

## Ribosomal Protein S6 Associates with Alphavirus Nonstructural Protein 2 and Mediates Expression from Alphavirus Messages

Stephanie A. Montgomery,<sup>1,2\*</sup> Peter Berglund,<sup>3</sup> Clayton W. Beard,<sup>1,2</sup> and Robert E. Johnston<sup>1,2</sup>

Department of Microbiology and Immunology<sup>1</sup> and Carolina Vaccine Institute,<sup>2</sup> University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892<sup>3</sup>

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Although alphaviruses dramatically alter cellular function within hours of infection, interactions between alphaviruses and specific host cellular proteins are poorly understood. Although the alphavirus nonstructural protein 2 (nsP2) is an essential component of the viral replication complex, it also has critical auxiliary functions that determine the outcome of infection in the host. To gain a better understanding of nsP2 function, we sought to identify cellular proteins with which Venezuelan equine encephalitis virus nsP2 interacted. We demonstrate here that nsP2 associates with ribosomal protein S6 (RpS6) and that nsP2 is present in the ribosome-containing fractions of a polysome gradient, suggesting that nsP2 associates with RpS6 in the context of the whole ribosome. This result was noteworthy, since viral replicase proteins have seldom been described in direct association with components of the ribosome. The association of RpS6 with nsP2 was detected throughout the course of infection, and neither the synthesis of the viral structural proteins nor the presence of the other nonstructural proteins was required for RpS6 interaction with nsP2. nsP1 also was associated with RpS6, but other nonstructural proteins were not. RpS6 phosphorylation was dramatically diminished within hours after infection with alphaviruses. Furthermore, a reduction in the level of RpS6 protein expression led to diminished expression from alphavirus subgenomic messages, whereas no dramatic diminution in cellular translation was observed. Taken together, these data suggest that alphaviruses alter the ribosome during infection and that this alteration may contribute to differential translation of host and viral messages.

The *Alphavirus* genus contains over 25 recognized viruses with a wide geographic distribution. Venezuelan equine encephalitis virus (VEE) is a New World alphavirus that is maintained in nature by cycling between a mosquito vector and susceptible vertebrate hosts. VEE is responsible for periodic outbreaks of disease in humans and equines and is classified as a select agent, making it a prominent pathogen among the alphaviruses.

Alphaviruses possess a genome of single-stranded message-sense RNA that is approximately 11.5 kb in length. The alphavirus genome is organized such that the 5' two-thirds of the genome encodes four nonstructural proteins (nsP1 through nsP4), whereas the 3' one-third encodes three mature structural proteins (capsid, E2, and E1) that are expressed at high levels after transcription from an internal 26S subgenomic mRNA promoter. The viral genome appears to be similar to cellular mRNAs, since it contains 5' and 3' untranslated regions, with a 5'-terminal methylguanylate cap and a 3'-terminal polyadenylate tail. Upon release into a host cell, the viral genomic RNA is directly translated by the cellular translation machinery. The nonstructural proteins are synthesized as two polyproteins, termed P123 and P1234. P123 results when translation terminates at an opal termination codon between nsP3 and nsP4. When the translation machinery reads through the opal, the larger P1234 is produced. Ultimately, both precursors are cleaved into the four mature nonstructural proteins by the

carboxyl-terminal protease domain of nsP2. Negative-sense viral RNA is synthesized early in infection by both the P123 intermediate along with nsP4, as well as the P23 intermediate, along with nsP1 and nsP4 (27, 63). Upon further processing, the four mature individual nonstructural proteins comprise a viral replication complex that utilizes the negative-sense RNA as a template to synthesize full-length positive-sense genomic RNA as well as the subgenomic RNA expressed from the internal 26S promoter (for a review, see reference 58).

Nonstructural protein 2 (nsP2) is an essential member of the viral replication complex. It is a multifunctional protein, as several roles in the viral replication cycle have been ascribed to it. The N-terminal domain of nsP2 contains helicase activity that is believed to be involved in the unwinding of duplex RNA formed during replication, and the C-terminal domain functions as the nonstructural proteinase that cleaves the polyprotein precursor (58). In addition, nsP2 is involved in the regulation of negative-strand RNA synthesis (51).

nsP2 also possesses poorly understood auxiliary functions that may affect the outcome of infection. Although the replication cycle occurs in the cytoplasm, nsP2 is the only alphavirus nonstructural protein that is present in both the cytoplasm and the nucleus of infected mammalian cells (3, 34, 45; S. A. Montgomery and R. E. Johnston, unpublished data). Although the role of nuclear nsP2 remains elusive, disruption of nsP2 nuclear localization compromises the ability of the virus to spread in the brain (11). Moreover, although alphaviruses characteristically cause lytic infection in mammalian host cells, mutations outside the nuclear localization domain have been identified that permit the establishment of persistent, noncytopathic infections. Interestingly, independent studies carried out in various

\* Corresponding author. Mailing address: Department of Microbiology and Immunology, CB 7292, Mary Ellen Jones Bldg., University of North Carolina at Chapel Hill, Chapel Hill, NC 27599. Phone: (919) 966-4026. Fax: (919) 843-6924. E-mail: smontgo@med.unc.edu.

alphaviruses have all mapped critical mutations affecting persistence to nsP2 (10, 14, 46, 64). The localization of nsP2 to the nucleus and the identification of several mutations in nsP2 that confer persistence in mammalian cells suggests that there are undefined functions of nsP2 mediated through interactions that affect host cell survival and determine the outcome of viral infection.

Clues to understanding these auxiliary functions can come from the identification of their interactions with cellular factors. To date, few cellular factors involved in the alphavirus life cycle have been identified. Early studies with Sindbis virus, the prototype alphavirus, first suggested that specific cellular factors are likely required for alphavirus replication (2, 3, 22, 28, 55). In infected cells, the capsid protein transiently associates with ribosomes, with this interaction between host and viral proteins believed to promote uncoating of the virus (61, 66). In addition, it is predicted that there are many specific interactions between alphavirus RNAs and host cellular proteins. The alphavirus genome contains four conserved sequence elements. Nucleotide divergence in these elements may have been suppressed as a result of these regions of RNA specifically interacting with cellular proteins, since mutation in the interacting RNA element would require a compensatory mutation in the host factor (36). Multiple cellular proteins are believed to bind the untranslated regions of alphavirus RNA and modulate viral replication. For instance, the La autoantigen binds the 3' end of the alphavirus negative-sense RNA, a region that serves as a viral promoter for progeny genome synthesis (24, 35, 38–40). Although alphaviruses likely interact specifically with many cellular factors during their replication cycle, cellular proteins are excluded from budding viral particles (42, 43).

We report here that ribosomal protein S6 (RpS6), the major phosphoprotein of the ribosome (16, 23, 48), interacts specifically with alphavirus nsP2. Characterization of this and other virus-host interactions will lead to a better understanding of how alphaviruses co-opt cellular functions for their own use during replication and ultimately how such interactions contribute to the pathogenesis of alphaviruses.

#### MATERIALS AND METHODS

**Cells and infections.** BHK-21 cells (ATCC, passage 55-65) were incubated at 37°C under 5% CO<sub>2</sub> and maintained in alpha minimal essential medium (Gibco) containing 10% donor calf serum, 10% tryptone phosphate broth, 0.29 mg of L-glutamine per ml, 100 U of penicillin per ml, and 0.5 mg of streptomycin per ml. For electroporation, BHK-21 cells were cultured overnight in medium containing 10% fetal bovine serum (FBS), harvested when subconfluent, and prepared for electroporation as previously described (8, 29). HEK-293 cells (ATCC, passage 30-45) were incubated at 37°C under 5% CO<sub>2</sub> and maintained in Dulbecco modified Eagle medium (Gibco) supplemented with 10% FBS, 100 U of penicillin per ml, and 0.5 mg of streptomycin per ml.

For VEE replicon particle (VRP) and VEE infection, six-well plates were seeded 18 h prior to infection at  $4 \times 10^5$  cells per well for BHK-21 cells and  $1 \times 10^6$  cells per well for HEK-293. For infection, the medium was removed, and cell monolayers were infected at a multiplicity of infection (MOI) of 10 (unless otherwise indicated) in a minimal volume of phosphate-buffered saline (PBS) supplemented with 1% donor calf serum and Ca<sup>2+</sup>/Mg<sup>2+</sup>. After 1 h of adsorption at 37°C, complete growth medium was added back to each well.

**Virus and replicon particles.** The assembly of the full-length VEE cDNA clone pV3000 that was derived from the natural Trinidad donkey VEE isolate has been described previously (9). Virus stocks were produced by electroporating in vitro-transcribed RNA of pV3000 into BHK-21 cells as previously described (8). Virus particles were harvested from the supernatant at 24 h postinfection (hpi) after a significant cytopathic effect was evident. Virus stocks then were clarified by centrifugation ( $10,000 \times g$ , 30 min, 4°C) and further concentrated by

pelleting the clarified virus preparations through 20% (wt/vol) sucrose in low-endotoxin PBS at  $72,000 \times g$  for 5 h at 4°C. Virus titers were determined by plaque assay on BHK-21 cells.

VRPs expressing green fluorescent protein (GFP), human ribosomal protein S6 (RpS6), or a C-terminally FLAG (DYKDDDDK) epitope-tagged human RpS6 (RpS6-FLAG) were packaged by using a split helper system as previously described (47). Briefly, three RNA transcripts were co-electroporated into BHK-21 cells: the replicon genome, which contains the four VEE nonstructural genes and the heterologous gene expressed from the viral 26S promoter, and two defective helper RNAs, which provide either the wild-type capsid or the wild-type glycoprotein genes but lack the virus-specific packaging signal. Since VRP replicon genomes lack the viral structural protein genes, infectious VRPs undergo only one round of infection. After packaging, VRPs were harvested, concentrated through a sucrose cushion, and resuspended, and titers were determined on BHK-21 cells either by immunofluorescence (GFP-VRP) or immunocytochemistry (RpS6-VRP and RpS6-FLAG-VRP) using sera containing antibody to the VEE nonstructural proteins. Titers were expressed as infectious units (IU) per ml.

**Plasmid constructs.** For the synthesis of the replicon transcript for GFP-VRP, the wild-type replicon plasmid pV5005 was used which contains the gene for GFP mutant 2 (7) located directly downstream of the 26S promoter, replacing VEE structural protein genes (30). For the synthesis of RpS6-VRP, the wild-type replicon plasmid pVRpS6 was used with the human ribosomal protein S6 (obtained from the Japanese National Institute of Technology and Evaluation, clone AK093634/FLJ36315/THYMU2005240) cloned directly downstream of the 26S promoter. The FLAG tag sequence (DYKDDDDK) was cloned immediately at the C terminus of RpS6 in pVRpS6 for the synthesis of RpS6-FLAG-VRP. For in vitro translations, genes were expressed from pCI-neo-based (Promega) plasmids. To insert heterologous genes into the pCI-neo vector, the multicloning site of pCI-neo was used, and a Kozak translational enhancer and translation start codon were cloned upstream of the gene of interest, while a translation stop codon was cloned immediately downstream of the gene. For pCI-neo-nsP2, pCI-neo-nsP1, and pCI-neo-nsP3, the wild-type viral nonstructural protein gene was PCR amplified from pV5005. For pCI-neo-humRpS6 and pCI-neo-humRpS6-FLAG, the human RpS6 gene was PCR amplified from clone AK093634. For pCI-neo-SINnsP2, the Sindbis virus nsP2 gene was PCR amplified from Sindbis virus TR339. For pCI-neo-SFVnsP2, the Semliki Forest virus nsP2 gene was PCR amplified from SFV4. For pCI-neo-mosRpS6, the *Aedes albopictus* RpS6 gene was PCR amplified from pGEMTEasy-C710RP6 (courtesy of Ann M. Fallon, University of Minnesota).

**Protein analysis. (i) Examination of protein synthesis.** To monitor protein synthesis in VEE-, VRP-, or mock-infected cells, cell monolayers were washed with PBS at various time points postinfection and starved of methionine and cysteine by providing Eagle minimal essential medium lacking methionine and cysteine (Sigma). After 1 h of incubation at 37°C, the medium was replaced with starvation medium supplemented with 33  $\mu$ Ci of [<sup>35</sup>S]methionine-<sup>35</sup>S]cysteine (Pro-Mix; Amersham Pharmacia)/ml. Immediately after incubation for 1 h at 37°C, monolayers were harvested in NP-40 lysis buffer as described above. Equal volumes of lysate from each time point were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on an 8% gel. To measure <sup>35</sup>S incorporation, cells that had been previously transfected with siRNAs were pulsed for 1 h with [<sup>35</sup>S]methionine-<sup>35</sup>S]cysteine. Total cell lysate was precipitated with 10% trichloroacetic acid, bound to a glass filter, washed, and quantitated by using a scintillation counter.

**(ii) Immunoprecipitation experiments.** For each immunoprecipitation sample, lysate was prepared from a confluent well of a six-well tissue culture plate. Unless otherwise indicated, at 8 hpi cell monolayers were rinsed once with PBS and lysed using 200  $\mu$ l of NP-40 lysis buffer (170 mM NaCl, 50 mM Tris, 15 mM EDTA, and 0.2% NP-40 plus complete protease inhibitor cocktail tablets [Roche]) per well. After incubation for 5 min, cell lysates were scraped into microfuge tubes, and lysates were cleared by centrifugation for 2 min at 4°C at  $12,000 \times g$ . Samples were frozen at  $-80^\circ\text{C}$ . The cell lysate (175  $\mu$ l) was cleared by adding 40  $\mu$ l of protein A-Sepharose (Sigma), followed by agitation at 4°C for at least 2 h. The protein A-Sepharose was removed by centrifugation, and lysates were further cleared by mixing and agitation with 40  $\mu$ l of protein A-Sepharose and 1  $\mu$ l of isotype-matched normal antisera at 4°C. After at least 2 h of incubation, the protein A-Sepharose was removed by centrifugation, and the lysates were transferred to new tubes containing 2  $\mu$ l of specific antibody and agitated overnight at 4°C. The lysates were then transferred to new tubes containing 40  $\mu$ l of protein A-Sepharose and agitated for 2 h at 4°C. After removal of the lysate, the beads were washed three times in NP-40 lysis buffer and once in 50 mM Tris (pH 7.6). Protein gel loading buffer was added to the beads before

heating to 95°C for 5 min. The dissociated protein was loaded onto a 10% SDS-PAGE gel.

(iii) **Matrix-assisted laser desorption ionization–time of flight/mass spectrometry (MALDI-TOF/MS) sequencing.** Proteins were identified by a combination of peptide mass fingerprinting and the sequence tag approach using an ABI 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer, according to previously described methods (4, 41).

(iv) **Western blotting.** Protein preparations were separated by 10% SDS-PAGE and transferred to an Immun Blot polyvinylidene difluoride membrane (Bio-Rad) in transfer buffer (48 mM Tris, 39 mM glycine, 10% methanol) at 12 V for 60 min. The membranes were blocked for 1 h at room temperature in 5% dry milk (for anti-RpS6 antibodies) or 5% normal goat antiserum (anti-nsP2) in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween 20) and then probed overnight at 4°C with primary antibodies diluted in their respective blocking buffer (anti-RpS6 [Cell Signaling] and anti-nsP2 [a gift from AlphaVax, Inc.]). Phospho-specific antibodies were diluted in 5% bovine serum albumin in TBST. Subsequently, membranes were washed three times in TBST and then probed with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit-HRP [Ambion] and anti-goat-HRP [Novus]) for 1 h at room temperature. Blots were washed three times in TBST, followed by the detection of HRP-conjugated secondary antibody by chemiluminescence using ECL detection reagents (Amersham Pharmacia). Blots were exposed to film and developed. Antibodies used throughout the present study include VEE anti-nsP2 (a gift from AlphaVax, Inc.); anti-FLAG (Sigma); anti-RpS6, anti-pRpS6 (Ser235/236), and anti-pRpS6 (Ser240/244; Cell Signaling); anti-actin (Santa Cruz); and anti-tubulin (Sigma). VEE anti-nsP1 and VEE anti-nsP3 were generated by intramuscular DNA injection of pCI-neo-nsP1 or pCI-neo-nsP3 into mice. Mice were injected with a total of 100 µg equally divided between each hind leg every 4 weeks for a total of four injections.

(v) **Phosphorimager scans.** After separation of <sup>35</sup>S-labeled proteins on an SDS-PAGE gel, the gels were fixed for 30 min (50% water, 40% methanol, 10% acetic acid), dried, and exposed to a phosphor screen overnight. Signal was detected by scanning with a Storm 840 Phosphorimager (Amersham Pharmacia). Protein quantification was performed by using an ImageQuant (Molecular Probes).

**In vitro transcriptions and in vitro protein synthesis.** To prevent run-on transcription, all DNA plasmids were linearized prior to in vitro transcription by NotI digestion at a unique site located at the 3' end of the replicon genome or immediately downstream of the inserted gene of interest for pCI-neo-based constructs. Linearized DNA was gel purified (QIAGEN) and subsequently used as a template for T7 mMessage mMachine in vitro transcription according to manufacturer's directions (Ambion). In vitro rabbit reticulocyte lysate nuclease-free translations (Promega) were performed according to the manufacturer's recommendations in the presence of [<sup>35</sup>S]methionine-[<sup>35</sup>S]cysteine.

**Immunofluorescence microscopy.** BHK-21 cells grown on coverslips were infected with SFV-NP at an MOI of 0.5. At 8 hpi, cells were fixed with 3.2% paraformaldehyde for 20 min at room temperature and permeabilized with 1% NP-40 in PBS for 5 min, and coverslips were incubated in blocking buffer (5% donkey serum and 0.1% Brij58 in PBS) for 30 min. Samples were then processed for immunofluorescence by using rabbit polyclonal anti-S6-phospho-Ser240 (Cell Signaling) and the mouse monoclonal anti-NP antibody IC5-3A8 diluted 1:100 and 1:30, respectively, as primary antibodies. The secondary antibodies used were donkey anti-rabbit immunoglobulin (FITC) and anti-mouse immunoglobulin (Cy3). Images were obtained by using a Bio-Rad MR1024 confocal microscopy system where the fluorescent signals were mapped to green and red, respectively.

**Nuclear purification.** A total of  $5 \times 10^7$  cells were harvested per sample and resuspended in 350 µl of hypotonic buffer (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride). After incubation on ice for 15 min, NP-40 was added to a final concentration of 1%. Nuclei were pelleted by centrifugation at  $1,000 \times g$  for 10 min at 4°C. The supernatant was collected as the cytosolic fraction. The nuclear pellet was resuspended in a 25% solution of Optiprep (Sigma) and put over 30 and 35% layers of Optiprep. The gradient was then spun for 20 min at  $10,000 \times g$  at 4°C. Nuclei were collected, washed, pelleted, and lysed with nuclear extraction buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 25% glycerol) with the salt concentration adjusted to 400 mM with 5 M NaCl. The insoluble nuclear fraction was removed by centrifugation at  $12,000 \times g$  for 10 min.

**Polysome gradients.** Polysome gradients were performed as previously described (37). Briefly, mock- or VRP-infected cells were treated with 0.1 mg of cycloheximide (Sigma) per ml immediately before being harvested. Cytoplasmic extract was made and loaded onto a 10 to 40% continuous sucrose gradient. The

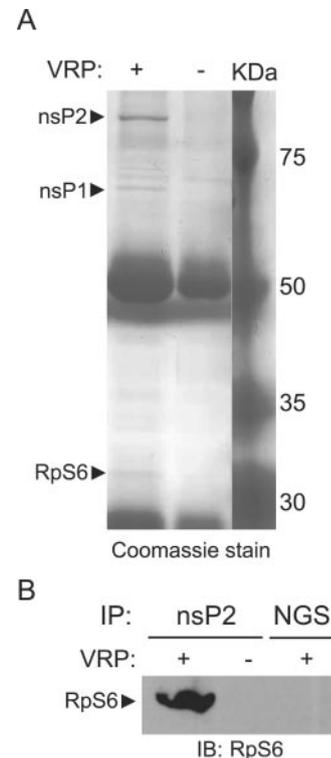


FIG. 1. RpS6 coimmunoprecipitates with nsP2 in vivo. (A) Coomassie blue-stained gel of VRP-infected cell lysates immunoprecipitated with anti-nsP2 antibody. GFP-VRP (5005-3000) was used to infect BHK-21 cells at an MOI of 10 IU/cell. At 24 hpi, cells were lysed in NP-40 lysis buffer. Approximately  $8 \times 10^5$  cell equivalents were immunoprecipitated anti-nsP2 antibody, separated on an 8% SDS-PAGE gel, and stained with Coomassie blue to visualize proteins that coimmunoprecipitate with nsP2. As labeled, nsP2, nsP1, and RpS6 were identified by MALDI-TOF/MS. (B) Immunoblot for human RpS6 on GFP-VRP- or mock-infected lysates that were immunoprecipitated by using anti-nsP2 antibody. Normal goat serum (NGS) serves as an isotype-matched control for immunoprecipitation. RpS6 is specifically detected in VRP-infected lysates immunoprecipitated for nsP2.

gradients were centrifuged for 105 min at  $17,000 \times g$  in a Beckman SW41 rotor at 4°C. Fractions were collected from the top of the gradient by using a FRAC-100 chromatography system.

**siRNA experiments.** One day prior to transfection, HeLa cells were seeded at  $7 \times 10^4$  cells in a 12-well plate in antibiotic-free Dulbecco modified Eagle medium supplemented with 10% FBS and 1% L-glutamine. Cultures were transfected with 10 pmol of either an RpS6 or nontargeting small interfering RNA (siRNA) pool (Dharmacon) in 300 µl of OptiMEMI and Lipofectamine 2000 (Invitrogen) mixture according to the manufacturer's recommendations. After 2 h of incubation at 37°C, 1 ml of medium was added. After 48 h, the medium was removed, and transfected cells were infected with GFP-VRP. At 18 hpi postinfection cells were treated with trypsin and washed with cold fluorescence-activated cell sorting (FACS) buffer (PBS, 0.5% human serum albumin). Cells were fixed with PBS-1% formaldehyde before FACS analysis. FACS data were obtained by using a FACScan flow cytometer (BD Biosciences) and analyzed by using FlowJo software (TreeStar).

## RESULTS

**Ribosomal protein S6 is associated with VEE nsP2 in infected cells.** Since nsP2 likely plays an important role in determining the outcome of infection in the cell, we sought to identify cellular factors that interacted with nsP2. We used

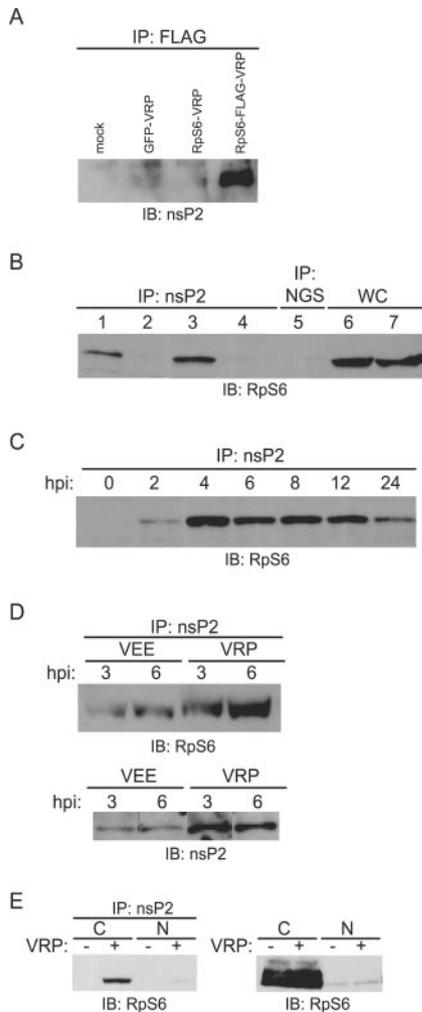


FIG. 2. Characterization of the RpS6-nsP2 interaction in vivo. Unless otherwise stated, cells were infected at an MOI of 10 IU/cell with GFP-VRP (5005-3000) or mock infected and lysed in NP-40 lysis buffer at 8 hpi. Lysates were then immunoprecipitated using anti-nsP2 antibody and subsequently subjected to immunoblot analysis using anti-RpS6 antibody. (A) Demonstration of the interaction in the reciprocal manner. HEK-293 cells were mock infected or infected with either GFP-VRP, RpS6-VRP, or RpS6-FLAG-VRP. At 8 hpi, the cells were lysed in NP-40 lysis buffer, and lysates were immunoprecipitated with anti-FLAG antibody and subsequently examined by immunoblot for nsP2. nsP2 coimmunoprecipitates specifically in VRP-infected lysates immunoprecipitated for the FLAG tag present on the RpS6 fusion protein. (B) The interaction between RpS6 and nsP2 occurs in multiple cell types. Lanes 1 and 2 contain samples using HEK-293 (human) cell lines and lanes 3 to 7 are from BHK-21 (hamster) cell lines. Samples in lanes 1, 3, 5, and 6 are infected with VRP; lanes 2, 4, and 7 contain mock-infected samples. As shown, the samples in lanes 1 to 4 were immunoprecipitated with anti-nsP2 antibody, the sample in lane 5 was immunoprecipitated with isotype-matched normal goat sera (NGS), and lanes 6 and 7 contain a direct load of whole-cell lysate (WC). (C) RpS6 coimmunoprecipitates with nsP2 throughout the course of infection. HEK-293 cells were infected and samples were harvested over a time course at the time points indicated. The nsP2-RpS6 interaction is continuously detected. (D) In the upper panel, RpS6 coimmunoprecipitates with nsP2 in VEE-infected cells. HEK-293 cells were infected at an MOI of 10 PFU/cell with VEE virus (V3000) or at 10 IU/cell with GFP-VRP (5005-3000). The presence of the viral structural proteins in VEE virus does not affect the ability of RpS6 to specifically coimmunoprecipitate with nsP2. In the lower panel, total cell lysates from VEE- or VRP-infected cells at 3 and

Venezuelan equine encephalitis replicon particles (VRP), which are nonpropagating viral vectors that express all four of the viral nonstructural proteins, replicate their genomic RNAs, and transcribe their subgenomic mRNA but lack genes encoding the viral structural proteins. Mammalian BHK-21 cells were infected with VRP expressing green fluorescent protein (GFP-VRP; also referred to as 5005-3000) at an MOI of 10, and at 24 hpi a cytoplasmic extract was prepared. Cytoplasmic proteins were immunoprecipitated using antibody against VEE nsP2, and the immunoprecipitate was separated on an SDS-PAGE gel. Coomassie blue-stained proteins were identified by MALDI-TOF/MS sequencing. From this analysis, ribosomal protein S6 (RpS6) was identified as a protein that specifically coimmunoprecipitated with nsP2 in VRP-infected lysates (Fig. 1A). We then confirmed the association of RpS6 with nsP2 by analyzing nsP2 immunoprecipitates by immunoblot with antibodies to RpS6 (Fig. 1B).

To detect the nsP2-RpS6 interaction in the reciprocal manner, that is, to determine whether nsP2 coimmunoprecipitated with anti-RpS6, a VRP was engineered that expressed human RpS6 with a C-terminal FLAG epitope tag (RpS6-FLAG-VRP), such that every VRP-infected cell would express a FLAG-tagged version of RpS6. Since VRP infection induces a global inhibition of protein synthesis (15, 29, 65; Montgomery and Johnston, unpublished), encoding the epitope-tagged version of human RpS6 on a viral message allows its continued synthesis after infection. RpS6-FLAG-VRP infection resulted in an ~5-fold excess of FLAG-RpS6 over endogenous RpS6 (data not shown). Cytoplasmic proteins from cells infected with RpS6-FLAG-VRP were immunoprecipitated using  $\alpha$ -FLAG antibody (Sigma) and subsequently subjected to Western blot analysis for nsP2. Indeed, nsP2 coimmunoprecipitated with the FLAG-tagged RpS6, confirming that we could detect the interaction in a reciprocal experiment (Fig. 2A).

**Characterization of the association of RpS6 with nsP2.** To further understand the novel association of RpS6 with nsP2, we examined the in vivo characteristics of the complex. Immunoprecipitation for nsP2, followed by immunoblotting for RpS6, revealed that the two proteins were associated not only in VRP-infected BHK-21 hamster cells but also in VRP-infected the human cell lines HEK-293 (Fig. 2B) and HeLa (not shown), demonstrating that the interaction occurred across species. The association of RpS6 with nsP2 was detected within several hours of infection, and it was stably present throughout the course of infection (Fig. 2C). It is noted that the appearance of the coimmunoprecipitating RpS6 occurs at a time after

6 hpi were immunoblotted for nsP2. Note that more nsP2 has accumulated at each time point in the VRP-infected lysates, which corresponds to more RpS6 coimmunoprecipitating with nsP2 from VRP-infected lysates. (E) For the left panel, nuclear and cytoplasmic fractions were prepared from GFP-VRP- or mock-infected cells before immunoprecipitation for nsP2. The RpS6 that coimmunoprecipitates with nsP2 is predominantly cytoplasmic. For the right panel, equivalent amounts of crude cytoplasmic and nuclear lysates are subjected to immunoblot analysis using anti-RpS6 antibody, revealing that in both VRP-infected and mock-infected lysates there is much less RpS6 present in the nucleus compared to that in the cytoplasm.

infection similar to when nsP2 synthesis and accumulation is first visualized (data not shown). Furthermore, RpS6 coimmunoprecipitated with nsP2 in cells infected with VEE virus (V3000; Fig. 2D), demonstrating that the association was not an artifact of using VRP in the original experiment and that the presence of the viral structural proteins does not affect the appearance of the complex. Taken together, these data demonstrate the generality of the interaction. However, although both VEE nsP2 and RpS6 localize to both the cytoplasm and the nucleus (13, 54; Montgomery and Johnston, unpublished), the complex containing nsP2 and RpS6 that we have observed is an interaction that predominantly occurs in the cytoplasm (Fig. 2E).

**RpS6 and nsP2 associate in vitro.** The nsP2 and RpS6 association was assessed in vitro in the absence of other viral proteins and many cellular proteins. nsP2 and human RpS6 (Japanese National Institute of Technology and Evaluation) were cloned individually into the pCI-neo mammalian expression vector, and then the mRNA was transcribed and cotranslated in the presence of [<sup>35</sup>S]methionine-[<sup>35</sup>S]cysteine in a nuclease-treated rabbit reticulocyte lysate translation reaction. RpS6 and nsP2 coimmunoprecipitated with anti-nsP2 antibody after in vitro synthesis, demonstrating that the presence of the other viral nonstructural proteins was not required for the interaction (Fig. 3A). Cotranslation of nsP2 and RpS6 with a C-terminal FLAG tag expressed from the pCI-neo mammalian expression vector and immunoprecipitation with antibody against the FLAG epitope also demonstrated the interaction in vitro (Fig. 3B).

**nsP2 proteins from multiple alphaviruses associate with RpS6.** The nsP2 protein from other alphaviruses also associates with RpS6. The nsP2 from Sindbis virus TR339 (SINnsP2) or Semliki Forest virus SFV4 (SFVnsP2) was cotranslated with RpS6-FLAG using the pCI-neo-based plasmid system. The in vitro translations were immunoprecipitated using antibody against the FLAG epitope. Similar to VEE nsP2, both SINnsP2 and SFVnsP2 coimmunoprecipitated with FLAG-RpS6, suggesting that the association of RpS6 and nsP2 is common throughout the *Alphavirus* genus (Fig. 3B).

**VEE nsP2 associates with RpS6 from mosquitoes.** Although eukaryotic RpS6 is well conserved, in *Aedes* and *Anopheles* mosquitoes there is a unique C-terminal extension on RpS6 (19, 20). This ~100-amino-acid tail is located immediately downstream of the cluster of serine residues on RpS6 that are the sites of phosphorylation. Although the function of this extension is unknown, it has sequence similarity to histone H1 (67). Outside of these mosquito species, no such extension has been reported for RpS6.

Both *Aedes* (12) and *Anopheles* (59) mosquitoes are competent vectors for VEE and thus may be potential vectors to spread the pathogen. Since RpS6 may play a modified role in mosquito ribosomes, we wanted to examine whether mosquito RpS6 associated with nsP2. The full-length RpS6 from *Aedes albopictus* was cotranslated with VEE nsP2 in vitro. Immunoprecipitation using antibody against VEE nsP2 revealed that *Aedes* RpS6 associates with nsP2 (Fig. 3C). Thus, the interaction of alphavirus nsP2 with RpS6 likely occurs in mosquito cells despite the altered RpS6.

**Other possible members of the nsP2-RpS6 complex.** Since RpS6 is a constituent of the 40S ribosomal subunit, we wanted

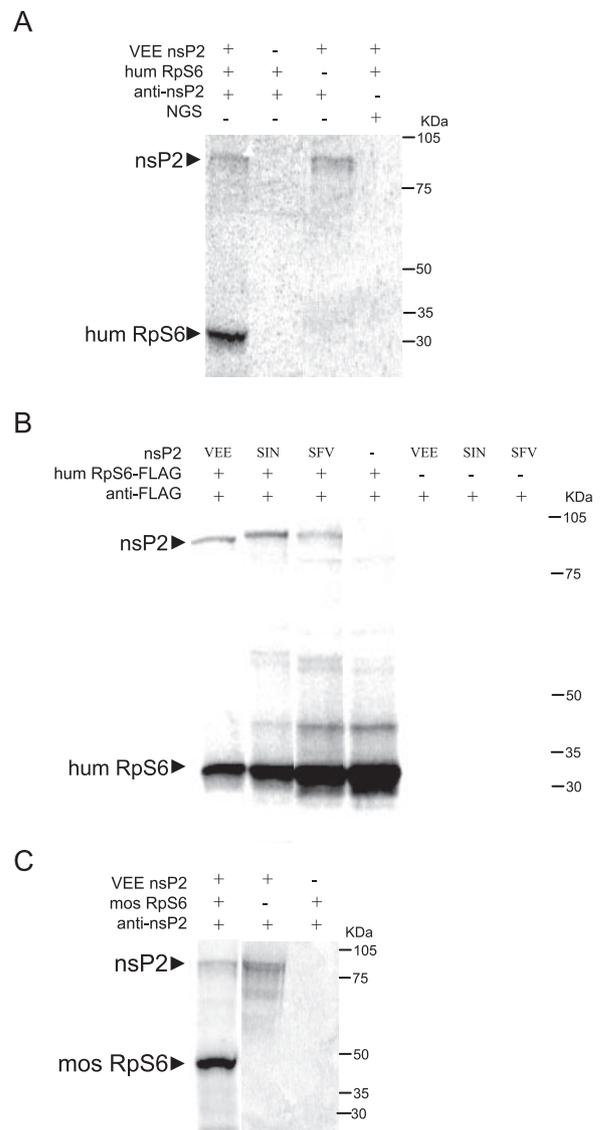
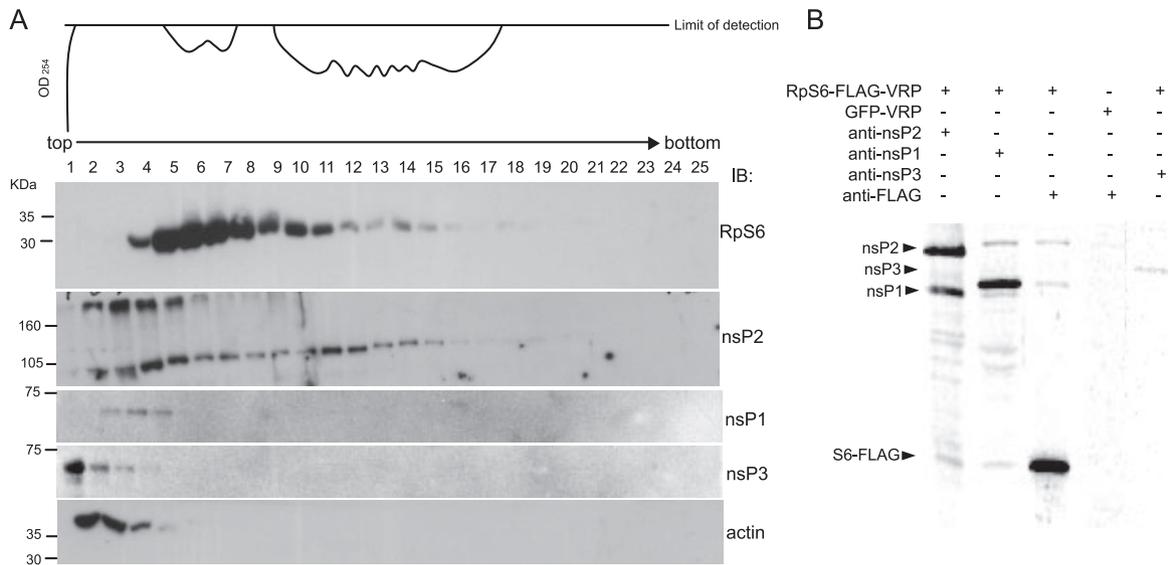


FIG. 3. In vitro interactions of nsP2 and RpS6. (A) nsP2 and human RpS6 interact when the two proteins are synthesized together in vitro. nsP2 and RpS6 were translated alone or together from individual pCI-neo-based plasmids in the presence of <sup>35</sup>S label and subsequently immunoprecipitated using anti-nsP2 antibody or isotype-matched antibody (NGS). The specific coimmunoprecipitation RPS6 with nsP2 demonstrates that the two proteins interact in the absence of other viral proteins. (B) Similar to VEE, nsP2 from SIN and SFV interact with human RpS6. nsP2 from either VEE, SIN, or SFV was cotranslated with RpS6-FLAG. Immunoprecipitation using anti-FLAG antibody reveals that all of the alphavirus nsP2s coimmunoprecipitate with RpS6-FLAG. (C) Mosquito RpS6 associates with nsP2. RpS6 from either human (hum RpS6) or *Aedes albopictus* (mos RpS6) was cotranslated with VEE nsP2. Subsequent immunoprecipitation using anti-VEE nsP2 antibody showed that mosquito RpS6 coimmunoprecipitates with nsP2.

to determine whether nsP2 associated with RpS6 in the context of the whole ribosome. Therefore, lysates from GFP-VRP-infected HEK-293 cells were centrifuged over a polysome sucrose gradient in order to examine whether nsP2 cosedimented with RpS6 in ribosome-containing fractions. When polysome gradient fractions were analyzed by immunoblotting, the dis-



**FIG. 4.** Other proteins associated with the complex containing nsP2 and RpS6. (A) RpS6 and nsP2 are present in the same fractions of a polysome gradient. HEK-293 cells were infected with GFP-VRP at an MOI of 5. At 8 hpi, cells were lysed and lysates were run over a 10 to 40% continuous sucrose gradient. An aliquot of each fraction of the polysome gradient was examined by immunoblotting using anti-RpS6, anti-nsP2, anti-nsP1, anti-nsP3, or anti-actin antibody. The polysome profile of this gradient, as monitored by measuring the optical density at 254 nm, is shown. The presence of RpS6 marks fractions that contain ribosomes, whereas the presence of actin demonstrates fractions containing most other cellular proteins. Note that nsP2, but not nsP1 and nsP3, comigrates with RpS6. The upper band in the nsP2 blot is the P123 precursor. (B) nsP1 coimmunoprecipitates with RpS6 and nsP2. HEK-293 cells were infected with RpS6-FLAG-VRP or GFP-VRP (as indicated) at an MOI of 10, and protein synthesis was monitored by using  $^{35}\text{S}$  label. At 8 hpi, cell lysates were made and subsequently used for immunoprecipitation. Immunoprecipitation with anti-nsP2 antibody reveals that nsP1 and RpS6-FLAG coimmunoprecipitate with nsP2, immunoprecipitation with anti-nsP1 antibody reveals that nsP2 and RpS6-FLAG coimmunoprecipitate with nsP1, and immunoprecipitation with anti-FLAG antibody reveals that nsP1 and nsP2 coimmunoprecipitate with RpS6-FLAG. Cells infected with GFP-VRP and immunoprecipitated with anti-FLAG antibody show that nsP1 and nsP2 coimmunoprecipitation is specific. There was no coimmunoprecipitation of the other radiolabeled proteins when lysates were immunoprecipitated with anti-nsP3 antibody.

tribution of nsP2 was essentially identical to the distribution of RpS6 (Fig. 4A), suggesting that nsP2 might associate with ribosomes. Some nsP2 remains at the top of the gradient with other cellular proteins, such as actin, which is expected for nsP2 that is present in viral replication complexes rather than associated with ribosomes. Similarly, the nonstructural polyprotein precursor remains at the top of the gradient. When the distribution of two other nonstructural proteins, nsP1 and nsP3, was examined, neither were present throughout the gradient. This suggests that the association of nsP2 with RpS6 being detected is not occurring with nsP2 that is present in the viral replication complex.

We also examined whether the other viral nonstructural proteins were associated with the complex containing RpS6 and nsP2. Alphavirus infection induces a global shutoff of host translation, but proteins encoded on viral messages are synthesized at high levels, allowing specific radiolabeling of viral protein. Thus, HEK-293 cells were infected with RpS6-FLAG-VRP and radiolabeled with [ $^{35}\text{S}$ ]methionine-[ $^{35}\text{S}$ ]cysteine after infection. Immunoprecipitation of such radiolabeled lysates allowed the visualization of other members of this complex. Lysates immunoprecipitated with antibody against the FLAG epitope revealed that both nsP2 and nsP1 coimmunoprecipitated with RpS6 (Fig. 4B). Confirming this observation, immunoprecipitation with antibody to nsP1 yielded bands corresponding to nsP2 and RpS6-FLAG in addition to nsP1, and immunoprecipitation with antibody to nsP2 yielded a band for

nsP2, as well as bands corresponding to nsP1 and RpS6-FLAG. RpS6-FLAG did not coimmunoprecipitate when antibody to nsP3 was used.

Taken together, these data pose several possibilities. It is possible that nsP2 directly associates with RpS6 and that nsP1 is able to indirectly associate with RpS6 through an interaction with nsP2. Conceivably, this indirect association of nsP1 with RpS6 is not maintained in the context of a whole ribosome or when subjected to polysome purification. Alternatively, it is possible that nsP2 and RpS6 interact both as a soluble complex and within the context of the ribosome and that nsP1 is present only in the soluble complex.

**RpS6 phosphorylation is diminished after alphavirus infection.** RpS6 is the major phosphoprotein of the 40S ribosomal subunit, and its phosphorylation tightly correlates with an actively translating ribosome. To examine RpS6 in the context of alphavirus infection, we looked at the phosphorylation state of RpS6 in cells infected with alphavirus replicons. Although the total amount of RpS6 in a VRP-infected cell remains unaltered, within hours of infection with GFP-VRP the population of phosphorylated RpS6 becomes greatly diminished (Fig. 5A). SFV infection similarly causes a dramatic decrease in the amount of phosphorylated RpS6 present in the cell (Fig. 5B and C). When cells were infected with SFV-NP, a replicon expressing influenza virus NP, a protein that localizes to the nucleus, the amount of total RpS6 was unaltered, but phosphorylated RpS6 disappeared within hours of infection (Fig.

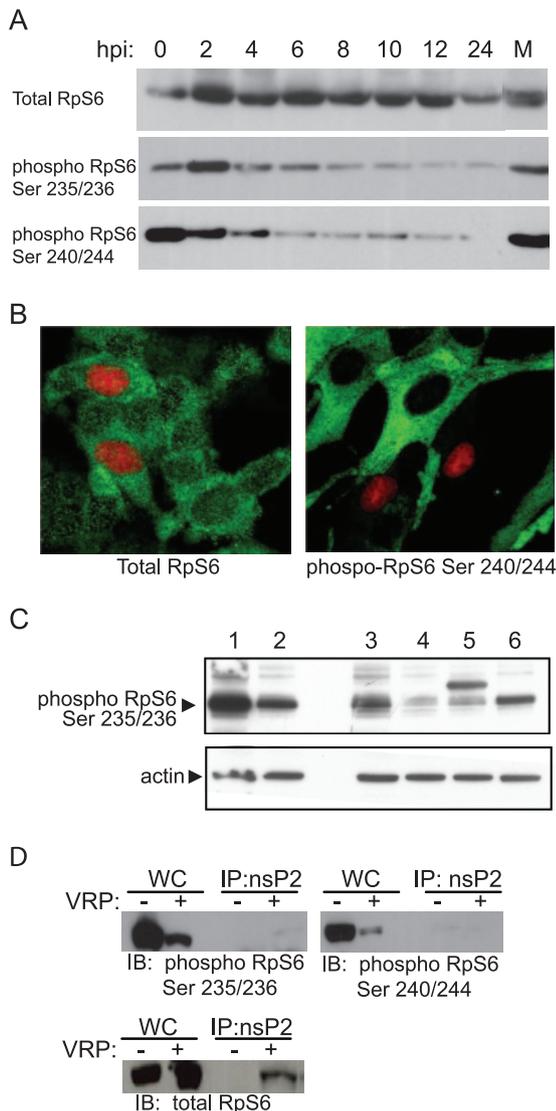


FIG. 5. VRP infection and RpS6 phosphorylation. (A) RpS6 phosphorylation is diminished within hours of VRP infection. Western blots for total RpS6 (upper panel) and phosphorylated RpS6 at Ser235/236 (middle panel) or Ser 240/244 (lower panel) are shown. Lysates are from GFP-VRP-infected (MOI of 10) HEK-293 cells at the time points indicated or from mock-infected (M) cells. (B) Similar to VRP infection, SFV4 reduces the amount of phosphorylated RpS6. Cells infected with SFV-NP, an SFV replicon that expresses influenza virus NP, at 8 hpi. NP, a nuclear protein, is visualized by Cy3 staining (red). In the left panel, total RpS6 is FITC stained (green); in the right panel, phospho-RpS6 (Ser240/Ser 244) is FITC stained. (C) Lysates of SFV-NP-infected cells at 14 hpi were immunoblotted for phospho-RpS6 (Ser240/Ser 244), demonstrating diminished RpS6 phosphorylation after SFV infection. Lane 1, cell lysates with phosphorylated RpS6 after 6 h of incubation in medium containing 20% fetal calf serum; lane 2, cell lysates with diminished amount of phosphorylated RpS6 after incubation in medium containing 0% fetal calf serum; lane 3, mock infection; lane 4, lysates from SFV-NP-infected cells; lane 5, cells infected with SFV-capsid enhancer-NP (note the nonspecific capsid band above RpS6 band); lane 6, cells infected with influenza virus. The lower gel shows actin. SFV replicon infection reduces phosphorylated RpS6 (lanes 4 and 5), whereas influenza virus infection does not diminish phosphorylated RpS6 as greatly (lane 6). (D) The population of RpS6 that associates with nsP2 is predominantly unphosphorylated. An immunoblot for total RpS6 and phosphorylated RpS6 on HEK-293 cell lysates immunoprecipitated with anti-nsP2 antibody is shown. Cells

5B). Likewise, RpS6 phosphorylation was diminished after infection with an SFV replicon expressing NP as well as when NP was overexpressed through its fusion to the SFV capsid translation enhancer (56). However, infection with influenza virus does not alter RpS6 phosphorylation, demonstrating that these alterations in RpS6 were due to the alphavirus replicon rather than presence of influenza virus NP. Note that RpS6 localizes to both the cytoplasm and the nucleus, whereas phosphorylated RpS6 is cytoplasmic.

Since RpS6 phosphorylation is diminished after infection, we examined the phosphorylation status of the population of RpS6 that associates with nsP2. Lysates from GFP-VRP-infected cells were immunoprecipitated with nsP2 antibody and then analyzed by Western blotting with the phospho-specific RpS6 antibodies. The population of RpS6 that coimmunoprecipitates with nsP2 is predominantly not phosphorylated at Ser235/236 or Ser240/244 (Fig. 5D), which is consistent with the majority of RpS6 after alphavirus infection being unphosphorylated. Thus, alphavirus infection causes cellular RpS6 to become unphosphorylated, and the RpS6 associated with nsP2 is not phosphorylated.

**RpS6 is required for efficient expression from alphavirus messages.** To examine the consequence of the interaction between RpS6 and nsP2, we used siRNA directed against RpS6 to knock down levels of RpS6 prior to alphavirus infection. HeLa cells were either transfected with a pool of siRNA against RpS6 messages, a nontargeting siRNA pool, or were mock transfected. In order to allow cellular protein levels to diminish, cells were incubated for 48 h with siRNA before being infected. Cells were infected at an MOI of 5 with GFP-VRP, which expresses GFP from the alphavirus subgenomic promoter. At the time of infection, RpS6 protein levels were reduced by 30 to 50%, but the amount of tubulin ( $t_{1/2} = 18$  h) remained stable, suggesting that a diminished population of RpS6 did not have a severe effect on host translation at the time of infection (Fig. 6A). To analyze expression of the virally encoded GFP, cells were harvested at 18 hpi and analyzed by FACS. Although a similar percentage of cells were infected in each transfection group, cells treated with siRNA against RpS6 displayed a median GFP intensity diminished by >10-fold (Fig. 6B and C). To more closely examine the status of host protein synthesis under conditions of diminished RpS6, [<sup>35</sup>S]methionine-[<sup>35</sup>S]cysteine incorporation was measured 48 h after the cells were transfected with siRNA. There was only a minimal decrease in overall host translation under conditions of re-

were infected at an MOI of 10 IU/cell with GFP-VRP (5005-3000) or were mock infected. At 8 hpi the cells were lysed in NP-40 lysis buffer, and a tripled amount of VRP- or mock-infected lysate was immunoprecipitated with anti-nsP2 antibody. The VRP- and mock-infected immunoprecipitates were then equally divided among three gels and analyzed by immunoblotting with one of two phospho-specific RpS6 antibodies, anti-RpS6 Ser235/236 or anti-RpS6 Ser 240/244, as well as an antibody that recognizes the total amount of RpS6. The RpS6 that coimmunoprecipitates with nsP2 is predominantly unphosphorylated at Ser235/Ser 236 or Ser 240/Ser 244. Note that the amount of phosphorylated RpS6 in VRP-infected cells is diminished compared to the amount of phosphorylated RpS6 in mock-infected cells (compare the whole-cell lysates labeled WC).

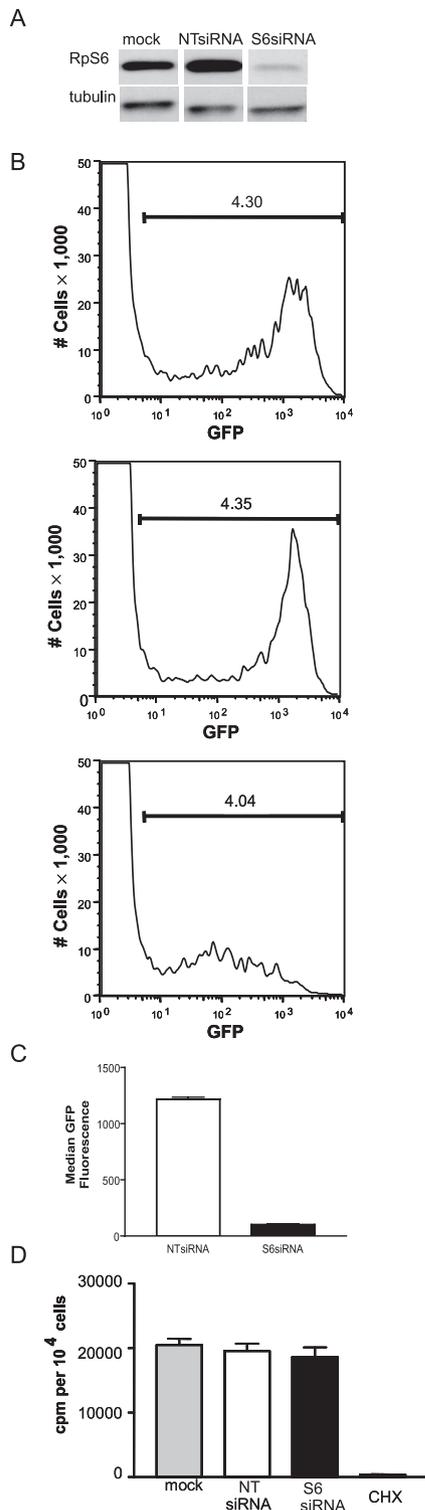


FIG. 6. Diminished RpS6 protein level reduces expression of virally encoded protein. (A) HeLa cells were mock transfected or transfected with either a nontargeting siRNA pool (NTsiRNA) or an RpS6 siRNA pool (S6siRNA). At 48 h posttransfection, cell lysates were collected and immunoblotted for RpS6 or tubulin. (B) At 48 h after HeLa cells were transfected with siRNA, they were infected at an MOI of 5 with GFP-VRP. At 18 hpi the cells were harvested and subjected to FACS analysis. The numbers on the profiles (4.30, 4.35, and 4.04) indicate the percentage of cells infected as determined by GFP fluorescence. Although similar percentages of cells were infected in the

duced RpS6 protein levels (Fig. 6D). Taken together, this demonstrates that proteins expressed from alphavirus messages require RpS6 for optimal synthesis of viral proteins.

## DISCUSSION

The pathogenesis of alphaviruses is likely determined by many specific virus-host interactions, but the identity of such complexes remains largely unknown. We pursued an unbiased approach designed to identify host proteins that interacted with nsP2 and found that such an interaction occurs with RpS6, the major phosphoprotein of the ribosome. Few viral proteins have been described as associating with subunits of the ribosome and, to our knowledge, this is the first demonstration of an alphavirus nonstructural protein interacting with a specific component of the ribosome.

The results presented here strongly suggest that the specific interaction between VEE nsP2 and RpS6 (i) occurs in mammalian and mosquito cells, (ii) is apparent within 2 h after infection, (iii) remains detectable throughout the course of infection, (iv) occurs in VRP- as well as VEE-infected cells, and (v) involves the unphosphorylated form of RpS6 associated with ribosomes. The nsP2 of Sindbis virus, the prototype alphavirus, as well as Semliki Forest virus, also interact with RpS6. Thus, this interaction is likely not a transient occurrence or limited to VEE, but rather its prevalence throughout infection suggests that it plays a fundamental role in mediating an effective alphavirus infection.

We also have shown that nsP2 cofractionates with RpS6 in a polysome gradient, which suggests that nsP2 associates with the whole ribosome. In our initial identification of proteins that coimmunoprecipitated with nsP2, multiple proteins were present, but it was only possible to positively identify some of these. Thus, it is possible that other ribosomal proteins were present but remained unidentified. It has been reported that the nsP2 of Semliki Forest virus is associated with cellular fractions containing ribosomes (49). A complete picture of how alphaviruses modify ribosomes and ribosomal constituents remains unclear. However, nsP2 binding the ribosome could contribute to selective translation of viral messages or even specific cellular messages in alphavirus-infected cells.

nsP1 also was associated with RpS6, but we did not observe any other nonstructural proteins associating with RpS6. nsP1 is an essential component of the viral replication complex. The

mock-transfected (upper panel), nontargeting siRNA-transfected (middle panel), and RpS6 siRNA-transfected (lower panel) groups, RpS6 knockdown led to diminished fluorescence. The FACS profiles shown are representative of 10 samples. (C) Median GFP intensity of cells infected with GFP-VRP after transfection with NTsiRNA (□) or S6siRNA (■). The intensity of virally encoded GFP is decreased 10-fold with the diminished RpS6 level. The data are representative of triplicate experiments. (D) At 48 h after transfection with siRNA, cellular translation was measured by using a 1-h pulse with [<sup>35</sup>S]methionine-[<sup>35</sup>S]cysteine. Extracts were analyzed by trichloroacetic acid precipitation and quantitation by a scintillation counter. The results are averages of quadruplicate samples and representative of three experiments. Host protein synthesis is minimally reduced after transfection with RpS6 siRNA, whereas cycloheximide treatment (CHX) dramatically reduces <sup>35</sup>S-label incorporation.

nsP1 protein has both guanine-7-methyltransferase and guanylyltransferase activity, which is required to cap the viral RNAs (1, 33, 52). nsP1 is required for the initiation of negative-sense RNA synthesis (18, 62) and is also responsible for the interaction of the alphavirus replicase with membranes (25). It is not known whether nsP1 interacts with RpS6 directly or indirectly through an association with nsP2. The absence of nsP1 throughout the polysome gradient could be the result of nsP1 interacting with nsP2 that is bound to RpS6, but the indirect nsP1 association with RpS6 is not maintained in the context of an assembled ribosome. Alternatively, there could be two distinct complexes being detected: one which contains nsP2, RpS6, and nsP1, and a second in which nsP2 binds the ribosome but not through an association with RpS6. Clearly, further study of complexes containing the various nsPs and cellular proteins is needed.

RpS6 is a component of the 40S subunit and is the major phosphoprotein of the ribosome, containing five serine residues that are phosphorylated. Ribosomes with the highest degree of RpS6 phosphorylation have an advantage in mobilizing into polysomes, although RpS6 phosphorylation alone may not be sufficient for polysome formation (21, 32, 53). Phosphorylation of RpS6 tightly correlates with an increase in translation in the cell, particularly of TOP messages (which contain a 5'-terminal oligopyrimidine tract). TOP mRNAs encode proteins involved in cell growth, cell cycle progression, and the translational machinery, including all of the ribosomal proteins and elongation factors. Interestingly, alphavirus infection leads to a loss in the appearance of newly synthesized ribosomes in the cytoplasm of infected cells (65). We have shown that after alphavirus infection there is a greatly diminished population of phosphorylated RpS6; since all of the ribosomal machinery is encoded on TOP messages (31), it is possible that the lack of newly synthesized ribosomes after alphavirus infection is a consequence of diminished translation of TOP mRNAs after infection.

However, recent work has brought the role of RpS6 phosphorylation in TOP message translation into question (44, 50, 57, 60), since blocking RpS6 phosphorylation does not lead to reduced translation of TOP messages. To date, the role of phosphorylated RpS6 remains obscure, and an understanding of RpS6 function is far from complete. RpS6 may have roles distinct from those as a part of the ribosome, as *Drosophila* RpS6 mutants develop melanotic tumors in lymph glands (26), and RpS6 is found in a free, phosphorylatable form in the nucleus of mammalian cells (13).

Nonetheless, diminished RpS6 phosphorylation tightly correlates with decreased levels of cellular translation. Interestingly, polioviruses and herpesviruses actually preserve RpS6 phosphorylation and translation of TOP messages despite using other strategies to induce a generalized host shutoff (5, 17). Conversely, although alphavirus infection rapidly induces a global translation inhibition, there is diminished RpS6 phosphorylation within hours of infection. Accordingly, the population of RpS6 that associates with nsP2 is predominantly not phosphorylated. It is possible that the association of nsP2 with RpS6 prevents RpS6 phosphorylation or causes its dephosphorylation. Perhaps nsP2 is only able to associate with the dephosphorylated form of RpS6, or dephosphorylated RpS6 may promote the translation of alphavirus messages. The role

of dephosphorylated RpS6 in alphavirus infection clearly warrants further study.

In mosquito cells, alphaviruses establish a persistent infection and are able to translate viral messages without dramatically altering the translation of cellular messages. The RpS6 present in mosquitoes contains a unique C-terminal tail (19, 20), suggesting that RpS6 plays an altered role in the mosquito ribosome. Although alphaviruses have a dramatically different effect on host translation in mosquito cells and mosquito RpS6 is divergent, nsP2 is able to associate with mosquito RpS6. Thus, it is possible that the interaction of RpS6 with nsP2 might cause viral messages to be favored by the ribosome for translation in an infected cell. Alternatively, if the interaction of nsP2 with RpS6 leads to inhibition of translation of cellular messages in mammalian cells, it is possible that the extension on mosquito RpS6 modifies the nsP2-RpS6 association such that the effect of nsP2 binding is altered.

When RpS6 levels were diminished, we found that a similar number of cells were infected by GFP-VRP, which is in direct contrast to what others have observed for internal ribosome entry site-containing viruses (6). Although a similar number of cells were positive for the virally expressed GFP, the median fluorescence intensity of GFP in cells transfected with siRNA targeting RpS6 was reduced >10-fold in comparison to non-targeting siRNA transfected controls. However, cellular translation was only mildly affected by diminished levels of RpS6. Although the levels of positive-sense and negative-sense viral messages have not been analyzed in cells with diminished RpS6, our data suggest that optimal expression of alphavirus genes is dependent upon RpS6. It is curious that diminution of RpS6 potentially reduces translation from viral messages but not cellular messages. Of course, in the natural context of an alphavirus-infected cell, it is the viral messages that predominantly occupy ribosomes and are efficiently translated. Thus, in the context of alphavirus infection, RpS6 may serve as a factor to promote the selective expression of viral message, and this may function in concert with other mechanisms that alphaviruses use to inhibit the translation of host proteins to allow the virus to efficiently utilize elements of the host cell.

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