to inhibit luciferase activity. The fusion of GFP to any of these capsid constructs resulted in the nuclear accumulation of GFP, suggesting that capsid residues 55 to 75 may contain an NLS (data not shown). Therefore, for these experiments, a construct consisting of GFP fused to a simian virus 40 T antigen NLS served as a control. A reduction in luciferase activity, relative to that in the control, was observed when luciferase was expressed in the presence of GFP–full-length capsid, GFP-capsid N terminus (amino acids 1 to 126), and GFP fused to the region of amino acids 55 to 75, suggesting that the expression of amino acids 55 to 75 of the capsid is sufficient to suppress gene expression, although not to the same extent as the full-length capsid (Fig. 2C). The expression of the constructs was confirmed by Western blotting using an anti-GFP antibody. These experiments demonstrated a slight decrease in the expression of the construct consisting of GFP fused to amino acids 55 to 75 relative to the expression of the other constructs (data not shown).

An EEEV replicon expressing a capsid with amino acids 55 to 75 deleted is not cytotoxic. We previously demonstrated that an EEEV replicon harboring the capsid gene is more toxic to cells than a replicon without the capsid gene (4). We also demonstrated that stable selection for replicon-containing cells harboring the full-length capsid gene results in the deletion of sequences encoding either the capsid N terminus or the entire capsid, presumably because these mutations decrease toxicity (4). Thus, to determine whether amino acids 55 to 75 of the capsid were required for cytotoxicity and the inhibition of host cell gene expression when coexpressed with the viral nonstructural proteins, we deleted the corresponding region from the EEEV replicon harboring the capsid gene. We then evaluated the ability of this replicon to establish persistent replication. Equal amounts of in vitro-transcribed replicon RNAs were introduced into BHK cells by electroporation (Fig. 3A), and 24 h after electroporation, selection with puromycin was performed. Interestingly, cells containing replicon RNA without the capsid gene or the EEEV replicon harboring the mutant capsid gene formed colonies more efficiently than cells infected with the replicon carrying the full-length capsid gene (data not shown). To evaluate potential differences in luciferase expression from these replicons, RNAs were introduced into cells by electroporation and, 24 h later, equal numbers of cells were lysed and luciferase activity was evaluated. The activity was decreased only when luciferase was expressed in the context of the replicon encoding the full-length capsid; however, the luciferase activity was restored when the region encoding amino acids 55 to 75 was deleted from the capsid gene (Fig. 3B).

The deletion of capsid amino acids 65 to 69 results in virus with delayed replication in mammalian (Vero) cells but not mosquito (C710) cells. To assess the importance of the capsid region of amino acids 55 to 75 for virus replication, we evaluated the effect of deleting amino acids 55 to 75 or 65 to 69 in the context of a full-length EEEV infectious clone-derived virus. Both EEEV capsid mutants were viable but replicated less efficiently than the parental EEEV in Vero cells, regardless of the MOI (Fig. 4A and C). Differences in replication kinetics between the capsid mutant virus with the deletion of amino acids 55 to 75 (Δ55-75) and the parental virus were

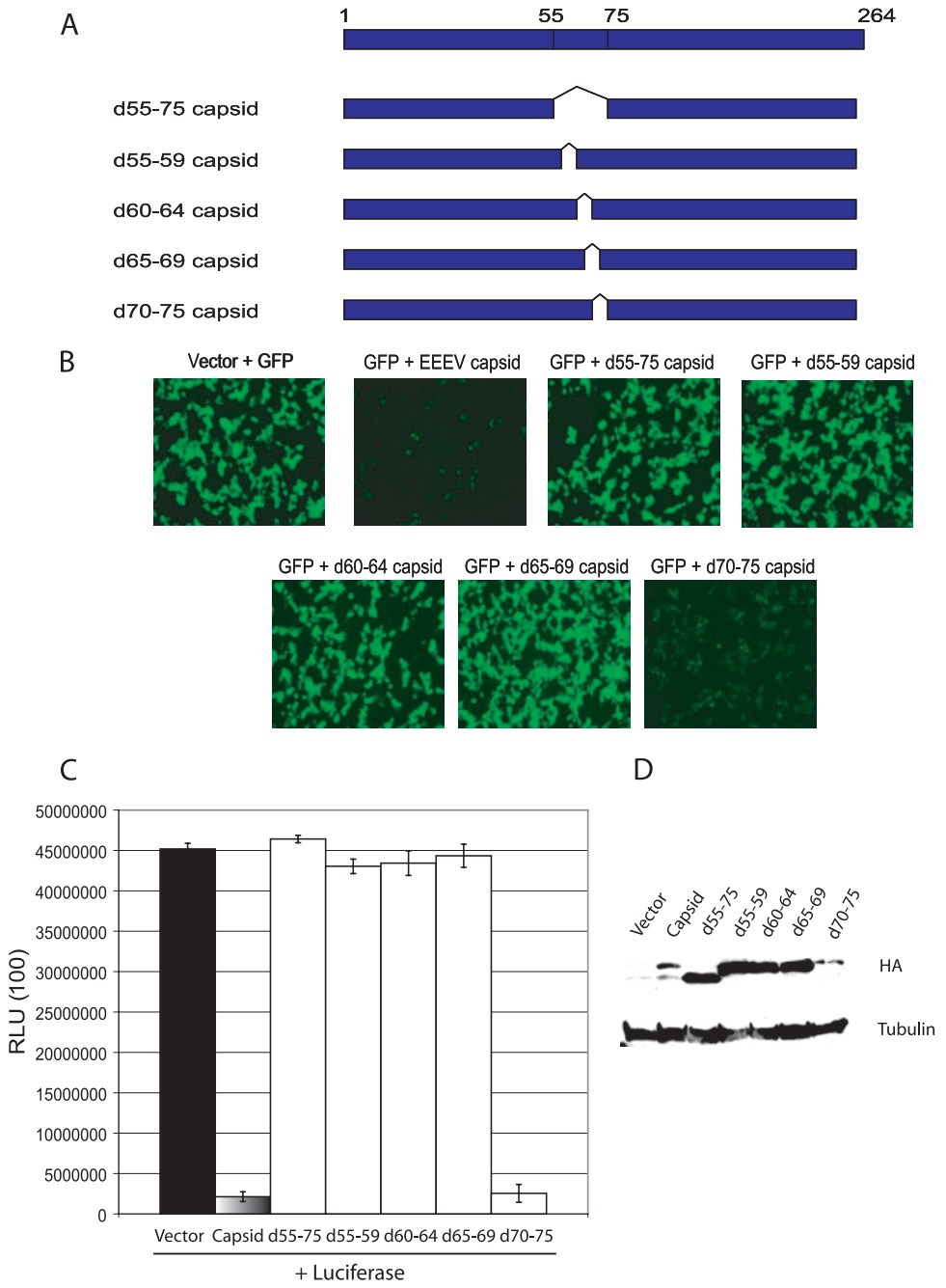

FIG. 1. Amino acids 55 to 75 of the EEEV capsid protein are necessary for the inhibition of gene expression. (A) Schematic representation of the EEEV capsid deletion mutant proteins generated in this study. An HA tag was introduced at the N terminus of the sequence corresponding to the coding region to confirm expression. (B) 293T cells were cotransfected with a GFP reporter plasmid and either an empty vector or a plasmid expressing either the full-length capsid or one of the capsid mutant proteins. Twenty-four hours posttransfection, the cells were examined for GFP expression by microscopy. (C) An experiment was performed as described in the legend to panel B, except that a reporter plasmid expressing firefly luciferase protein was used and cells were harvested and assayed for luciferase activity. The black and white bars and the error bars represent the means ± the standard errors for samples from three separately transfected wells. The experiment was repeated at least twice with consistent results. (D) Western blotting was performed to detect the expression of the full-length capsid and capsid deletion mutant proteins (by detecting the HA tag). The expression of α-tubulin was also measured as a loading control.

observed as early as 6 h postinfection (hpi), and these differences were more evident at 24 and 36 hpi. An MOI of 0.1 resulted in 100-fold-lower levels of the mutant virus ($P < 0.01$) than of the parental virus (Fig. 4C). In contrast, when C710 mosquito cells were infected with the parental and capsid mutant viruses, no significant differences in replication between the parental and Δ65-69 capsid mutant viruses were observed ($P > 0.05$) (Fig. 4B and D). However, the Δ55-75 capsid mutant replicated less efficiently than the parental or Δ65-69 capsid mutant virus. The ability of the Δ65-69 mutant to replicate

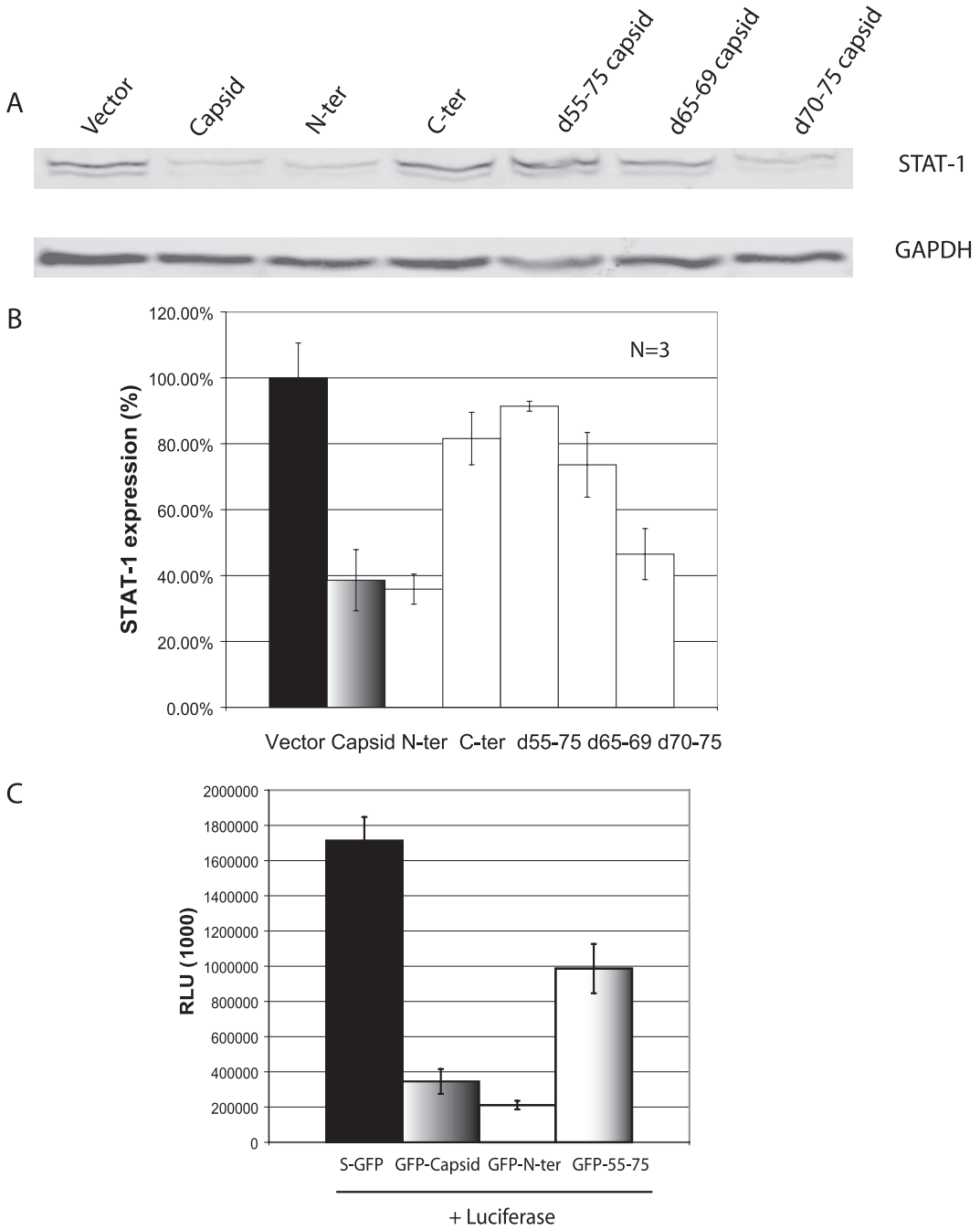

FIG. 2. The expression of amino acids 55 to 75 of the EEEV capsid is sufficient to inhibit gene expression. (A) 293T cells were transfected with an empty vector or a plasmid encoding the full-length capsid, the capsid N terminus (N-ter), the capsid C terminus (C-ter), or one of the capsid deletion mutant proteins. One day after transfection, cells were treated with IFN, and 12 h after treatment, cell lysates were obtained and analyzed by Western blotting for the expression of STAT-1 and GAPDH as a loading control. (B) The quantification of STAT-1 expression was performed using a phosphorimager. The STAT-1 expression level in the vector-transfected cells was set as 100%. (C) 293T cells were cotransfected with a firefly luciferase expression plasmid and a plasmid encoding either GFP fused to a simian virus 40 NLS (S-GFP), the full-length capsid fused to GFP, the region of amino acids 55 to 75 of the capsid fused to GFP (GFP-55-75), or the N terminus of the capsid fused to GFP. Twenty-four hours posttransfection, the cells were lysed and assayed for luciferase activity. The bars and error bars represent the means ± the standard errors for samples from three separately transfected wells. The expression of the constructs was confirmed using anti-GFP antibodies and tubulin as a loading control.

comparably to wild-type virus suggests that the mutation in the capsid does not impair the ability of the capsid to function in virus replication.

Subcellular localization of the capsid in EEEV-infected cells. It has been shown previously that the capsid protein of Semliki Forest virus associates with the nucleus (34), and our data suggest that EEEV capsid residues 55 to 75 can mediate the nuclear accumulation of GFP (see above). Therefore, we examined the subcellular location of the capsid in parental and capsid mutant EEEV-infected cells to determine if the dele-

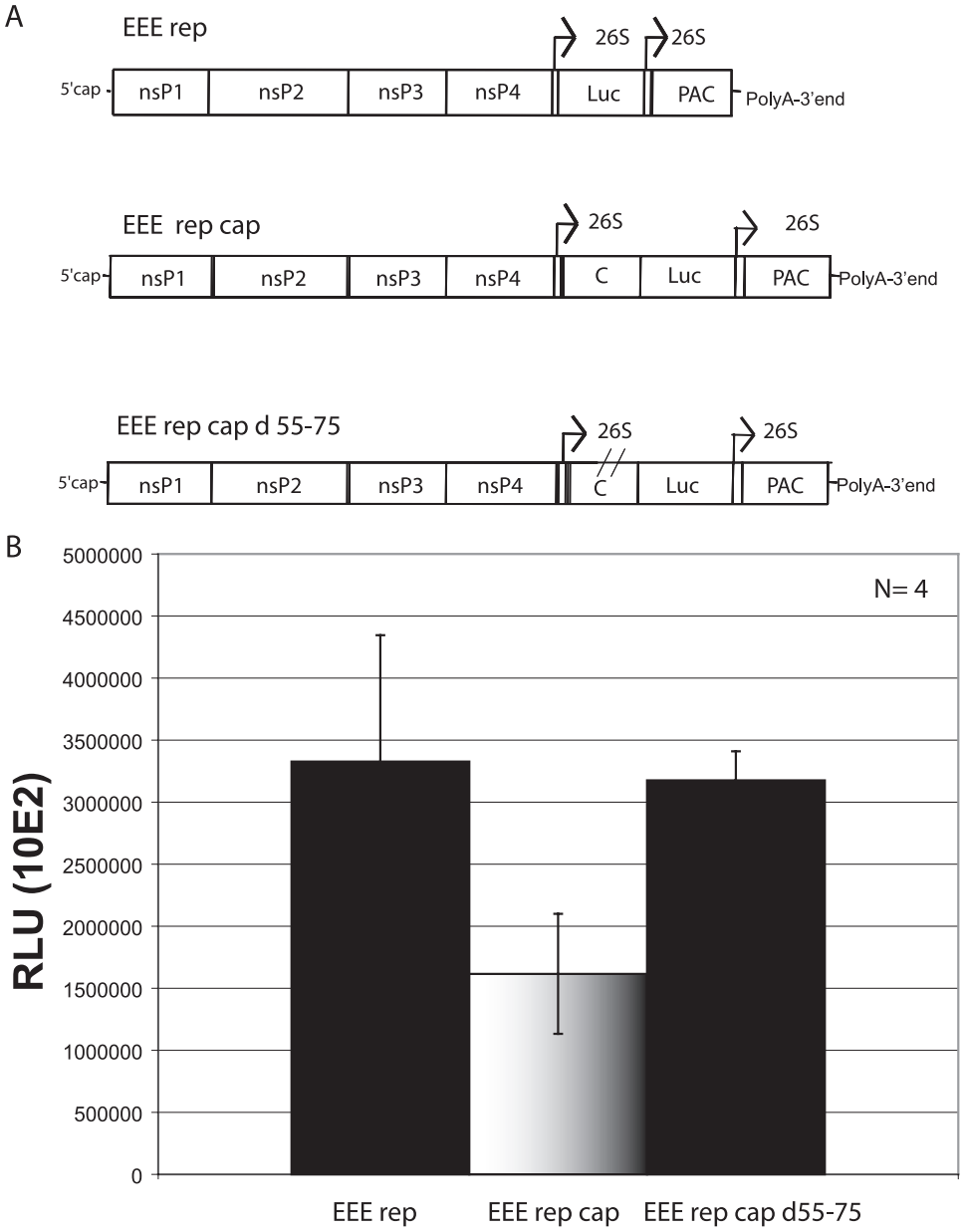

FIG. 3. An EEEV replicon expressing a capsid with the region of amino acids 55 to 75 deleted is not cytotoxic. (A) Schematic representation of the EEEV replicons used in this study. EEE rep, EEEV replicon lacking the capsid gene; EEE rep capsid, EEEV replicon encoding the capsid; EEE rep cap d55-75, EEEV replicon with the deletion of the sequence encoding amino acids 55 to 75 within the capsid gene; nsP1 to nsP4, genes for nonstructural proteins 1 to 4; Luc, luciferase gene; PAC, puromycin acetyltransferase gene (encoding puromycin resistance); C, capsid gene; arrows labeled 26S, 26S subgenomic promoters. (B) Equal amounts of in vitro-transcribed replicon RNAs were introduced into BHK cells by electroporation, and 24 h after electroporation, equal numbers of cells were lysed and assayed for luciferase activity.

tion of amino acids 55 to 75 and/or 65 to 69 within the capsid altered the subcellular localization of the protein. Microscopic examination using an EEEV capsid-specific antibody revealed that the full-length capsid and capsid mutant proteins were found primarily in the cytoplasm at 24 hpi (data not shown). However, there was obvious nuclear localization at 8 hpi. Interestingly, the wild-type capsid appeared to be concentrated in a region of the nucleus suggestive of the nucleolus (Fig. 5). Thus, in the context of virus infection, these mutations do not detectably alter the nuclear/cytoplasmic distribution of the capsid protein. Interestingly, previous work had shown a perinuclear location of a GFP-capsid fusion produced by a VEEV replicon (23); however, at the time points examined, we did not observe similar localization of the EEEV capsid in infected cells.

Δ55-75 and Δ65-69 capsid mutant EEEVs are more sensitive to the antiviral action of IFN than the parental virus. We previously demonstrated that the expression of the EEEV capsid protein can suppress IFN responses (4). To evaluate whether the capsid mutant viruses were less efficient in suppressing the antiviral action of IFN than the parental virus,

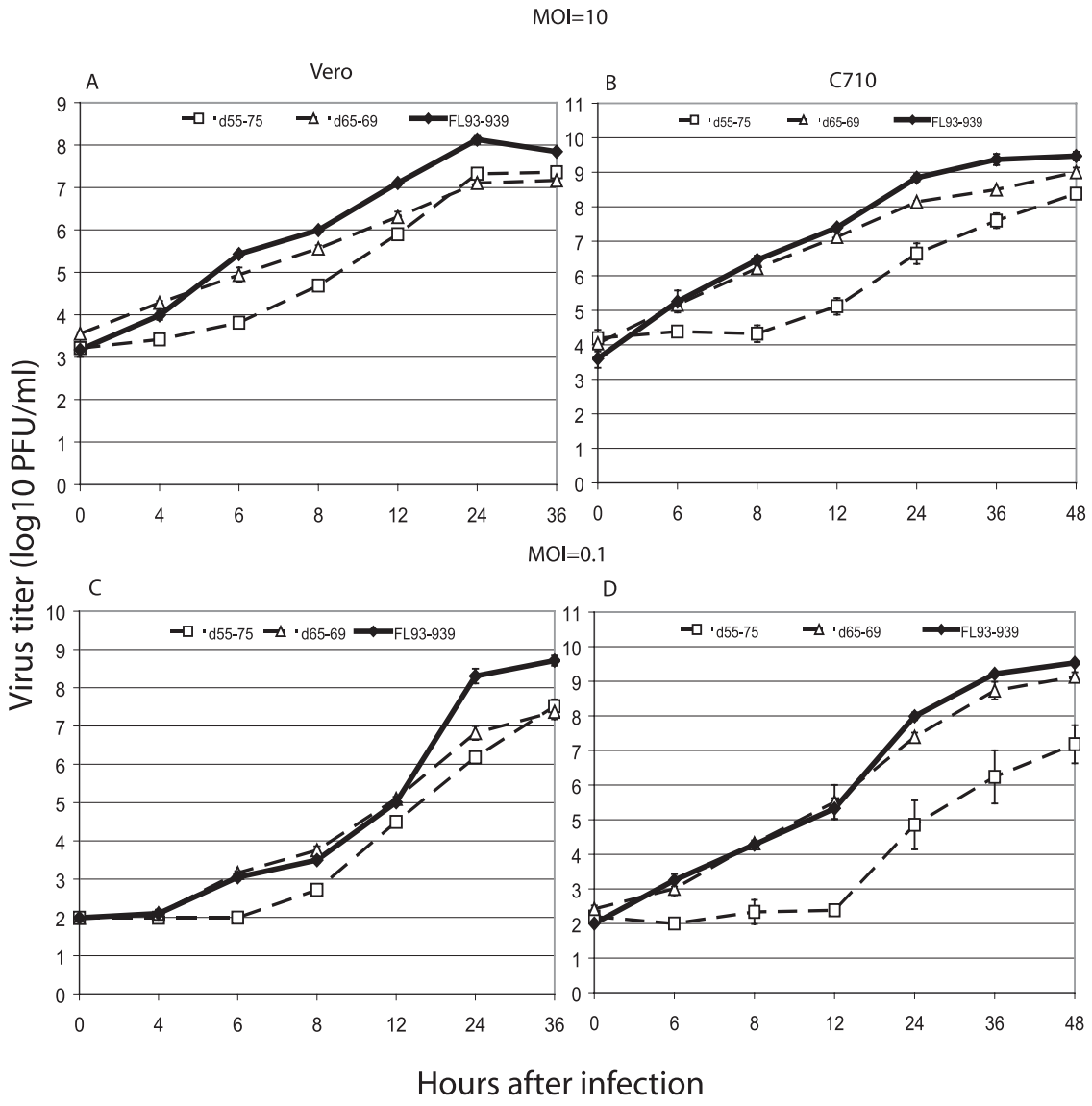

FIG. 4. Capsid mutant viruses replicate less efficiently than parental EEEV in Vero cells, but Δ65-69 is not impaired in mosquito cells. Vero cells and C710 cells were infected with parental FL93-939 or the Δ55-75 or Δ65-70 capsid deletion mutant, and at the indicated time points, supernatants were harvested and virus titers were determined by plaque assays (n = 3). The error bars indicate standard errors of the means.

Vero cells were infected with EEEV FL93-939 and the capsid mutant viruses (MOI = 2) and, 1 h after infection, cells were mock treated or treated with 1,000 IU of IFN-β. A reduction in virus replication in the IFN-treated cells infected with the Δ55-75 and Δ65-69 capsid mutant EEEVs relative to that in the mock-treated cells was observed as early as 8 hpi; however, no significant difference in replication between the IFN-treated and mock-treated cells infected with the parental EEEV was observed (Table 1). These modest differences between wild-type and mutant EEEVs became more pronounced at 12 hpi. Thus, the deletion of amino acids 55 to 75 or 65 to 69 increased EEEV sensitivity to IFN.

Δ55-75 and Δ65-69 capsid mutant EEEVs induce the phosphorylation of eIF-2α to a lesser extent than the parental EEEV. We previously showed that the infection of cells with EEEV induces the phosphorylation of the translation initiation factor eIF-2α in infected cells and that the eIF-2α phosphorylation levels correlate with the efficiency with which the capsid induces the shutoff of gene expression (4). To determine whether the Δ55-75 and Δ65-69 capsid mutant viruses were still capable of inducing the phosphorylation of eIF-2α, we infected Vero cells (MOI = 0.1) and assayed them for the presence of phosphorylated eIF-2α at 12 and 24 h after infection. Decreased levels of phosphorylated eIF-2α in cells infected with the capsid mutant viruses compared to those in cells infected with the parental virus were observed at 12 and 24 hpi. eIF-2α phosphorylation was detectable only in parental virus-infected cells and not in mutant virus-infected cells at 12 hpi (data not shown). At 24 hpi, eIF-2α phosphorylation was detectable in all infected cultures, but the mutant virus-infected cells exhibited reduced phosphorylation relative to that in the parental virus-infected cells (Fig. 6). This decrease in phosphorylation

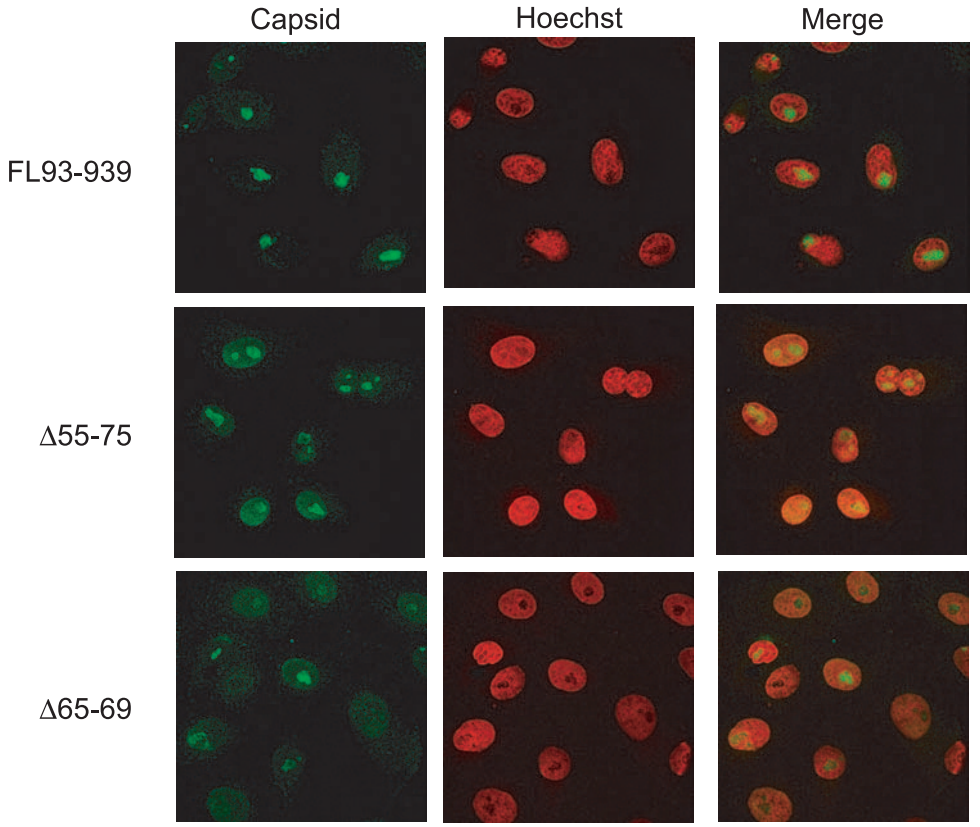

FIG. 5. Subcellular localization of EEEV capsid proteins from wild-type and mutant viruses in Vero cells. Vero cells were infected with parental and capsid mutant EEEVs, and confocal microscopy studies were performed using anti-EEEV capsid-specific antibodies. The capsid proteins from strain FL93-939 (top row) and mutants Δ55-75 (middle row) and Δ65-69 (bottom row) were concentrated in the nuclei 8 hpi.

levels correlated with the decreased levels of replication of the capsid mutant viruses.

Δ55-75 and Δ65-69 capsid mutant EEEVs are attenuated in mice. To evaluate the virulence of the Δ55-75 and Δ65-69 capsid mutant EEEVs, 6-week-old NIH Swiss mice were infected subcutaneously with 1,000 PFU of wild-type or mutant viruses, and viremia and mortality were monitored daily. Twenty-four hours postinfection, low viremia titers were observed in animals infected with the capsid mutant viruses. However, the viruses were rapidly cleared from the circulation; viremia was undetectable at 48 and 72 hpi, results differing significantly from the viremia titers obtained for animals infected with the parental, wild-type EEEV ($P < 0.05$; one-way analysis of variance) (Fig. 7A). Importantly, none of the animals infected with the capsid mutant viruses showed any signs of illness, and all survived infection. In contrast, five of six animals infected with the parental virus succumbed to infection (Fig. 7B), demonstrating the necessity of capsid region 55 to 75 for efficient virus replication and virulence in vivo. Mice inoculated subcutaneously with mutant virus doses as high as 10^5 PFU survived infection and showed no signs of disease (data not shown), indicating that the attenuation of the capsid mutant viruses was dose independent.

DISCUSSION

The inhibition of host cell protein expression is a characteristic of alphavirus infections (31, 47). Studies with Sindbis and Semliki Forest viruses, both Old World alphaviruses, have shown that the shutoff of protein synthesis in infected cells occurs as early as 2 to 4 hpi (46, 54). Several hypotheses have been proposed as potential mechanisms. First, it has been

TABLE 1. Replication of wild-type and capsid mutant EEEVs in Vero cells mock treated or treated with 1,000 IU of IFN-β

Virus	Virus titer (log_{10} PFU/ml ± SE) at 8 hpi		Log_{10} reduction in virus titer at 8 hpi	Virus titer (log_{10} PFU/ml ± SE) at 12 hpi		Log_{10} reduction in virus titer at 12 hpi
	Without IFN treatment	With IFN treatment		Without IFN treatment	With IFN treatment	
FL93-939	5.73 ± 0.06	5.8 ± 0.03	0	7.3 ± 0.01	7.1	0.2
Δ55-75	4.8 ± 0.2	4.5 ± 0.2	0.3	6.4 ± 0.2	5.5	0.9
Δ65-69	5.6 ± 0.04	5.5 ± 0.04	0.1	6.7 ± 0.2	6.1 ± 0.2	0.6

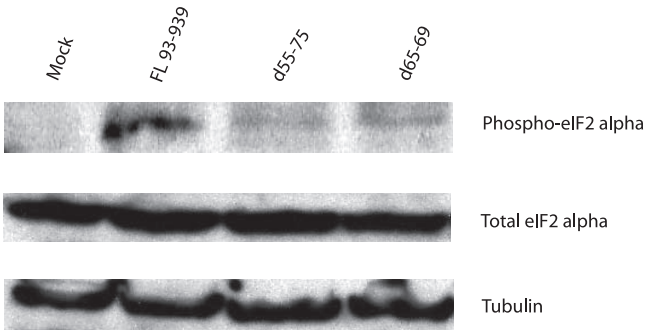

FIG. 6. Parental and capsid mutant EEEVs induce the phosphorylation of eIF-2α. Vero cells were mock infected or infected with EEEV strain FL93-939 or the Δ55-75 or Δ65-69 capsid mutant virus (MOI = 0.1), and at 24 hpi, cells were lysed and Western blotting was performed to detect the phosphorylated eIF-2α (phospho-eIF2 alpha), total eIF-2α, and α-tubulin as a loading control. Similar results were observed at 12 hpi (data not shown).

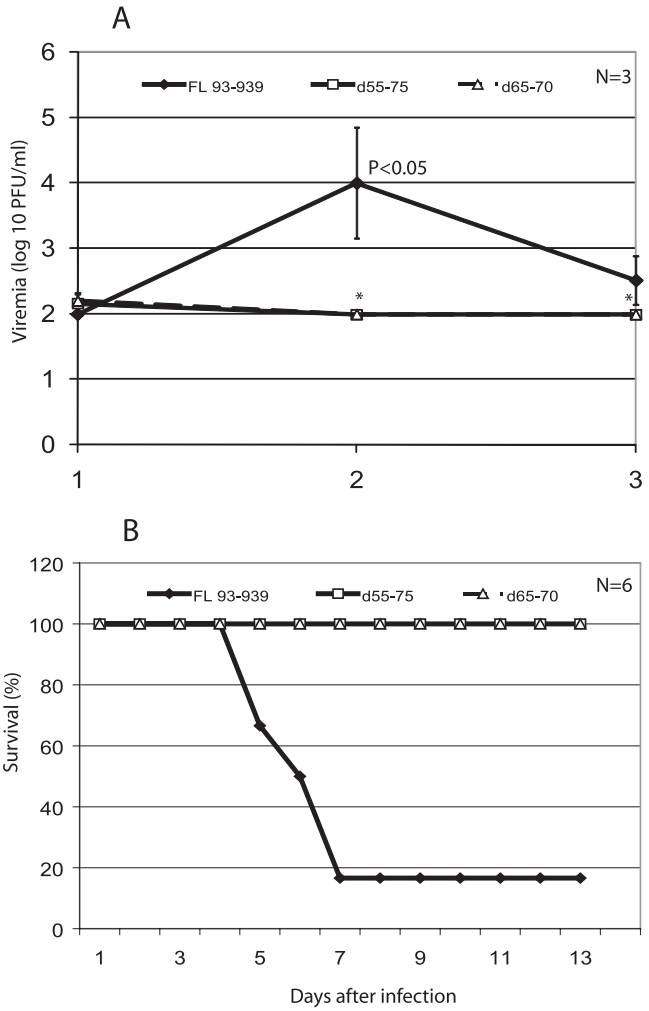

FIG. 7. Capsid mutant viruses are completely attenuated in mice. Six-week-old mice were infected subcutaneously with 1,000 PFU of wild-type parental FL93-939 virus or Δ55-75 or Δ65-70 capsid mutant virus. (A) Viremia generated in the animals as measured by plaque assays ($n = 3$) at the indicated days postinfection. Asterisks indicate that the titer was below the limit of detection (2 $\log_{10}$ PFU/ml). (B) Survival rates ($n = 6$).

suggested that the 26S subgenomic mRNAs outcompete cellular mRNAs for a component necessary for the initiation of protein synthesis (31, 54). Second, it has been proposed that the synthesis of virus-specific RNA in the cytoplasm inhibits cellular protein synthesis because of the consumption of cytoplasmic ribonucleoside triphosphates, which leads to a decrease in the ribonucleoside triphosphate pool available for protein synthesis (54). However, studies using Sindbis and Semliki Forest virus replicons have shown that the inhibition of protein synthesis occurs in the absence of the structural proteins and, also, under conditions in which the level of the viral subgenomic RNA is too low to be detected by ^{35}S protein labeling (21, 40). The inhibition of protein synthesis induced by these Old World alphaviruses appears to involve the viral nsP2 protein, since mutations in the corresponding gene result in replicons capable of establishing persistent replication in host cells and in viruses less able to inhibit host protein synthesis than wild-type viruses (1, 9, 17, 20, 22, 40). More recently, we and others have identified remarkable differences in the mechanisms by which the New World versus Old World alphaviruses inhibit host cell gene expression (4, 25). In contrast to the Old World alphaviruses, which appear to use nsP2 for the inhibition of protein synthesis, the New World alphaviruses EEEV and VEEV appear to use the capsid protein for the inhibition of host gene expression (4, 25). Interestingly, Old World alphaviruses have been suggested previously to inhibit protein expression by two different mechanisms: the inhibition of transcription and the inhibition of translation (24, 27). However, those studies did not provide clear evidence for the inhibition of transcription and showed evidence only of a reduction in endogenous RNA levels, which may also be caused by other mechanisms, such as the inhibition of mRNA processing and/or RNA degradation. In our previous study, we also showed that endogenous RNA levels for IFN-inducible genes are decreased in cells transfected with the EEEV capsid gene (4). Thus, further studies are necessary to determine how the capsid and nsP2 cause a reduction in RNA levels.

The inhibition of gene expression is not unique to alphavirus infections. For example, the NSs protein of Bunyamwera virus inhibits the phosphorylation of the RNA polymerase II (pol II) C-terminal domain, specifically that of serine 2, which is a process necessary for mRNA elongation and 3′-end processing (48). Rift Valley fever virus inhibits cellular RNA synthesis by targeting transcription factor II H-dependent transcription (32). Poliovirus and vesicular stomatitis virus have also been shown to target specific components of the RNA pol II transcriptional machinery (16, 55, 56, 58, 59). Additionally, the ribonucleoprotein complex of influenza virus was shown previously to bind the C-terminal domain of RNA pol II, triggering an inhibitory effect on RNA pol II elongation (12), and the influenza A virus NS1 protein can inhibit the processing of host cell mRNAs (36). Several studies have also demonstrated that the inhibition of host gene expression can serve as a mechanism by which RNA viruses evade host innate immunity (8, 10, 19, 22, 35, 36, 41, 50).

Although alphaviruses replicate in the cytoplasm, the EEEV capsid localizes to the nucleus early after infection, as does the

nsP2 protein of Old World alphaviruses (9, 18, 38, 42, 43). This observation suggests that both the capsid and nsP2 affect some host nuclear function(s), such as transcription, mRNA processing, and/or the targeting of RNA for degradation. In this study, we further characterized the inhibition of protein synthesis mediated by the capsid protein and identified a 21-amino-acid region necessary for the inhibition of gene expression. Evidence supporting the importance of amino acids 55 to 75 for the inhibitory effect of the capsid was also obtained when the mutant proteins were fused to GFP and expressed in cells in the presence of a luciferase plasmid. A reduction in luciferase activity was observed when luciferase was expressed along with a plasmid encoding amino acids 55 to 75 fused to GFP, the full-length capsid fused to GFP, or the N terminus of the capsid fused to GFP. However, it should be noted that the inhibition of gene expression observed with GFP fused to amino acids 55 to 75 was not as pronounced as that observed with the full-length capsid–GFP fusion, as determined by measuring luciferase activity. Other evidence supporting the importance of the capsid amino acids 55 to 75 in the inhibition of gene expression was obtained with EEEV replicons. It was reported previously that EEEV replicons harboring the capsid gene are cytotoxic to host cells and that stable selection for cells expressing EEEV replicons with the capsid gene consistently results in selection for replicons with the capsid gene deleted in part or in its entirety (4, 25). In the present study, we deleted the region encoding amino acids 55 to 75 from the EEEV replicon harboring the capsid gene and evaluated the ability of the replicon to establish persistent replication. The resultant EEEV replicon was as efficient as the EEEV replicon without the capsid gene in establishing persistent replication and yielded luciferase activity levels similar to those expressed from the EEEV replicon lacking the capsid gene.

Having demonstrated a critical role for amino acids 55 to 75 in the capsid-mediated inhibition of host gene expression and cytotoxicity, we assessed the importance of this region for virus replication. Deletions of capsid amino acids 55 to 75 and 65 to 69 were generated in full-length cDNA clones of the virulent EEEV strain FL93-939 (3). Interestingly, both mutants were viable but attenuated for replication in Vero cells, producing 100-fold-lower titers than the parental virus by 24 h after low-multiplicity infection (Fig. 7); however, the replication of the Δ65-69 capsid mutant in mosquito cells was not affected. These results correlate with those in recent reports that suggest the inhibition of nuclear import by the capsid protein of VEEV in mammalian but not mosquito cells (6, 23). Whether this cell-specific difference applies to the capsid protein of EEEV deserves further investigation. The attenuation of the mutants in Vero cells is notable given that these cells do not produce IFN-α/β. We previously demonstrated that the EEEV capsid can inhibit IFN-α/β antiviral responses (4). Consistent with this finding, the mutation of the capsid in the context of a replicating virus increased the sensitivity of virus replication to IFN-β added after virus infection. Whereas wild-type virus was relatively resistant to the antiviral effects of IFN-β, the mutant viruses showed modestly increased sensitivity to the antiviral effects of IFN-β (Table 1). The virus replication defect in Vero cells suggests that the capsid may also suppress additional, IFN-α/β-independent antiviral responses. Despite the absence of IFN-α/β genes, Vero cells retain the signaling pathways that activate IFN regulatory factor 3 in response to virus infection. Because a number of genes can be activated directly by IFN regulatory factor 3, such responses may account for the attenuation seen in the Vero cells (7, 28, 57). The reduced replication of the Δ65-69 capsid mutant was observed only in Vero cells and not in mosquito cells, suggesting that the deletions within the capsid protein did not affect nucleocapsid formation and/or other essential capsid functions. In contrast, the Δ55-75 capsid mutant replicated poorly in both Vero and C710 cells, suggesting a possible defect in some aspect of the viral replication cycle that deserves further investigation. The Sindbis virus capsid has been divided into region I, region II, and region III. Within the amino terminus are regions I and II. Region I, rich in basic amino acids, is involved in binding to genomic RNA and also contains an alpha helix (helix I) important for nucleocapsid assembly. Region II is involved in the recognition of the viral genomic RNA, and the carboxy-terminal region III contains the protease domain (30, 33, 37, 39, 49, 51, 53). Further studies are needed to determine whether the Δ55-75 capsid mutation affects region I or II functions in EEEV. Further studies should also address the cellular or viral factors that may be interacting with and exporting the capsid from the nucleus to the cytoplasm. Alternatively, the protein may possess an export signal that regulates its subcellular localization during viral infection.

In mice, the EEEV capsid deletions profoundly affected viral pathogenesis, essentially eliminating viremia and rendering the mutants avirulent at a dose at which the parental virus is 80% lethal. These data, coupled with those described above, strongly suggest that the inhibition of gene expression is critical for EEEV pathogenesis.

The identification of these mutations, which dramatically affect virulence in mice, raises the possibility that these or similar mutations may allow the development of attenuated EEEV strains for use as vaccines. Additional studies will be required to further address this possibility. The mechanism by which the capsid inhibits gene expression remains to be determined but is presumed to involve the interaction of the capsid with host cell factors. Given that amino acids 55 to 75 were sufficient, when fused to GFP, to inhibit gene expression, the relevant host cell factor(s) should interact with this domain. Defining such a host cell factor(s) may provide information helpful for identifying potential targets for therapeutic intervention.

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