Venezuelan Equine Encephalitis Virus Nonstructural Protein 2 in the Host Cell

by

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ABSTRACT

STEPHANIE A. MONTGOMERY: Venezuelan Equine Encephalitis Virus Nonstructural Protein 2 in the Host Cell
(Under the direction of Robert E. Johnston)

Venezuelan equine encephalitis virus (VEE) is a naturally emerging disease threat and a previously developed biological weapon, making it an important pathogen of the Alphavirus family. Unfortunately, little is known about how the viral proteins interact with the host cell at the molecular level. The viral nonstructural protein 2 (nsP2), a required member of the alphavirus replication complex, has undefined activities that are critical in determining the outcome of alphavirus infection. To gain insight into auxiliary functions of nsP2, cellular proteins in the mammalian host cell with which nsP2 interacted were examined. In VEE and other alphaviruses, nsP2 is associated with ribosomal protein S6 (RpS6), an essential component of the 40S ribosomal subunit. The interaction of RpS6 with nsP2 occurred throughout the course of infection and did not require the presence of the other viral proteins. Moreover, reducing the cellular level of RpS6 protein caused diminished expression from alphavirus subgenomic messages, whereas it did not dramatically alter cellular translation. The subcellular localization of VEE nsP2 was examined in order to gain insight into its activity in the mammalian host cell. VEE nsP2 was found both in the cytoplasm and the nucleus of mammalian cells during infection and also when expressed in the absence of other viral proteins. The C-terminal third of VEE nsP2 can localize EGFP exclusively to the nucleus of mammalian cells, and contains the sequence PGK MV, which is
homologous to the nuclear localization signal of Semliki Forest virus nsP2. Mutation in this sequence diminished, but did not eliminate, localization to the nucleus, suggesting that this sequence contributes to the nuclear localization of VEE nsP2. Furthermore, VEE nsP2 specifically interacted with the nuclear import protein karyopherin-α 1, suggesting that during infection nsP2 is transported to the nucleus by karyopherin-α 1. Additionally, a novel leucine-rich nuclear export signal was identified in VEE nsP2, and nuclear export mediated by this signal occurred via the CRM1 pathway. Taken together, these results establish that nsP2 interacts with essential cellular components and is actively directed to multiple subcellular compartments, suggesting that nsP2 mediates a critical interplay of the virus with the host cell.
To Nate

I admire your brilliance and have enjoyed sharing this journey with you as my colleague. Your enthusiasm for science and pursuit of adventure in life are inspirational. Without hesitation, you have embraced my passions and found joy in watching me be consumed by my work. You are my biggest fan, and I will forever be indebted to you for your endless support.
ACKNOWLEDGMENTS

There are several individuals without whom I would not have been able to conduct my dissertation research. All of this work was carried out in the laboratory of Dr. Robert E. Johnston. I am indebted to Dr. Johnston for having faith in a first year graduate student and the project that she proposed, though it did not follow along any of the established lines of investigations in his lab. His insight and support for the project have been immensely helpful. I would also like to acknowledge all of the members of my thesis committee – Dr. Steven L. Bachenheimer, Dr. Dennis T. Brown, Dr. Blossom Damania, Dr. Aravinda de Silva – for their willingness to offer me their time, assistance, and knowledge. I appreciate the contributions of all of the past and present members of the Carolina Vaccine Institute, especially the Johnston and Heise labs. I greatly benefited from the procedural expertise and excellent service of several UNC core facilities, particularly the Genome Analysis Facility, the Lineberger Comprehensive Cancer Center Nucleic Acids Core Facility, the Michael Hooker Proteomic Center, and the Microscopy Services Laboratory. I acknowledge my parents, David and Andrea Miller, for their devotion to my education early in my life, and their brave support of my pursuit of science later in my life. Lastly, throughout all of my endeavors, my husband Nate has genuinely and selflessly offered his enthusiasm and encouragement, for which I am immensely grateful.
PREFACE

When I decided to carry out my graduate studies in the laboratory of Dr. Robert Johnston, he and Dr. Nancy Davis agreed to meet with me in order to assist me in choosing a project for my dissertation research. Since I fully was expecting them to sit down with me and discuss the last NIH grant, pointing out which aims were not being actively worked on by any current laboratory members, it was much to my surprise when Dr. Johnston began the meeting by asking,

“So, what interests you?”

Admittedly, that was the appropriate question for my new mentor to ask, as I had gained all of my prior research experience in labs that studied bacteria, as opposed to his career, which was built on studying viruses. After getting past my initial shock, I managed to compose a response describing how I was intrigued in the dramatic differences in pathogenesis of Venezuelan equine encephalitis virus in the mosquito vector and the mammalian host. In particular, I was fascinated by the observation that the virus was able to block the synthesis of proteins in mammals but did not have this affect on mosquitoes. If I initially had been shocked by Dr. Johnston’s question, I was equally surprised by his response to this idea,

“Sure, you can work on that.”

Skeptical of my own proposition, I countered the support with a barrage of questions. “Do we have the resources to conduct the necessary studies?” “Is it acceptable for me to
begin a course of investigation that lacks specific funding?” “Do we have the necessary expertise?” Drs. Johnston and Davis assured me that my fledgling idea was feasible and could be developed into an appropriate project for my graduate work.

Like the progression of most graduate studies, I never fully addressed the question that I originally set out to answer. But, what my initial idea unveiled was that there was a lack of knowledge concerning many fundamental areas of alphavirus biology, including at the cellular level and particularly in the field of New World alphaviruses such as Venezuelan equine encephalitis virus. Dr. Mark Heise had walked in to that meeting and advised me to read up on the viral protein nsP2, which was implicated in inhibiting protein synthesis in mammals. And, so began my studies on nsP2.

I would soon learn that the function of nsP2 in the replication cycle had been fairly well studied, especially with Old World alphaviruses. However, there was also a body of evidence suggesting that nsP2 had additional, undescribed roles important in the outcome of infection that had yet to be described. So, I ultimately decided to pursue two fundamental questions that would help us better understand such auxiliary functions of nsP2: What host proteins interact with nsP2? Where is nsP2 found in the host cell? I regret that the question of the different effect that alphaviruses have on protein synthesis between mammalian and mosquito infection remains unanswered. Nonetheless, the work presented in this dissertation characterizes critical activities of Venezuelan equine encephalitis virus nsP2, which establishes fundamental knowledge about the multifunctional protein and will be valuable for future studies addressing its behavior in mammalian and mosquito cells.
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>AUR</td>
<td>Aura virus</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
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<tr>
<td>BiFC</td>
<td>Bimolecular fluorescence complementation assay</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>BSL</td>
<td>Bio-safety level</td>
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<tr>
<td>CHX</td>
<td>Cyclohexamide</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effects</td>
</tr>
<tr>
<td>cpm</td>
<td>Count/s per minute</td>
</tr>
<tr>
<td>CRM1</td>
<td>Chromosome region maintenance protein 1</td>
</tr>
<tr>
<td>CSE</td>
<td>Conserved sequence element</td>
</tr>
<tr>
<td>DCS</td>
<td>Donor calf serum</td>
</tr>
<tr>
<td>DI</td>
<td>Defective interfering</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothriotol</td>
</tr>
<tr>
<td>dsRNA</td>
<td>Double-stranded RNA</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent-assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>H</td>
<td>Hour/s</td>
</tr>
<tr>
<td>hpi</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>HeLa</td>
<td>Henrietta Lacks</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPI</td>
<td>Hours post infection</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immune deficiency virus</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IU</td>
<td>Infectious units</td>
</tr>
<tr>
<td>kB</td>
<td>Kilobase</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>KPNA</td>
<td>Karyopherin-alpha</td>
</tr>
<tr>
<td>LD₅₀</td>
<td>Lethal dose, 50%</td>
</tr>
<tr>
<td>LMB</td>
<td>Leptomycin B</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix Assisted Laser Desorption/Ionization Time-of-Flight</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
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<tr>
<td>MAY</td>
<td>Mayaro virus</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>MIN</td>
<td>Minute/s</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliters</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>NES</td>
<td>Nuclear export signal</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>nsP1</td>
<td>Nonstructural protein 1</td>
</tr>
<tr>
<td>nsP2</td>
<td>Nonstructural protein 2</td>
</tr>
<tr>
<td>nsP3</td>
<td>Nonstructural protein 3</td>
</tr>
<tr>
<td>nsP4</td>
<td>Nonstructural protein 4</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>P123</td>
<td>Nonstructural proteins 1-3 precursor</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFU</td>
<td>Particle forming units</td>
</tr>
<tr>
<td>PKR</td>
<td>Double Stranded RNA-activated Protein Kinase R</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RpS6</td>
<td>Ribosomal protein S6</td>
</tr>
<tr>
<td>RRV</td>
<td>Ross river virus</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S6K1</td>
<td>Ribosomal protein S6 kinase 1</td>
</tr>
<tr>
<td>SIN</td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>spp.</td>
<td>Species (plural)</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TOP</td>
<td>terminal oligopyrimidinetract</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VEE</td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>VRP</td>
<td>VEE replicon particles</td>
</tr>
<tr>
<td>WEE</td>
<td>Western equine encephalitis virus</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
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</table>
CHAPTER ONE

BACKGROUND AND INTRODUCTION
1.1 VENEZUELAN EQUINE ENCEPHALITIS VIRUS (VEE)

The Discovery of VEE and History of Outbreaks

Venezuelan equine encephalitis virus (VEE) was first recognized during the mid-1930s as a disease of horses, donkeys, and mules in South America. In 1938, VEE was first isolated from the brain of a post-mortem horse that succumbed to VEE infection during an outbreak in Yaracuy State, Venezuela (146). It was not until the late 1950s and 1960s that VEE was recognized as the causative agent of human disease, when human disease was noted concurrently with an outbreak in horses (145).

While there are no epidemiological records of suspected VEE outbreaks during the nineteenth century, retrospective examination of epidemiological reports suggests that VEE outbreaks may have begun during the 1920s before VEE was identified (144). For much of the twentieth century, there were sporadic outbreaks of VEE throughout the Americas. Of note, hundreds of thousands of horses and humans became infected during an outbreak in Peru in the 1940s and in Columbia during the 1960s (146). As of 1973, there were no documented cases of VEE for nearly twenty years, which prompted speculation that epizootic strains had gone extinct. However, in 1992 one of the largest recorded outbreaks of VEE began in Venezuela, and was followed by disease in Colombia and Mexico (90, 104, 149). Many factors are believed to have limited disease incidence during this time, including lifelong immunity in surviving infected horses and insufficient mosquito populations (136, 145).

The VEE Transmission Cycle
VEE circulates both in enzootic and epizootic transmission cycles (Figure 1.1). In the enzootic cycle, enzootic strains (subtype I, varieties D-F, and subtypes II-VI) of VEE continuously circulate between mosquito vectors and small rodent hosts that inhabit wet tropical forest or shaded swamp habitats in Mexico, Central America, and South America (13, 145). The principal vertebrate reservoir of enzootic strains of VEE is believed to be *Proechimys* spp. and *Sigmodon* spp. and the primary enzootic mosquito vector is *Culex* spp. mosquitoes (52, 145, 156, 157). The natural transmission cycle of VEE differs from that of other alphaviruses; whereas wild rodents are the enzootic reservoir of VEE, wild birds are the host reservoir for other alphaviruses (123).

Occasionally, and primarily in South and Central America, there are outbreaks of disease caused by epizootic strains (subtype I, varieties A-B and C) of VEE (145, 146). In the epizootic cycle, equines serve as the principal amplification host, and multiple species of mosquitoes serve as vectors that spread disease (10, 134, 137, 150). Because infected equines develop a high-titer viremia, the virus is transferred when a female mosquito feeds on a viremic equine. The infected mosquito vector spreads disease, infecting naïve hosts through its bite when taking subsequent bloodmeals, which is how humans become infected with VEE during the epizootic disease cycle. While some humans develop high-titer viremia, they are considered an incidental host in the VEE transmission cycle (145).

Classification and Geographic Distribution

VEE is a member of the *Togaviridae* family of viruses. The *Togaviridae* were initially recognized as viruses that possess a lipid envelope, coming from the Latin word *“toga”* meaning a Roman cloak (119). Within the *Togaviridae* family, VEE is one of the
nearly thirty recognized members of the alphavirus genus (12). Aside from the alphaviruses, the rubiviruses are the only other members of the Togaviridae, and they are not as widespread as the alphaviruses. Members of the alphavirus genus have been formally identified on every continent except for Antarctica, though an alphavirus of elephant seals, which widely range throughout Antarctica, has been identified (63).

Alphaviruses are grouped according to their geographic localization in either the “Old World”, which encompasses Europe, Asia, Australia, and Africa, or the “New World”, comprised of the Americas (129, 148). VEE is widely distributed throughout the Americas, having been isolated in South, Central, and North America (129). The Old World alphaviruses contain the Semliki Forest (SF) and Sindbis (SIN) groups, while the New World alphaviruses contain the VEE/Eastern equine encephalitis (EEE) group. The pathogenesis of these groups differs, as the Old World alphaviruses cause arthritic disease while the New World alphaviruses induce encephalitic disease.

While the origin of the two groups of alphaviruses remains unclear, multiple hypotheses have been formed to explain their origin based upon examination of alphavirus phylogeny. One theory proposes the alphaviruses to have originated in the New World and spread to the Old World in two distinct events, one giving rise to the SIN group and another to the SF group (70). An extension of this theory proposes that the alphaviruses could have originated in either the New or the Old World and been spread to the other hemisphere by migratory birds or marine animals around 2,000 to 3,000 years ago (63, 147).

Epizootic and Enzootic Strains and the Emergence of VEE
In the late 1950s, VEE was isolated in the absence of an outbreak from wild rodents in sylvatic and swamp habitats in Central and South America (145, 146). Studies of these enzootic strains of VEE revealed that they are antigenically distinct from strains isolated during equine outbreaks of disease, and enzootic strains were unable to generate viremia in equines in experimentally infected horses (145). Systematic studies of VEE in the 1960s found that isolates from outbreaks belonged to specific serotypes, which were originally designated antigenic subtype I, varieties A, B, and C (145). More recent serological tests and genetic analyses found varieties A and B indistinguishable, and they are now grouped together as variety IAB (144). Since subtypes IAB and IC have only been isolated during outbreaks, they are considered the “epizootic strains”. Conversely, VEE “enzootic strains”, which have been isolated from swamps and sylvatic habitats, are varieties ID, IE, and IF (144).

There are two major factors that influence VEE emergence: enzootic vs. epizootic strains and host range. That is, there are both viral and host determinants that are responsible for a shift from the enzootic to the epizootic cycle, as the strains of VEE differ between enzootic and epizootic cycles and each utilizes different vertebrate and arthropod hosts (145). In general, the cycling of VEE between mammalian and mosquito hosts means that it must adapt to two different hosts and thus places selective pressure on VEE. However, there are molecular differences between enzootic and epizootic strains of VEE, which is evidenced by the two types of strains being antigenically distinct. The molecular determinants that are responsible for the epizootic potential of VEE are beginning to be elucidated, and one critical determinant involves a pattern of positively-charged residues in the E2 glycoprotein on the surface of the virion (9, 54, 138). However, alterations in the habitat and ecology of host
reservoirs also affect pathogen transmission and can lead to sudden VEE emergence, as the host range differs in enzootic and epizootic strains of VEE (13).

1.2 VEE PATHOGENESIS AND VIRULENCE

VEE Infection of Mammals

In humans, VEE infection causes an acute infection that presents as a wide range of clinical symptoms, from asymptomatic to fatal encephalitis. Disease symptoms in humans most commonly include headache and fever and may also include rash and arthralgia (49, 98, 123). After an incubation period of 1-6 days in humans, the acute phase of VEE disease ensues and lasts 4-6 days. Occasionally, the disease in humans is biphasic with a recurrence 4-8 days after initial onset (146). Human mortality rates are usually less than 1%, but are accompanied by diffuse congestion, adema, and hemorrhaging of the lungs, gastrointestinal tract, and brain (25, 146). Severe neurologic disease can occur in up to 14% of cases in children, with mortality in these cases reaching 20% (55, 137).

After becoming infected with VEE, equines are typically asymptomatic for 1-3 days before developing a peak titer of $10^5$-10$^7$ LD$_{50}$/mL (55). While infected humans develop viremic titers similar to those of horses, they are not believed to amplify the epizootic cycle because of their lesser exposure to mosquitoes (146). In equines, signs of infection include fever, anorexia, and depression, which appear about 2 days post-infection. If encephalitis ensues in the infected horse, it occurs within 5-10 days of infection and death occurs soon thereafter; the development of encephalitis in equines correlates with the magnitude of
viremia (55). VEE mortality in equines can exceed 50%, and the virus can be isolated post-mortem from the brain and sometimes the spleen and blood (146).

The clinical diagnosis of VEE in both humans and equines is difficult because there are a wide range of clinical signs, and the disease presents similarly to other arthropod-born viral diseases endemic to the same regions. Although it is difficult to discriminate early in an epizootic cycle, outbreaks of VEE in humans are concurrent with outbreaks of disease in equines in the region. In a human patient, a suspected case of VEE is confirmed diagnostically by either detection of VEE-specific IgM antibodies by capture ELISA, seroconversion by examination of IgG, or isolation of virus or viral RNA from a patient’s serum or pharyngeal swab (146). Although similar tests can be used to diagnose equines, diagnosis of VEE in equines can be difficult, as often by the time that clinical signs present, viremia has already subsided.

In contrast to VEE infection of humans and equines, small rodents that are natural reservoir hosts of VEE show minimal to no signs of disease when infected with VEE, even when experimentally inoculated by intracranial injection (13). In laboratory experiments, within 3 days of being subcutaneously inoculated with VEE, reservoir host rodents become viremic with a viral titer sufficient to infect enzootic mosquito vectors (118, 150). Similar to laboratory mice, the draining lymph node may be the initial sight of VEE replication in reservoir mammals, and high titers are later found in the spleen (13). Seroconversion occurs by day 7 and antibodies can be detected over 1 year later, which could act to limit VEE circulation in nature (13).

*Mouse Model of VEE Infection*
VEE infection in mammals has been modeled in the laboratory through experimental infection of adult laboratory mice. Virus that is delivered through the bite of a mosquito in nature is mimicked in the laboratory by subcutaneous inoculation with a needle. This mouse model of infection leads to the onset of a biphasic disease in mice that is similar to that of VEE-infected horses (43). After the subcutaneous inoculation of $1 \times 10^3$ PFU of VEE, local Langerhans’ cells in the skin recognize and capture the virus and deliver it to the draining lymph node. VEE replicates in the draining lymphnodes, resulting in viral spread to all of the major lymphoid organs and a significant viremia in the first, or peripheral, phase of infection (78). The peripheral infection is cleared within 3 to 4 days after inoculation of the mouse (48). In the second phase, VEE invades the central nervous system through the olfactory and trigeminal nerves, giving the virus access to the neurons of the central nervous system where it replicates and induces a fatal encephalitis (15).

The mouse model of infection has been crucial in identifying viral determinants of pathogenesis and elucidating the host response to VEE. The advent of a full-length cDNA clone of VEE has allowed the construction of viral mutants with restricted spread in the mouse at different stages in the course of infection (15, 19, 21, 48, 155). Additionally, variations in the site of inoculation and amount of inoculum allow the identification of viral determinants of pathogenesis. The advent of mice harboring targeted mutations in genes of interest has proven to be another powerful tool in examining the response of the mammalian host to VEE infection (155).

Dissemination in the Mosquito Vector
In both the enzootic and epizootic transmission cycles, VEE is spread by a mosquito vector which acquires and transmits the virus during engorgement. When the mosquito feeds on an infected host, VEE is ingested with the bloodmeal. The bloodmeal is delivered to the mosquito midgut, where the virus initially replicates. Within two days of infection, the virus disseminates via the hemocoel, which allows it to reach secondary organs and tissues. Importantly, VEE replicates in the salivary glands, where viral particles bud from infected cells and accumulate in the saliva (143). Virus becomes quite concentrated in the saliva by 8 days post-infection, and salivation during subsequent engorgements passes the virus to subsequent hosts. Once infected with VEE, a persistent, lifelong infection is established in the mosquito. Although the lifespan of the mosquito is not shortened by carrying the virus, there are cytopathic effects in cells of the midgut and the salivary glands (8, 65, 143).

**Disease Treatment and Control**

Although VEE is a naturally emerging pathogen and a potential bioterrorism weapon, currently there is neither a licensed human vaccine nor an antiviral treatment regime for VEE infection. The earliest VEE vaccines were made in South America from formalin-inactivated virus that was isolated from the tissues of experimentally infected mice. To develop a live, attenuated VEE vaccine, a virulent strain was passaged eighty-three times in guinea pig heart cell culture in 1961 (7). The resulting attenuated strain, termed TC-83, is the best vaccine for effectively protecting equines from VEE infection, which is crucial in halting VEE outbreaks. To prevent epizootic disease outbreaks, it is critical to protect horses in Mexico, Colombia, and Venezuela, the region believed to harbor strains that are the progenitors of epizootic strains (146). As with the control of any arboviral disease, curbing the mosquito vector
population is also important, as decreased mosquito populations correlate with diminished disease incidence.

1.3 THE BIOLOGY OF ALPHAVIRUSES

*Genome Organization and Elements*

The VEE genome is a single-stranded, message-sense (also called “positive-sense” or “plus-sense”) RNA of approximately 11,400 nucleotides. The viral genome resembles cellular mRNAs, as it possesses 5’ and 3’ untranslated regions (UTR), with a 5’ terminal methylguanylate cap and a 3’ terminal polyadenylate tail. The VEE genome is comprised of two distinct regions: the 5’ two-thirds of the genome, which encodes the four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4), and the remaining 3’ one-third, which encodes the three structural proteins (capsid, E2, and E1) (Figure 1.2). The structural proteins are expressed at high levels as a subgenomic message from an internal 26S mRNA promoter. The subgenomic RNA is identical to the analogous portion of the genomic RNA, and is also capped and polyadenylated (119).

Within the genome there are four regions that are highly conserved across alphavirus, termed *cis*-acting conserved sequence elements (CSEs). For CSE 1, located in the 5’ UTR, and CSE 2, located in the early portion of the 5’ translated region, it is believed that the secondary structure of the regions are just as critical as their nucleotide sequence (129). CSE 3 is located at the junction of the nonstructural and structural coding regions in a domain that serves as a promoter for subgenomic RNA synthesis (71), and CSE 4 is in the 3’ UTR immediately upstream of the poly(A) tract (99). Mutations to these regions affect viral
growth and they are required for RNA replication (62, 71, 88, 89, 102). Cellular and viral proteins have been described as binding to the CSEs, presumably functioning to promote viral RNA replication (91-93). Indeed, specific interactions of viral and host proteins with the CSEs may suppress nucleotide divergence within the region, as mutation to the interacting RNA element would require a compensatory mutation in the host factor (89). Many other specific interactions are predicted to occur between alphavirus RNAs and host cellular proteins (129).

**Structure of the Virion**

The single-stranded RNA genome is encapsidated by capsid that is arranged in a T = 4 icosahedral protein shell (119). A tight-fitting lipid bilayer, which is derived from the host cell plasma membrane from which the particle budded, envelopes the icosahedral shell and contains heterodimers of the viral glycoproteins E2 and E1. The surface of the virion is made up of sets of three heterodimers which arrange as 80 trimers, also in a T=4 icosahedral lattice (129). The resulting VEE virion is spherical and 70 nm in diameter (94).

**Entry and disassembly**

The precise cellular receptor(s) for alphaviruses remains elusive. Since alphaviruses have a wide host range that encompasses mosquito to mammalian cells, they likely either use multiple receptors or a ubiquitous cell-surface molecule as their receptor (129). Laminin and heparin sulfate, both of which are widely-found molecules, have been described as alphavirus receptor (60, 128, 139). However, these receptors may not function in nature, but rather may be the consequence of laboratory adaptation (60). Regardless of the identity of the host
receptor, the viral glycoprotein E2 binds the receptor, as antibodies against E2 neutralize viral infectivity and E2 mutants are unable to bind cells (28, 120). Binding of the cellular receptor at 37°C leads to conformational change in the glycoprotein spike that exposes new epitopes (32, 80).

The alphavirus glycoproteins do not remain at the surface of the infected cell. Alphaviruses appear to be able to enter a cell via endocytosis by clathrin-coated vesicles (129). The low pH environment present in endosomes causes a conformational rearrangement of the glycoproteins, allowing the envelope to fuse with the membrane of the endosome (129). However, recently, it was found that alphavirus disassembly does not require acid pH, the disassembly of the icosahedral protein shell, or the fusion of the virus membrane with the host cell membrane (95). Rather, alphaviruses appear to directly penetrate the cellular plasma membrane through the reorganization of the glycoproteins to form a pore (94). This pore may also form in the targeted cellular membrane and serve as a path for entry of viral RNA (95). Once uncoated, the genome is able to be translated (119).

*Replication of the VEE Genome*

Once released into a host cell, the message-sense viral genomic RNA, which appears similar to eukaryotic mRNAs, is directly translated by the cellular translation machinery. The nonstructural proteins are translated as two polyproteins, P123 and P1234 (22, 72). The smaller P123, which contains nsP1, nsP2, and nsP3, is formed if the ribosome stalls at the opal stop codon positioned between nsP3 and nsP4, and translation ceases. The larger P1234, which also contains nsP4, is synthesized if there is read-through of the stop codon, which is estimated to occur in 10-20% of translations (22, 72, 122). The polyprotein precursors are
cleaved to form the mature nonstructural proteins by nsP2, which contains a papain-like proteinase domain. P1234 can be autoproteolytically cleaved \textit{in cis} into P123 and nsP4, which, along with more frequent synthesis of P123, results in the accumulation of P123 early during infection (129). Several hours into infection, cleavage at the nsP1/nsP2 site occurs \textit{in trans} to release a mature nsP1, and the resulting proteins readily cleave the nsP2/nsP3 site \textit{in trans}. At this point in infection, the presence of proteins that efficiently cleave the nsP2/nsP3 site also leads to the cleavage P1234 into P12 and P34 (129).

Alphavirus RNA replication occurs in association with cellular membranes (6, 37). Within the initial hours of infection, accumulation of P123 and P23 is critical for the production of negative-sense (or “minus-strand”) viral RNA, which is synthesized by two complexes, the P123 intermediate and nsP4, and the P23 intermediate with nsP1 and nsP4 (66, 122, 140). While positive-sense RNA is also produced early in infection, the synthesis of the negative-strand RNA wanes after 3 h while the production of positive-sense RNA remains steady throughout infection. A replication complex comprised of the four mature individual nonstructural proteins that utilizes the negative-sense RNA as template is responsible for the synthesis of both the positive-sense genomic RNA and the subgenomic RNA that is expressed at high levels from the internal 26S promoter (76, 103). The production of 26S mRNA in the infected cell serves to further amplify the viral structural protein genes.

\textit{Nonstructural Proteins}

Several functions have been ascribed to the four individual nonstructural proteins. nsP1 has guanine-7-methyltransferase and guanyltransferase activities, which are presumed
to be responsible for capping the viral RNA (1, 81, 117). It is also required for the initiation and/or continuation of minus-strand RNA synthesis (50, 141). nsP1 binds membranes, which may serve to associate the replication complex with membranes, and its ability to associate with membranes seems to be mediated through the addition of palmitates to nsP1 (1, 64). nsP2, a proteinase and helicase, is discussed in detail below. nsP3, the most poorly understood nonstructural protein, is a phosphoprotein (73, 96) and large deletions within its C-terminus are tolerated (21). nsP4 is the viral polymerase, essential for the synthesis of all viral RNA molecules (5, 50, 66, 114). Synthesized only when there is read-through of the opal codon, the concentration of nsP4 is well-regulated and it is rapidly degraded (23, 74).

Structural Proteins, Assembly, and Release

The 26S subgenomic RNA encodes the structural proteins, which are translated as a single polyprotein that is processed into individual structural proteins by both nascent viral proteins and host proteins in the endoplasmic reticulum (ER). Capsid, the first structural protein to be translated, is freed from the nascent polyprotein by autoproteolytic cleavage (16, 119, 124). In the cytoplasm, a cis-acting capsid recognizes a packaging signal contained within the genomic RNA to specifically encapsidate full-length genomic RNA to form nucleocapsids (151). Failure of proper nucleocapsid formation, such as encapsidation of incomplete or fragmented genomic viral RNA, ultimately results in the formation of defective interfering (DI) particles.

Continued translation of the 26S mRNA produces a nascent polyprotein containing PE2, E1, and 6K. This polyprotein contains signal sequences directing its insertion into the ER (129). E3 and 6K are both short proteins that serve as signal peptides. 6K serves as a
signal sequence for E1 and remains associated with E1 and E2 in the membrane (119). The components of the polyprotein move together through the secretory pathway, from the ER to the Golgi, and ultimately to the plasma membrane (119). In the ER, oligosaccharides which are later trimmed in the Golgi apparatus, are added to PE2 and E1; consequently, the composition of the glycoprotein modifications on E2 and E1 are dependent upon the cell type in which they were synthesized, as mosquito and mammalian glycoprotein modifications differ (59). Host cell enzymes, including signalases and furin-type proteases, process the structural polyprotein into the individual proteins PE2, 6K, and E1 in the ER (119). A host cell furin-type protease in the Golgi apparatus ultimately cleaves E3, releasing E2 (79, 84, 113, 142). Although the cleavage of E3 is not required for assembly and budding of progeny virions, it is required for the production of infectious progeny (100, 109).

Ultimately, the transmembrane glycoproteins interact with the nucleocapsids at the plasma membrane (119). This interaction attracts additional glycoproteins, which forms a coat around the nucleocapsid, resulting in an enveloped particle that buds from the plasma membrane (11, 41). Capsid-capsid interactions are stabilized by interaction with the glycoproteins, and assembly of nucleocapsid may occur in synchrony with viral budding (4, 33, 125, 158).

1.4 VEE REPLICON PARTICLES (VRP)

While the advent of a full-length infectious cDNA VEE clone alone was a major advancement, it also served as a tool for the development of VEE replicon particles (VRP),
which are non-propagating viral vectors that were designed based on the VEE genome (19, 21, 101).

**Design and Packaging**

The VRP genome encodes all four of the viral nonstructural proteins, but lacks the genes encoding the viral structural proteins. Instead, a heterologous gene (or no gene at all) can be expressed from the internal 26S subgenomic promoter (Figure 1.3). In the replicon genome RNA, the 5’ and 3’ untranslated regions of the VEE genome are intact, and it contains a virus-specific signal for its packaging into progeny particles. Since all of the nonstructural proteins are present in the replicon genome, VRP replicate their genomic RNAs and transcribe their subgenomic mRNA, which encodes the heterologous gene. Because the structural genes are absent from the replicon RNA, two separate helper RNAs, which lack the virus-specific packaging signal, are supplied *in trans*. Each of the helper RNAs expresses genes encoding the structural proteins under the 26S promoter: one expresses capsid and the other the glycoproteins. The viral replication complex, comprised of the nonstructural proteins, synthesizes the structural proteins from the helper RNAs, but since the helper RNAs lack the virus-specific packaging signal, only the replicon RNA is packaged into VRP.

**Benefits of VRP**

When cells are infected with VRP, the replicon RNA is amplified and the heterologous gene is expressed, but no progeny are assembled and thus there are no subsequent rounds of infection (Figure 1.3) (101). Hence, an advantage of the VRP system is that it serves as a tool to allow the study of a single round of the alphavirus infection cycle.
Additionally, whereas VEE is a pathogen that requires handling in a BSL-3 laboratory, VRP can be handled in a BSL-2 laboratory, and thus pose less risk to laboratory workers using the agent. Moreover, the concurrent expression of a heterologous gene during a single round of infection has proven to be a useful way of marking cells or introducing foreign gene products during infection (78, 85). VRP also serve as an excellent tool for expressing high levels of protein, because VRP efficiently infect many cell types and the 26S promoter is able to express protein at high levels for a sustained period of time. It is estimated that efficient protein production lasts up to 75 h and can result in the heterologous protein constituting up to 25% of the total cellular protein (76). Indeed, the ability to efficiently infect various cell types and its high level of expression of the heterologous gene has contributed to the effectiveness of VRP as vaccine vectors (3, 14, 20, 26, 42, 51, 56, 86, 101, 121, 132).

1.5 NONSTRUCTURAL PROTEIN 2 (nsP2)

The most well characterized functions of nsP2 concern its essential activities in viral RNA replication. nsP2 has enzymatic activities and is a component of P123, which participates in the synthesis of negative strand viral RNA, and the fully-matured replication complex, which is responsible for the synthesis of positive strand viral RNA. Auxiliary functions, which are distinct from its role in the replication complex, also have been described for nsP2.

*Essential Functions in the Replication Complex*
Both the N-terminus and the C-terminus of nsP2 possess enzymatic functions. The N-terminal domain of nsP2 contains a nucleoside triphosphate (NTP)-binding domain associated with ATPase and GTPase activity that is dependent upon a required lysine residue (106). Since nsP2 also binds RNA and has sequence homology to an ancient family of helicases, it is implicated in unwinding of double-stranded RNA that forms during viral RNA replication (44, 45, 105). The C-terminal domain of nsP2 functions as the nonstructural proteinase that cleaves the polyprotein precursor (129). The crystal structure of the C-terminus of VEE nsP2 has been elucidated, revealing that it is comprised of two distinct domains (110). Mutagenesis analysis of the C-terminus revealed that two residues are necessary for catalytic function of the proteinase, a critical cysteine residue located approximately 60 residues upstream of a required histidine residue, giving it the properties of a papain-like proteinase (127). The proteinase recognizes the three specific, distinct cleavage sites that liberate each of the nonstructural proteins from the polyprotein precursor (129). The kinetics of the processing of the nonstructural protein precursor suggest that the context of the proteinase affects its preference for a particular cleavage site.

Mutations in nsP2 located outside of the required enzymatic domains demonstrate that nsP2 functions in the synthesis of viral RNA. The regulation of negative-strand RNA synthesis is mediated by nsP2, as there are temperature-sensitive mutants with mutations in nsP2 that fail to convert from negative- to positive-strand synthesis (24, 115). Additionally, nsP2 regulates the synthesis of the 26S subgenomic RNA and could be involved in initiation of synthesis of positive strand RNA at the subgenomic promoter (130). These activities may indicate a direct role in RNA synthesis or may derive from differential proteolytic processing which then results in an RNA replication phenotype.


**Evidence for Auxiliary Functions**

Over recent years, it has become evident that nsP2 carries out important functions during infection that are not directly involved with its functions in the replication complex, referred to as auxiliary functions. Early evidence that nsP2 possessed auxiliary function came from studies with SFV and SIN, where nsP2 was identified as the only alphavirus nonstructural protein that is present in both the cytoplasm and the nucleus of infected cells, even though the alphavirus replication cycle occurs exclusively in the cytoplasm (6, 107, 108). Not only does SFV nsP2 localize to the nucleus, but it also accumulates in the nucleolus, the site of ribosome assembly (107). The pentapeptide PRRRV defines the nuclear localization signal (NLS) in SFV nsP2, with the central positively-charged arginine crucial for its function. Though the role of nuclear nsP2 remains elusive, disruption of nsP2 nuclear localization reduced the ability of SFV to inhibit DNA synthesis in the mammalian host cell and compromises the ability of the virus to spread in the brain (31). Interestingly, in mosquito cells that display low CPE upon alphavirus infection, nsP2 does not localize to the nucleus, whereas it does localize to the nucleus of mosquito cell variants which display high CPE upon alphavirus infection (82, 83). Moreover, the mosquito cell nucleus, but not the mammalian cell nucleus, is required for efficient alphavirus infection (30).

There are also numerous observations that suggest that nsP2 is a critical determinant of the outcome of infection. Although alphaviruses characteristically cause lytic infection in mammalian host cells, several independent studies have identified mutations in nsP2 that confer the ability of alphaviruses to establish persistent, non-cytopathic infections (27, 34, 97, 152). Additionally, the proteinase activity of nsP2 may function in cleaving host proteins.
that are part of a host antiviral response (131). Finally, recent studies have suggested a difference in the nsP2 of Old World and New World alphaviruses, as mature nsP2 of the Old World alphaviruses SIN and SFV, but not that of VEE, cause transcription shutoff of the host, which could antagonize the host’s antiviral response (39, 40). Taken together, such observations indicate that nsP2 possesses undefined functions that are mediated through interactions with the host cell and determine the outcome of alphavirus infection.

1.6 ALPHAVIRUSES AND THE HOST CELL

Although few cellular factors involved in the alphavirus life cycle have been identified, some of the fundamental interactions that have been described between alphaviruses and the host cell are considered below.

Host Cell Receptors

To gain entry into host cells, alphaviruses must interact with a host cell receptor. Because alphaviruses have a broad host range, it is postulated that alphaviruses either utilize multiple receptors or that a ubiquitous surface protein functions as the host cell receptor. Cellular receptors are difficult to identify in the laboratory, as cell culture adaptation of viruses may identify molecules as receptors that do not function as such in nature.

While the cellular receptor recognized by alphaviruses remains elusive, some candidates have been identified. Mosquito cell culture models have identified laminin, a widely distributed, high-affinity receptor, as a molecule that allows VEE to enter mosquito cells, though this has not been verified in vivo in mosquitoes (77). Mammalian cell culture
models have also identified the high-affinity laminin receptor as a receptor for alphaviruses (128, 139). While laminin may serve as a receptor in cell culture, the highly specific infectivity of VEE for the mosquito vector suggests the presence of a co-receptor or the use of a different receptor in vivo (144). Another widely distributed molecule, heparin sulfate, has been described as an alphavirus receptor (60). Although heparin sulfate binding is likely a cell culture adaptation, the ability of SIN to bind heparin sulfate can contribute to alphavirus virulence in laboratory mice (111).

Host Proteins and the Replication Complex

Early studies with SIN first suggested that specific cellular factors are likely required for alphavirus replication (2, 6, 61, 67, 122). Although host factors are believed to be components of the replication complex, none have been identified unequivocally (6, 119). Moreover, since the synthesis of alphavirus RNA occurs on cellular membranes, the replication complex must interact with host proteins to anchor it, although the precise determinants are unknown (6, 37).

Several recent studies have looked for host factors that interact with nsP3, an essential member of the replication complex. In one such study, G3BP2 (Ras-GTPase-activating protein SH3-domain-binding) and 14-3-3 proteins were identified as host proteins in association with nsP3 (18). Both of these host proteins could be crucial in alphavirus infection, as G3BP2 can regulate translation, is associated with stress granules, and has been previously described in viral replication, while 14-3-3 proteins regulate cell signaling pathways (18). In another study, not only were 14-3-3 proteins identified as host proteins
that interacted with nsP3, but also cytoskeleton proteins, chaperones, elongation factor 1A, heterogeneous nuclear ribonucleoproteins, and some of the ribosomal proteins (36).

Viral replication is believed to be modulated by cellular proteins that bind the untranslated regions of alphavirus RNA. The La autoantigen was identified as a host factor that binds to the 3’ end of the alphavirus negative-sense RNA, a region that serves as a viral promoter for progeny genome synthesis (62, 88, 91-93).

**Shutoff of the Host Cell**

Many viruses interfere with the synthesis and stability of cellular proteins, DNA, and RNA, resulting in host cell shutoff. Shutoff, coupled with an increasing synthesis of viral products, results in the virus usurping the cellular machinery and dominating cellular biosynthetic pathways. Alphaviruses block transcription and translation through undefined, independent mechanisms (46). Interestingly, transcription shutoff appears to differ between Old World and New World alphaviruses, wherein nsP2 is responsible for transcription shutoff in Old World alphaviruses while capsid is responsible in New World alphaviruses (40).

For alphaviruses, which possess a message-sense RNA genome, translation is the first step in replication upon entering a cell; thus, the ability to efficiently usurp the translation machinery is crucial for successful replication. Alphaviruses rapidly inhibit host protein synthesis in the mammalian host cell, but persistently infect mosquito host cells without affecting host translation (154). The mechanism of alphavirus translation shutoff in mammalian cells is poorly understood. Several mechanisms by which alphaviruses might inhibit host protein synthesis have been proposed, including: (i) a reduction in intracellular
ribonucleoside triphosphate pools as a result of viral RNA synthesis (154); (ii) changes in the ionic environment of the cell that favor the translation of viral RNAs over that of host mRNAs (17, 154); and, (iii) competition between viral and host messages for host factors, including the ribosome (58, 154). In SFV, the capsid protein interferes with host mRNA binding of the 80S ribosomal initiation complex (135), and electroporation of high amounts of capsid protein alone into cells inhibited host cell protein synthesis (29). However, the observation that SFV capsid protein shuts off host protein synthesis is disputed by findings that in SIN and VEE replicon systems, in which the viral structural proteins are not synthesized, host cell translation is inhibited ((35); Appendix A.1). Studies in SIN demonstrated that the alphavirus inhibition of host translation is independent of the PKR-induced shutoff of protein synthesis that is a generalized innate cellular response to infection (47).

Interestingly, alphavirus infection leads to a termination in the appearance of newly synthesized ribosomes in the cytoplasm of infected cells (154). Although the mechanism is not understood, it is presumably a result of the inhibition of new protein synthesis; specifically, the synthesis of the ribosomal precursor RNA is preferentially inhibited (154).

Host Cell Response Against VEE

Replication of alphaviruses results in the formation of viral double-stranded RNA (dsRNA) replication intermediates, which are recognized by the host cell and induce IFN-α/β genes and other factors that are involved in the activation of IFN-induced antiviral proteins (133, 153). Early studies with VEE revealed the importance of type I interferon (IFN), a component of the innate immune response (53, 57). With VEE, there is a direct correlation
between virulence and resistance to IFN-α/β; in the extreme cases, the attenuated vaccine strain TC-83 is quite sensitive to IFN-α/β whereas epizootic stains are resistant to IFN-α/β (126). In fact, a substitution identified in the untranslated region of TC-83 that strongly contributes to its attenuation also confers sensitivity to IFN-α/β, demonstrating that the host IFN response is critical in combating VEE (155).

Another cellular protein that responds to dsRNA is a protein called double stranded RNA-activated Protein Kinase R, or PKR. PKR binds dsRNA, which induces its kinase activity that phosphorylates the translation initiation factor eIF2α, ultimately inhibiting host translation (38, 87). Indeed, there is increased permissivity to alphaviruses in the absence of PKR (112).

VEE infection also elicits apoptosis, or programmed cell death in mammalian cells, including neurons of the central nervous system, which become persistently infected with SIN (68, 69, 75, 116). VEE also appears to induce apoptosis in dendritic cells, some of the first cells infected within a host (86). Apoptosis of alphavirus-infected cells occurs within hours of infection and may prevent viral spread.

1.7 DISSERTATION OBJECTIVE

While VEE has been studied for nearly 70 years, there is a dearth of knowledge concerning the interaction of VEE with the eukaryotic cell at a molecular level. This deficit must be addressed in order to understand the pathogenesis of VEE and the fundamental differences between infections of its various host organisms. Of particular interest is how the multifunctional VEE protein nsP2 interacts with the mammalian host cell, as it is a key
determinant in establishing the outcome of infection. Accordingly, the objective of this
dissertation is to examine the behavior of VEE nsP2 within the host cell as a means to
ultimately understand its contribution to the pathogenesis of VEE at a molecular level.
Specifically, this dissertation aims to characterize the interaction of VEE nsP2 with a critical
host protein and define the means by which nsP2 subcellular compartmentalization within the
mammalian host cell is mediated. In Chapter 2, ribosomal protein S6 (RpS6) is identified as
a cellular protein with which nsP2 interacts during infection. The complex containing nsP2
and RpS6 is characterized, the effect that alphaviruses have on the state of RpS6 is assessed,
and the consequence that reduction of RpS6 has on infection is examined. In Chapter 3,
signals are defined that direct VEE nsP2 to both the nucleus and the cytoplasm of
mammalian cells. Furthermore, pathways by which VEE nsP2 is delivered into the nucleus
and expelled from the nucleus are identified. Further work that supplements these findings is
presented in the appendices.
FIGURE 1.1

enzootic

epizootic
**FIGURE 1.1: The VEE transmission cycle.** VEE is a mosquito-borne disease that has both enzootic and epizootic transmission cycles. In the enzootic cycle, the virus continuously cycles between mosquitoes and small wild rodents that inhabit wet, shady terrain. These rodents are the principal reservoir of VEE. Periodically, the epizootic cycle ensues, leading to outbreaks of febrile illness and neurologic disease in horses and humans. In the epizootic cycle, the viremic equines serve as amplifying hosts which continue the outbreak cycle. While humans become infected during epizootic outbreaks, they are considered incidental hosts.
FIGURE 1.2
**FIGURE 1.2:** The VEE genome. The VEE genome is a single strand of message-sense RNA of approximately 11.4 kB. Boxes represent genes encoding the denoted viral proteins, the bent arrow represents the subgenomic promoter, and solid lines represent untranslated regions of the genome. Not shown to scale.
FIGURE 1.3

A

B
FIGURE 1.3: VEE replicon particles (VRP). (A) VRP are non-propagating viral vectors that are synthesized by the co-electroporation of three separate RNAs: the replicon RNA and two defective helpers. The replicon RNA contains the four VEE nonstructural genes, the 5’ and 3’ ends of the VEE genome, the VEE packaging signal, and a heterologous gene which is expressed from the subgenomic 26S promoter. Defective capsid and glycoprotein helpers that lack a packaging signal are supplied in trans. Upon co-electroporation into cells, the RNAs are replicated by the viral nonstructural proteins, but only the replicon RNA, containing the heterologous gene, is packaged into particles. (B) The resulting VRP are able to infect cells, but because they lack the structural genes, they are unable to infect subsequent cells.
1.8 REFERENCES


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CHAPTER TWO

RIBOSOMAL PROTEIN S6 ASSOCIATES WITH ALPHAVIRUS NONSTRUCTURAL PROTEIN 2 AND MEDIATES EXPRESSION FROM ALPHAVIRUS MESSAGES

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2.1 ABSTRACT

Although alphaviruses dramatically alter cellular function within hours of infection, interactions between alphaviruses and specific host cellular proteins are poorly understood. While 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. In this report we demonstrate that nsP2 associates with ribosomal protein S6 (RpS6), and 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, as 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 lead to diminished expression from alphavirus subgenomic messages, while 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.
2.2 INTRODUCTION

The Alphavirus genus contains over 25 recognized viruses with a wide geographic distribution. Venezuelan equine encephalitis virus (VEE) is a New World alphavirus which 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 4), while the 3’ one-third encodes three mature structural proteins (capsid, E2, and E1) that are expressed at high levels following transcription from an internal 26S subgenomic mRNA promoter. The viral genome appears similar to cellular mRNAs, as 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, 62). 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. Reviewed in (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). Additionally, 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); Montgomery and Johnston, unpublished data). Though 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, non-cytopathic infections. Interestingly, independent studies carried out in various 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 (CSE). Nucleotide divergence in these elements may have been suppressed as a result of these regions of RNA specifically interacting with cellular proteins, as mutation to 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.
2.3 MATERIALS AND METHODS

Cells and Infections

BHK-21 cells (ATCC, passage 55-65) were incubated at 37°C under 5% CO₂ and maintained in alpha minimal essential medium (Gibco) containing 10% donor calf serum (DCS), 10% tryptose 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% CO₂ and maintained in Dulbecco’s modified Eagle medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin per mL, and 0.5 mg of streptomycin per mL.

For VEE replicon particle (VRP) and VEE infection, 18 h prior to infection 6 well plates were seeded 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% DCS and Ca⁺/Mg⁺. 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 previously described (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 hours post infection (hpi) after significant CPE was evident. Virus stocks then were clarified by centrifugation (10,000 × g, 30 min, 4°C) and further concentrated by pelleting the clarified virus preparations through 20% (wt/vol) sucrose in low-endotoxin phosphate-buffered saline (PBS) at 72,000 × g for 5 h at 4°C. Virus titers were determined by plaque assay on BHK-21 cells.

VEE replicon particles (VRP) expressing green fluorescent protein (GFP), human ribosomal protein S6 (RpS6), or a C-terminally FLAG (DYKDDDDK) epitope-tagged human RpS6 (RpS6-FLAG) were packaged 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 VRP undergo only one round of infection. After packaging, VRP are harvested, concentrated through a sucrose cushion, resuspended, and titered on BHK-21 cells either by immunofluorescence (GFP-VRP) or immunocytochemistry (RpS6-VRP, 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 \textit{in vitro} translations, genes were expressed from pCI-neo-based (Promega) plasmids. To insert heterologous genes into the pCI-neo vector, the multi-cloning 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 nsP2 gene was PCR amplified from Sindbis TR339. For pCI-neo-SFVnsP2, the Semliki Forest nsP2 gene was PCR amplified from SFV4. For pCI-neo-mosRpS6 the \textit{Aedes albopictus} RpS6 gene was PCR amplified from pGEMTEasy-C710RPS6 (courtesy of Ann. M. Fallon, U. Minnesota).

\textit{Protein Analysis}

\textit{(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 post-infection and starved of methionine and cysteine by providing Minimum Essential Medium, Eagle lacking methionine and cysteine (Sigma). After 1 h incubation at 37°C, the medium was replaced with starvation medium supplemented with 33 µCi/mL 35S-methionine/35S-cysteine (Pro-mix, Amersham Pharmacia). Immediately following 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 on an 8% SDS-PAGE gel. To measure 35S incorporation, cells that had been previously transfected with siRNAs were pulsed for 1 h with 35S-methionine/cysteine. Total cell lysate was precipitated with 10% trichloroacetic acid, bound to a glass filter, washed, and quantitated using a scintillation counter.

(ii) Immunoprecipitation Experiments

For each immunoprecipitation sample, lysate was prepared from a confluent well of a 6 well tissue culture plate. Unless otherwise indicated, at 8 hpi cell monolayers were rinsed once with PBS and lysed using 200µl per well of NP-40 lysis buffer (170 mM NaCl, 50 mM Tris, 15 mM EDTA, 0.2% NP-40 plus Complete protease inhibitor cocktail tablets [Roche]). After incubating for 5 min, cell lysates were scraped into microfuge tubes, and lysates were cleared by centrifugation for 2 minutes at 4°C at 12,000 × g. Samples were frozen at −80°C. Cell lysate (175 µL) was cleared by adding 40 µL of protein A-Sepharose (Sigma) and agitating at 4°C for at least 2 h. The protein A beads were removed by centrifugation, and lysates were further cleared by mixing and agitation with 40 µL of protein A-Sepharose and 1µL of isotype-matched normal antisera at 4°C. Following at least 2 h of incubation, the
protein A beads were removed by centrifugation, and the lysates were transferred to new tubes containing 2 µL of specific antibody and agitated overnight at 4°C. The lysates were then transferred to new tubes containing 40 µL of protein A-Sepharose and agitated for 2 h at 4°C. After removing the lysate, the beads were washed three times in NP-40 lysis buffer and one time in 50 mM Tris (pH 7.6). Protein gel loading buffer was added to the beads before heating to 95°C for 5 minutes. The dissociated protein was loaded onto a 10% SDS-PAGE acrylamide gel.

(iii) 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, following previously described methods (4, 41).

(iv) Western Blotting

Protein preparations were separated by 10% SDS-PAGE, and transferred to Immunoblot PVDF (Bio-Rad) membrane 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 Signalling], anti-nsP2 [gift of AlphaVax, Inc.]). Phospho-specific antibodies were diluted in 5% bovine albumin (BSA) in TBST. Subsequently, membranes were washed three times in TBST and then probed with appropriate HRP-conjugated secondary antibodies (anti-rabbit-
HRP [Ambion], anti-goat-HRP [Novus]) for 1 h at room temperature. Blots were washed three times in TBST followed by detection of HRP-conjugated secondary antibody by chemiluminescence using ECL detection reagents (Amersham Pharmacia). Blots were exposed to film and developed. Antibodies used throughout this work include: VEE anti-nsP2 (gift from AlphaVax, Inc.), anti-FLAG (Sigma), anti-RpS6, anti-pRpS6 (Ser235/236), 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 100µg divided between each hind leg every 4 weeks for a total of 4 injections.

(v) Phosphorimager Scans

After separation of $^{35}$S-labeled proteins on an SDS-PAGE gel, 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 using ImageQuant (Molecular Probes).

In Vitro Transcriptions and In Vitro Protein Synthesis

To prevent run-on transcription, prior to in vitro transcription all DNA plasmids were linearized by Not I 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 template for T7 mMMessage mMMachine in vitro transcription according to manufacturer’s directions (Ambion).
In vitro rabbit reticulocyte lysate nuclease free translations (Promega) were performed according to manufacturer’s recommendations in the presence of $^{35}$S-methionine/cysteine.

**Immunofluorescence microscopy.**

BHK-21 cells grown on cover slips were infected with SFV-NP at an moi of 0.5. At 8 hpi, cells were fixed with 3.2% PFA for 20 min at RT, permeabilized with 1% NP40 in PBS for 5 minutes, and cover slips were incubated in blocking buffer (5% Donkey serum, 0.1% BriJ58 in PBS) for 30 min. Samples were then processed for immunofluorescence 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. Secondary antibodies used were donkey anti-rabbit Ig (FITC) and anti-mouse Ig (Cy3). Images were obtained using a BioRad MR1024 confocal microscopy system where the fluorescent signals were mapped to green and red, respectively.

**Nuclear Purification**

$5 \times 10^7$ cells were harvested per sample and resuspended in 350 µL hypotonic buffer (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF]. After incubation on ice for 15 minutes, Nonidet P-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 a 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 MgCl₂, 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 × 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/mL cycloheximide (Sigma) immediately before being harvested. Cytoplasmic extract was made and loaded onto a 10 to 40% continuous sucrose gradient. The gradients were centrifuged for 105 min at 17,000 × g in a Beckman SW41 rotor at 4°C. Fractions were collected from the top of the gradient using a FRAC-100 chromatography system.

siRNA Experiments

One day prior to transfection, HeLa cells were seeded at 7×10⁴ cells in a 12 well plate in antibiotic-free DMEM medium supplemented with 10% FBS and 1% L-glutamine. Cultures were transfected with 10pmol of either an RpS6 or non-targeting siRNA pool (Dharmacon) in 300 µL OptiMEMI and Lipofectamine 2000 (Invitrogen) mixture according to manufacturer’s recommendations. After 2 h incubation at 37°C, 1 mL media was added. After 48 h, the medium was removed, and transfected cells were infected with GFP-VRP. At 18 h post-infection cells were trypsinized and washed with cold FACS buffer (PBS/0.5% human serum albumin). Cells were fixed with PBS/1% formaldehyde before FACS analysis. FACS data were obtained using a FACScan flow cytometer (BD Biosciences) and analyzed 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 employed Venezuelan equine encephalitis replicon particles (VRP), which are non-propagating 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 GFP-VRP (VRP expressing green fluorescent protein; 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 a SDS-PAGE gel. Coomassie stained proteins were identified by MALDI-TOF/MS sequencing. From this analysis, ribosomal protein S6 (RpS6) was identified as a protein that specifically co-immunoprecipitated with nsP2 in VRP-infected lysates (Figure 2.1A). We then confirmed the association of RpS6 with nsP2 by analyzing nsP2 immunoprecipitates by immunoblot with antibodies to RpS6 (Figure 2.1B).

To detect the nsP2-RpS6 interaction in the reciprocal manner, that is to determine whether nsP2 co-immunoprecipitated 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); unpublished data, Montgomery and Johnston), encoding the epitope-tagged version of human RpS6 on a viral message allows its
continued synthesis after infection. RpS6-FLAG-VRP infection resulted in approximately a 5-fold excess of FLAG-RpS6 over endogenous RpS6 (data not shown). Cytoplasmic proteins from cells infected with RpS6-FLAG-VRP were immunoprecipitated using α-FLAG antibody (Sigma) and subsequently subjected to Western blot analysis for nsP2. Indeed, nsP2 co-immunoprecipitated with the FLAG-tagged RpS6, confirming that we could detect the interaction in a reciprocal experiment (Figure 2.2A).

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 immunoblot for RpS6 revealed that the two proteins were associated not only in VRP-infected BHK-21 hamster cells, but also in VRP-infected human cell lines HEK-293 (Figure 2.2B) 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 (Figure 2.2C). It is noted that the appearance of the co-immunoprecipitating RpS6 occurs at a time after infection similar to when nsP2 synthesis and accumulation is first visualized (data not shown). Furthermore, RpS6 co-immunoprecipitated with nsP2 in cells infected with VEE virus (V3000; Figure 2.2D), 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 data), the complex containing
nsP2 and RpS6 that we have observed is an interaction that predominantly occurs in the cytoplasm (Figure 2.2E).

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, then the mRNA was transcribed and co-translated in the presence of $^{35}$S-methionine/$^{35}$S-cysteine in a nuclease-treated rabbit reticulocyte lysate translation reaction. RpS6 and nsP2 co-immunoprecipitated with anti-nsP2 antibody after in vitro synthesis, demonstrating that the presence of the other viral nonstructural proteins was not required for the interaction (Figure 2.3A). Co-translation 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 (Figure 2.3B).

nsP2 Proteins from Multiple Alphaviruses Associate with RpS6

The nsP2 protein from other alphaviruses also associates with RpS6. The nsP2 from Sindbis TR339 (SINnsP2) or Semliki Forest SFV4 (SFVnsP2) was co-translated 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, SINnsP2 and SFVnsP2 both co-immunoprecipitated with FLAG-RpS6, suggesting that the association of RpS6 and nsP2 is common throughout the alphavirus genus (Figure 2.3B).
**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 approximately 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 co-translated with VEE nsP2 in vitro. Immunoprecipitation using VEE nsP2 antibody revealed that *Aedes* RpS6 associates with nsP2 (Figure 2.3C). 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**

As RpS6 is a constituent of the 40S ribosomal subunit, we wanted 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 co-sedimented with RpS6 in ribosome-containing fractions. When polysome gradient fractions were analyzed by immunoblot, the distribution of nsP2 was essentially identical to the distribution of RpS6 (Figure 2.4A) 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 shut-off 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 $^{35}$S-methionine/cysteine after infection. Immunoprecipitation of such radiolabeled lysates allowed visualization of other members of this complex. Lysates immunoprecipitated with antibody against the FLAG epitope revealed that both nsP2 and nsP1 co-immunoprecipitated with RpS6 (Figure 2.4B). Confirming this observation, immunoprecipitation with antibody against nsP1 yielded bands corresponding to nsP2 and RpS6-FLAG in addition to nsP1, and immunoprecipitation with antibody against nsP2 yielded a band for nsP2 as well as bands corresponding to nsP1 and RpS6-FLAG. RpS6-FLAG did not co-immunoprecipitate when antibody against nsP3 was used (data not shown).

Taken together, these data pose several possibilities. It is possible that nsP2 directly associates with RpS6 and 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 the polysome.
purification. Alternatively, it is possible that nsP2 and RpS6 interact both as a soluble complex and within the context of the ribosome, and nsP1 is present only in the soluble complex.

RpS6 Phosphorylation is Diminished after Alphavirus Infection

RpS6 is the major phoshproteio 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 (Figure 2.5A). SFV infection similarly causes a dramatic decrease in the amount of phosphorylated RpS6 present in the cell (Figure 2.5B, 2.5C). When cells were infected with SFV-NP, a replicon expressing influenza NP, a protein that localizes to the nucleus, the amount of total RpS6 was unaltered but phosphorylated RpS6 disappeared within hours of infection (Figure 2.5B). Likewise, RpS6 phosphorylation was diminished after infection with an SFV replicon expressing NP as well as when NP was over-expressed 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 NP. Note that RpS6 localizes to both the cytoplasm and the nucleus, but phosphorylated RpS6 is cytoplasmic.

Finding that RpS6 phosphorylation is diminished after infection, the phosphorylation status of the population of RpS6 that associates with nsP2 was examined. Lysates from GFP-
VRP-infected cells were immunoprecipitated with nsP2 antibody and then analyzed by Western blot using the phospho-specific RpS6 antibodies. The population of RpS6 that co-immunoprecipitates with nsP2 is predominantly not phosphorylated at Ser235/236 or Ser240/244 (Figure 2.5D), 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 RpS6 prior to alphavirus infection. HeLa cells were either transfected with a pool of siRNA against RpS6 messages, a non-targeting 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 30-50%, 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 (Figure 2.6A). 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 (Figure 2.6B, 2.6C). To more closely examine the status of host protein synthesis under conditions of diminished RpS6, \(^{35}\)S-methionine/cysteine incorporation was measured 48 h after transfecting cells with siRNA. There was only a minimal decrease in overall host translation
under conditions of reduced RpS6 protein levels (Figure 2.6D). Taken together, this demonstrates that proteins expressed from alphavirus messages require RpS6 for optimal synthesis of viral proteins.
2.5 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: 1) occurs in mammalian and mosquito cells, 2) is apparent within 2 hours after infection, 3) remains detectable throughout the course of infection, 4) occurs in VRP as well as VEE infected cells, and 5) 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 co-fractionates with RpS6 in a polysome gradient, which suggests that nsP2 associates with the whole ribosome. In our initial identification of proteins that co-immunoprecipitated 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).
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 nsP1 protein has both guanine-7-methyltransferase and guanyltransferase activity, which is required to cap the viral RNAs (1, 33, 52). nsP1 is required for the initiation of negative-sense RNA synthesis (18, 63), and is also responsible for 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, 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), as blocking RpS6 phosphorylation did 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 employing other strategies to induce a generalized host shut-off (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 IRES-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 greater than 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
predominately occupy ribosomes and is 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 employ to inhibit translation of host proteins to allow the virus to efficiently utilize elements of the host cell.
2.6 ACKNOWLEDGMENTS

We are grateful to AlphaVax, Inc. for the gift of VEE nsP2 antibody. Proteomics analyses were performed by the UNC-Duke Proteomics Center, which was partially supported by a gift from an anonymous donor for research targeted to proteomics and cystic fibrosis, in memory of Michael Hooker. We thank Doug Lyles, John H. Connor, and Zackary Whitlow for generous assistance with polysome gradients. We are grateful to Ann M. Fallon (University of Minnesota) for sharing the full-length Aedes albopictus RpS6 clone with us. We are grateful to Timothy P. Moran for generous assistance. This work was supported by N.I.H. grant AI51990.
FIGURE 2.1

A

VRP: + -
nsP2
nsP1
Rp86

Coomassie stain

B

IP: nsP2 | NGS
VRP: + - +
Rp86

IB: RP36
FIGURE 2.1: Rps6 co-immunoprecipitates with nsP2 in vivo. (A) Coomassie 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 x 10^5 cell equivalents were immunoprecipitated anti-nsP2 antibody, separated on an 8% SDS-PAGE gel and coomassie stained to visualize proteins that co-immunoprecipitate with nsP2. As labeled, nsP2, nsP1, and Rps6 were identified by MALDI-TOF mass spectophotometry. (B) Immunoblot for human Rps6 on GFP-VRP or mock infected lysates that were immunoprecipitated using anti-nsP2 antibody. Normal goat sera serves as an isotype-matched control for immunoprecipitation. Rps6 is specifically detected in VRP-infected lysates immunoprecipitated for nsP2.
FIGURE 2.2

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IB: nsP2

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IB: RpS6

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IB: RpS6
FIGURE 2.2: 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 the indicated time points post-infection. 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, cells were lysed in NP-40 lysis buffer and lysates were immunoprecipitated with anti-FLAG antibody and subsequently examined by immunoblot for nsP2. nsP2 co-immunoprecipitates 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 1-2 contain samples using HEK-293 (human) cell lines and lanes 3-7 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, samples in lanes 1-4 were immunoprecipitated with anti-nsP2 antibody, 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 co-immunoprecipitates with nsP2 throughout the course of infection. HEK-293 cells were infected and samples were harvested over a timecourse, at time points indicated. The nsP2-RpS6 interaction is continuously detected. (D) Upper panel: RpS6 co-immunoprecipitates 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 co-immunoprecipitates with nsP2. Lower panel: Total cell lysates from VRP- or VEE-infected
cells at 3 and 6 hpi immunoblotted for nsP2. Note that more nsP2 has accumulated at each time point in VRP-infected lysates, which corresponds with more RpS6 co-immunoprecipitating with nsP2 from VRP-infected lysates. (E) **Left panel:** Nuclear and cytoplasmic fractions were prepared from GFP-VRP- or mock-infected cells before immunoprecipitation for nsP2. The RpS6 that co-immunoprecipitates with nsP2 is predominantly cytoplasmic. **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 as compared to the cytoplasm.
FIGURE 2.3: *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 $^{35}$S-label and subsequently immunoprecipitated using anti-nsP2 antibody or isotype-matched antibody (NGS). The specific co-immunoprecipitation 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 co-translated with RpS6-FLAG. Immunoprecipitation using anti-FLAG antibody reveals that all of the alphavirus nsP2s co-immunoprecipitate with RpS6-FLAG. (C) Mosquito RpS6 associates with nsP2. RpS6 from either human (hum RpS6) or *Aedes albopictus* (mos RpS6) was co-translated with VEE nsP2. Subsequent immunoprecipitation using anti-VEE nsP2 antibody reveals mosquito RpS6 co-immunoprecipitates with nsP2.
FIGURE 2.4

A

B

| RpS6-FLAG-VRP | + | + | + | - | + |
| GFP-VRP      | - | - | - | - | - |
| anti-nsP2    | - | - | - | + | - |
| anti-nsP1    | + | - | - | - | - |
| anti-nsP3    | - | - | - | - | + |
| anti-FLAG    | - | + | + | + | - |
FIGURE 2.4: Other proteins associated with the complex containing nsP2 and RpS6.

(A) RpS6 and nsP2 are present in the same fractions of a polysomes 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 10-40% continuous sucrose gradient. An aliquot of each fraction of the polysomes gradient were examined by immunoblot using anti-RpS6, anti-nsP2, anti-nsP1, anti-nsP3, or anti-actin antibody. The polsone profile of this gradient, as monitored by the OD\textsubscript{254}, 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 co-migrates with RpS6. Upper band in the nsP2 blot is the P123 precursor.

(B) nsP1 co-immunoprecipitates 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 \textsuperscript{35}S-label. At 8 hpi, cell lysates were made and subsequently used in immunoprecipitation. Immunoprecipitation using α-nsP2 antibody reveals that nsP1 and RpS6-FLAG co-immunoprecipitate with nsP2; immunoprecipitation using α-nsP1 antibody reveals that nsP2 and RpS6-FLAG co-immunoprecipitate with nsP1; immunoprecipitation using α.FLAG antibody reveals that nsP1 and nsP2 co-immunoprecipitate with RpS6-FLAG. Cells infected with GFP-VRP and immunoprecipitated using α-FLAG antibody, showing that nsP1 and nsP2 co-immunoprecipitation is specific. There was not co-immunoprecipitation of other radio-labeled proteins when lysates were immunoprecipitated with α-nsP3 antibody.
FIGURE 2.5

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Total RpS6
phospho-RpS6 Ser 240/244

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D

VRP:  
WC: + -  
IP: nsP2 + -  
IB: phospho RpS6 Ser 235/236  
IB: total RpS6  

WC: + -  
IP: nsP2 + -  
IB: phospho RpS6 Ser 240/244  

82
**FIGURE 2.5: 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*). Lysates are from GFP-VRP-infected (moi of 10) HEK-293 cells at timepoints indicated or mock-infected (M) cells. (B) Similar to VRP infection, SFV4 reduces the amount of phosphorylated RpS6. Cells infected with SFV-NP, a SFV replicon that expresses influenza NP, at 8 hpi. NP, a nuclear protein, is visualized by Cy3 staining (red). *Left panel*, total RpS6 is FITC stained (green); *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 incubation in media containing 20% FCS; Lane 2, cell lysates with diminished amount of phosphorylated RpS6 after incubation in media containing 0% FCS; lane 3, mock infection; lane 4, lysates from SFV-NP infected cells; lane 5, cells infected with SFV-Capsid enhancer-NP (note 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), while influenza virus infection does not diminish phosphorylated RpS6 as greatly (lane 6). (D) The population of RpS6 that associates with nsP2 is predominantly unphosphorylated. Immunoblot for total RpS6 and phosphorylated RpS6 on HEK-293 cell lysates immunoprecipitated with α-nsP2 antibody. Cells were infected at an moi of 10 IU/cell with GFP-VRP (5005-3000) or mock-infected. At 8 hpi 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 divided amongst three gels, and analyzed by
immunoblot with one of two phospho-specific RpS6 antibodies, anti-RPS6 Ser235/236 or anti-RpS6 Ser 240/244, as well as an antibody that recognize the total amount of RpS6. The RpS6 that co-immunoprecipitates 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 as compared to the amount of phosphorylated RpS6 in mock-infected cells (Compare whole cell lysates labeled WC).
FIGURE 2.6: Diminished RpS6 protein level reduces expression of virally-encoded protein. (A) HeLa cells were mock-transfected or transfected with either a non-targeting siRNA pool (NTsiRNA) or an RpS6 siRNA pool (S6siRNA). At 48 h post transfection, cell lysates were collected and immunoblotted for RpS6 or tubulin. (B) 48 h after HeLa cells were transfected with siRNA, they were infected at an moi of 5 with GFP-VRP. 18 hpi cells were harvested and subjected to FACS analysis. Numbers on profiles (4.30, 4.35, and 4.04) indicate percentage of cells infected as determined by GFP fluorescence. Although a similar percentage of cells were infected in mock-transfected (upper panel), non-targeting siRNA transfected (middle panel), and RpS6 siRNA transfected (lower panel), RpS6 knockdown led to diminished fluorescence. FACS profiles shown are representative of ten samples. (C) Median GFP intensity of cells infected with GFP-VRP after transfection with NTsiRNA (white) or S6siRNA (black). Intensity of virally-encoded GFP is decreased 10-fold with diminished RpS6 level. Data is representative of triplicate experiments. (D) 48 h after transfection with siRNA, cellular translation was measured by 1 h pulse with $^{35}$S-methionine/cysteine. Extracts were analyzed by trichloroacetic acid precipitation and quantitation by scintillation counter. Results are average 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 $^{35}$S-label incorporation.
2.7 REFERENCES


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CHAPTER THREE

NUCLEAR IMPORT AND EXPORT OF VENEZUELAN EQUINE ENCEPHALITIS
VIEUS NONSTRUCTURAL PROTEIN 2

Stephanie A. Montgomery and Robert E. Johnston

This work has been submitted for publication in Journal of Virology.
3.1 ABSTRACT

Many RNA viruses, which predominantly replicate in the cytoplasm, have nuclear components that contribute to their life cycle or pathogenesis. We have investigated the intracellular localization of the multifunctional nonstructural protein 2 (nsP2) in mammalian cells infected with Venezuelan equine encephalitis virus (VEE), an important, naturally emerging zoonotic alphavirus. VEE nsP2 localizes to both the cytoplasm and the nucleus of mammalian cells in the context of infection and also when expressed alone. Through the analysis of a series of EGFP fusion proteins, a segment of nsP2 was identified that completely localizes to the nucleus of mammalian cells. Within this region, mutation of the putative nuclear localization signal PGKMOV diminished, but did not obliterate, the ability of the protein to localize to the nucleus, suggesting that this sequence contributes to the nuclear localization of VEE nsP2. Furthermore, VEE nsP2 specifically interacted with the nuclear import protein karyopherin-α 1, but not with karyopherin-α 2, 3, or 4, suggesting that karyopherin-α 1 transports nsP2 to the nucleus during infection. Additionally, a novel nuclear export signal was identified which included residues L526 and L528 in VEE nsP2. Leptomycin B treatment resulted in nuclear accumulation of nsP2, demonstrating that nuclear export of nsP2 is mediated via the CRM1 nuclear export pathway. Taken together, these results establish bidirectional transport of nsP2 across the nuclear membrane, which suggests that nsP2 shuttles between the cytoplasm and nucleus during infection.
3.2 INTRODUCTION

Venezuelan equine encephalitis virus (VEE), a New World member of the genus *Alphavirus* in the *Togaviridae* family, is a pathogen that is responsible for significant disease in equines and can cause a wide range of human symptoms, from inapparent infection to acute encephalitis. In nature, VEE is transmitted between susceptible hosts through a mosquito vector and is responsible for periodic outbreaks of widespread human and equine disease throughout the Americas.

The alphaviruses are enveloped viruses with an 11.5 kb genome of single stranded, message-sense RNA. The viral genome, which resembles cellular messages because it contains 5’ and 3’ untranslated regions, a 5’ terminal methylguanylate cap and a 3’ terminal polyadenylate tail, encodes four nonstructural proteins (nsP1 through 4) and three mature structural proteins (capsid, E2, and E1). Upon infection, this RNA genome is directly translated in the cell to produce the nonstructural polyproteins P1234 and P123, depending on whether there is read-through at the opal stop codon present between nsP3 and nsP4 in most alphaviruses (8, 31). The polyprotein is processed by nsP2, which contains a protease domain in its carboxy-terminus, to form the four individual nonstructural proteins, nsP1-4 (21). Early during infection, negative-sense viral RNA is synthesized by two complexes, the P123 intermediate and nsP4, and the P23 intermediate with nsP1 and nsP4 (30, 58, 66). In the late stage of infection, the four mature individual nonstructural proteins constitute a replication complex that synthesizes full-length positive-sense genomic RNA from the negative-sense template and also a subgenomic RNA that is expressed at high levels from the internal 26S promoter (32, 46). The 26S subgenomic RNA encodes the structural proteins
and is translated as a polyprotein that is processed into individual structural proteins by both nascent viral proteins and host proteins in the endoplasmic reticulum. Alphavirus replication is reviewed in (59).

The alphavirus nsP2 protein is a quintessential multifunctional viral protein, with multiple critical activities in the replication cycle ascribed to it. nsP2 possesses multiple enzymatic activities and is required for a functional alphavirus replication complex. The N-terminus of nsP2 contains a nucleoside-triphosphate (NTP)-dependent helicase that is believed to function as an RNA helicase that unwinds double-stranded RNA that forms during replication (18, 48). This region of nsP2 also has RNA 5’ triphosphatase activity that is responsible for cleaving the 5’ end of the viral RNA prior to the addition of the methylguanylate cap (63). The C-terminus of nsP2 possesses a papain-like proteinase that is responsible for the cleavage of the nonstructural polyprotein precursor (21). This portion of nsP2 is also involved in the regulation of synthesis of the subgenomic 26S RNA and negative-strand RNA (9, 54, 55, 60). The crystal structure of the multifunctional C-terminus of VEE nsP2 has recently been elucidated, and it is comprised of two distinct domains (52).

Aside from providing essential functions in viral replication, nsP2 also may have auxiliary functions during infection that affect its outcome. Throughout the course of infection, nsP2 is found in association with ribosomes, and the nsP2 of both New World and Old World alphaviruses specifically interacts with ribosomal protein S6, though the function of this interaction with an essential host protein has yet to be determined (35, 44). Additionally, although alphaviruses characteristically cause a lytic infection of mammalian cells, several independent studies have identified mutations in nsP2 that allow the establishment of a persistent alphavirus infection (10, 15, 42, 67). Recent studies have
suggested a difference in nsP2 of Old World and New World alphaviruses, as mature nsP2 of the Old World alphaviruses Sindbis virus (SIN) and Semliki Forest virus (SFV), but not that of VEE, cause transcription shutoff of the host, which may have a direct function in antagonizing the host’s antiviral response (16, 17). Collectively, such observations suggest that there are undefined functions of nsP2 mediated through interactions with the host cell that affect the outcome of viral infection.

Similar to most positive-strand RNA viruses, the alphavirus replication cycle occurs in the cytoplasm, and it has been demonstrated that the mammalian cell nucleus is not required for alphavirus replication (11). In spite of this, approximately half of nsP2 is found in the nucleus of infected mammalian cells, and nsP2 is the only alphavirus nonstructural protein present in the nucleus (1, 19, 34, 41, 49, 50). Studies performed in SFV, an Old World alphavirus, identified PRRRV as the nuclear localization signal (NLS) in nsP2, and defined the central arginine residue in the sequence as essential for nsP2 nuclear localization. Moreover, SFV nsP2 localizes to the nucleolus, the cellular site of ribosome assembly, and a signal for nucleolar localization is present in the C-terminus of SFV nsP2 (49). The NLS is not essential for the viral life cycle, but disruption of the ability of nsP2 to localize to the nucleus compromises the ability of SFV to inhibit host DNA synthesis and to spread in the brain of an infected mouse (13, 47). To date, the molecular function of nuclear nsP2 remains elusive.

Some components and functions of eukaryotic cells are segregated to the nucleus, which is enveloped by a double-layered membrane. Translocation across this membrane barrier occurs at nuclear pores, through which bidirectional transport of macromolecules occurs. Theoretically, proteins less than 30 to 40 KDa are able to passively diffuse through
the nuclear pore complex (NPC), but larger proteins require active transport (4, 20, 39). The movement of larger proteins through the NPC is governed by importins and exportins, collectively termed karyopherins. The transport of these proteins into and out of the nucleus is an energy-dependent, signal-mediated process. Karyopherins specifically recognize nuclear localization signals (NLSs) or nuclear export signals (NESs) that dictate transport into or out of the nucleus, respectively, and are able to traverse the NPC with a cargo protein. The classic NLSs are termed monopartite or bipartite depending on how many stretches of basic amino acids are present; however, there are many examples of NLSs that do not fall into these categories (3, 27). The SFV NLS is a monopartite, or SV40 large-T antigen-like NLS. Just as NLSs confer the ability of a protein to be recognized by an importin and actively transported from the cytoplasm into the nucleus, NESs are recognized by exportins and allow proteins to be actively transported from the nucleus to the cytoplasm through the NPC (51). The most commonly identified NESs are short leucine-rich signals, although other hydrophobic residues such as isoleucine, valine, methionine, and phenylalanine can contribute to the signal as well (25, 37, 40).

While it once seemed counterintuitive for RNA viruses, and in particular positive-stranded RNA viruses, to enter the nucleus, there now are many examples of such viruses that possess a viral protein that interacts with the nucleus and/or nuclear trafficking. These include but are not limited to: coronaviruses, Dengue virus, Hepatitis C virus, Newcastle disease virus, Influenza virus, tobacco etch virus, poliovirus, Ebola virus, and HIV (2, 14, 22, 28). By targeting the nucleus or nuclear trafficking, a virus can recruit host factors for viral replication or evade the host antiviral response by inhibiting host gene expression, inhibiting
the export of host proteins or RNAs from the nucleus, and inducing cytoplasmic retention of nuclear proteins.

Although the nuclear localization of nsP2 has been examined in Old World alphaviruses, it has not been investigated in VEE, a New World alphavirus and important pathogen. In this study, we show that VEE nsP2 localizes to both the nucleus and the cytoplasm of mammalian cells during infection. The subcellular distribution of nsP2 can be reproduced by expression of nsP2 in the absence of other viral proteins, which serves as a useful tool to more closely examine signals that direct the localization of nsP2 within the host cell. We show that nsP2 possesses signals for both its import into and export from the nucleus. Furthermore, nsP2 interacts exclusively with karyopherin-α1, suggesting that the nuclear import of nsP2 is mediated by this cargo protein. Finally, inhibition of the CRM1-dependent nuclear export pathway led to nuclear accumulation of nsP2, indicating that nuclear export of nsP2 is mediated by CRM1. Evidence of nuclear import and export of VEE nsP2 suggests that the localization of nsP2 during alphavirus infection may represent a dynamic cycling of this multifunctional replicase protein between the cytoplasm and nucleus.
3.3 MATERIALS AND METHODS

Cells

BHK-21 (ATCC, passage 55-65), HEK-293 (ATCC, passage 30-45) and Vero (ATCC, passage 95-110) were grown at 37°C under 5% CO₂. BHK-21 cells were maintained in alpha minimal essential medium (Gibco) containing 10% donor calf serum (DCS), 10% tryptose phosphate broth, 0.29 mg of L-glutamine per mL, 100 U of penicillin per mL and 0.5 mg of streptomycin per mL. HEK-293 cells were maintained in Dulbecco’s Modified Eagle’s Medium (Gibco) containing 10% fetal bovine serum (FBS) and 100 U of penicillin per mL and 0.5 mg of streptomycin per mL. Vero cells were maintained in Minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate and 10% FBS.

Infection with VEE and VEE replicon particles

The construction of a full-length VEE cDNA clone pV3000 derived from the Trinidad donkey VEE isolate has been previously described (7). The preparation of purified VEE virus stocks has also been previously described (6). Briefly, VEE virus was produced by electroporating in vitro transcribed RNA of pV3000 into BHK-21 cells and harvesting virus particles from the supernatant 24 hours later. Virus stocks were purified first by centrifugation (10,000 × g, 30 min, 4°C) of the supernatant for clarification, and then further concentrated by pelleting the clarified virus preparations through 20% (wt/vol) sucrose in
low-endotoxin phosphate-buffered saline (PBS) at 72,000 \times 9 g for 5 h at 4°C. Plaque assays were performed on BHK-21 cells to determine viral titers as expressed in PFU per mL.

VEE replicon particles (VRP) expressing green fluorescent protein (GFP) were packaged using a split helper system as previously described (43). To synthesize VRP, three in vitro synthesized RNA messages 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. The supernatant was harvested 24 h post-electroporation, and subsequently concentrated through a 20% (wt/vol) sucrose cushion. After the particles were resuspended, VRP titers were determined on BHK-21 cells by immunofluorescence and expressed as infectious units (IU) per mL.

For VEE virus and VRP infections, BHK-21 or HEK-293 cells were seeded 18 h prior to infection at 1 \times 10^4 cells per well of an 8-well glass titer slide. BHK-21 cells were seeded on Lab-Tek™ glass slides (Nunc), but HEK-293 cells are fastidious and required Lab-Tek II CC²™ chamber slides (Nunc). For infection, the growth medium was removed, and cell monolayers were infected at a multiplicity of infection (MOI) of 1 in 80 μL of low-endotoxin phosphate-buffered saline (PBS) supplemented with 1% DCS and Ca²⁺/Mg²⁺. After incubation at 37°C for 1 h to allow for adsorption, 200 μL of the appropriate complete growth medium was added back to each well.

*Plasmid constructs*
The plasmid used to synthesize the replicon genome for packaging VRP was the wild-type replicon plasmid pV5005 which contains the gene for GFP mutant 2 (5) located directly downstream of the 26S promoter in place of the VEE structural protein genes (33). The K646D mutation, which replaces a lysine residue at VEE nsP2 position 646 with an aspartic acid, was made in the pV5005 plasmid by replacing the AAA codon with GAT.

All EGFP fusions were constructed using the pEGFP-C3 vector (Clontech). To insert heterologous genes into the pEGFP-C3 vector, the Xho I and Apa I sites of the multi-cloning site were used and a translational stop codon was added twice in tandem immediately following the inserted heterologous sequence. By always using the same restriction sites to insert the heterologous sequence, all fusion proteins had the same 5 amino acid (aa) linker, YSDLE, between EGFP and the inserted sequence. For all of the constructs containing sequence from the VEE viral nonstructural protein nsP2 gene, the sequence was PCR amplified from pV5005 (7). The following list of fusion plasmids used in this study describes the heterologous sequence inserted into pEGF-C3 by naming the plasmid and the inserted amino acids: EGFP-1-265nsP2, G1 to L265; EGFP-266-530nsP2, I266 to S530; EGFP-531-794nsP2, G531 to C794; EGFP-506-769nsP2, T506 to R769; EGFP-531-769nsP2, G531 to R769; EGFP-506-794nsP2, T506 to C794; EGFP-511-794nsP2, S511 to C794; EGFP-516-794nsP2, L516 to C794; EGFP-521-794nsP2, V521 to C794; EGFP-526-794nsP2, L526 to C794; EGFP-VEEterm–nsP2, N399 to C794. For EGFP-SFVterm–nsP2, N400 to C798 of the SFV nsP2 gene was PCR amplified from SFV4 (gift from P. Berglund; (35)).

The construction of pCI-neo-nsP2 has been previously described (35). Briefly, a consensus Kozak sequence and start codon were added to the 5’ end of the full-length nsP2 derived from pV5005 and inserted into the multi-cloning site of pCI-neo (Promega).
The panel of plasmids expressing karyopherin-α1, 2, 3, or 4 with an N-terminal FLAG epitope tag were the generous gift of P. Palese, and their assembly has been previously described (57, 65). The YFP protein fragment complement assay was the generous gift of S. Michnick (38). PCR-amplified full length nsP2 or aa 531-794 of nsP2 were cloned into the BspE I and Xba I sites at the 3’ end of Venus 1, and karyopherin-α1, PCR amplified from the plasmid described above, was inserted at the 5’ end of Venus 2 at the Not I and Cla I sites.

*Site-directed mutagenesis*

To construct mutations in the EGFP-nsP2 fusion proteins, complimentary sense and anti-sense primers were designed that contained the desired mutation(s) flanked by 12-14 nt of unmodified sequence. To construct the VEE C-terminus containing the SFV substitution encoding RRR, GGCAAAATG was mutated to GCAGAAAGG, and to construct the SFV C-terminus containing the VEE sequence substituting GKM, CGACGCAGG was mutated to GGAAAGATG. To construct the V521A mutation, the GTG codon was mutated to GCG; for L526A, CTC to GCC; and for L528A, CTG to GCG. Primers containing these mutations were used with the QuickChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. All mutations were confirmed by sequencing.

To assemble the K646D mutation into pVR21, an overlap PCR mutagenesis method was employed. To mutate the lysine residue to an aspartic acid, overlapping primers containing a GAT substitution for the parental AAA codon were used to generate a PCR product that was inserted between the unique BstE II and BsaBI sites present in pVR21. The identity of this insertion was confirmed by sequencing.
**Transfections**

One day prior to transfection, $1 \times 10^4$ HEK-293 cells were seeded in 200 µL per well of an 8 well slide such that the monolayers would be approximately 50% confluent when transfected. For transfections performed in 6 well plates, $5 \times 10^5$ HEK-293 were plated in 2 mL per well at 24 h prior to transfection. Transfections were carried out using FuGENE 6 (Roche) according to the manufacturer’s directions. Briefly, 3-6 µL of FuGENE 6 were added to 100 µL of Opti-MEM I reduced serum medium (Gibco) and incubated at room temperature for 5 min. DNA was then added to the mixture at a ratio of approximately 3 µL of transfection reagent to 1 µg of DNA. Following an incubation of 15 min at room temperature, 7 µL of the mixture was added to each well of an 8 well slide or 100 µL of the mixture was added to each well of a 6 well dish. Cells were fixed in 2% paraformaldehyde (PFA) or lysed at 36-48 hours post-transfection.

**Indirect immunofluorescence staining**

BHK-21 cells were fixed in 2% PFA for 35 minutes at room temperature, whereas HEK-293 cells were fixed overnight at 4°C, washed three times with PBS, and permeabilized. Cells were permeabilized in acetone at -20°C for 20 minutes, rehydrated in three PBS washes, and blocked for 1 h in 7.5% bovine serum albumin (BSA) in PBS. After excess blocking solution was removed in three PBS washes, monolayers were stained with anti-histone H1 antibody (Leinco Technologies) at a 1:50 dilution in 0.1% BSA in PBS for 1 h. After PBS washes, cells were stained in 0.1% BSA in PBS for 1 h with the appropriate anti-IgG secondary antibody directly conjugated to Alexa Fluor 594® or AlexaFluor 488® (Invitrogen) diluted 1:400. After a final series of PBS washes, cells were mounted under a
glass coverslip using a polyvinyl alcohol/glycerol mounting medium with 2% N-propyl
gallate with 0.25% 1,4-diazabicyclo[2,2,2,]octane; gift from the University of North Carolina
Microscopy Services Laboratory) to resist photobleaching. For DAPI (4′,6′-diamidino-2-
phenylindole) staining, coverslips were mounted using Vectashield containing DAPI (Vector
Labs). For nsP2 staining, an antibody recognizing VEE nsP2 (gift from AlphaVax, Inc.) was
used according to a previously described protocol (23).

*Nuclear purification*

Nuclei were purified from BHK-21 cells as previously described (35). Briefly, mock
or VRP-infected cells were harvested, resuspended in hypotonic buffer (20 mM HEPES, 10
mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl
fluoride [PMSF]), incubated on ice, and gently lysed using Nonidet P-40 at a final
concentration of 1%. Nuclei were pelleted at 1000 × g at 4°C and the supernatant was
collected as the cytoplasmic fraction. The nuclear pellet was resuspended in a 25% solution
of Optiprep (Sigma) and purified through layers of 30% and 35% Optiprep. Nuclei were
recovered from the Optiprep gradient, washed, pelleted, and lysed with nuclear extraction
buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol).
After lysis, the insoluble nuclear fraction was removed by centrifugation and the supernatant
was collected as the nuclear fraction. Whole cell lysates were made using RIPA lysis buffer
(50mM Tris HCl [pH 7.5], 150 mM NaCl, 5mM EDTA, 1% Triton X-100, 0.1% SDS, 1%
sodium deoxycholate plus complete protease inhibitor cocktail tablets [Roche]) and
clarifying lysates by centrifugation after lysis.
**Immunoblotting**

Protein samples were separated on a 10% SDS-PAGE gel and subsequently transferred to Immun-blot PVDF (Bio-Rad) membrane in transfer buffer (48 mM Tris, 39 mM glycine, 10% methanol) at 12 V for 60 min. Membranes were then blocked for 1 h in 5% dry milk in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl and 0.1% Tween-20) and incubated overnight at 4°C with primary antibodies diluted in blocking buffer (anti-GRP-78/BiP [Santa Cruz] at 1:750; anti-nsP2 [gift from AlphaVax, Inc] at 1:40,000; anti-EGFP [BD Biosciences] at 1:1,000; anti-FLAG [Sigma] at 1:1,000). After probing with primary antibody, the membranes were washed three times in TBST and probed with the appropriate HRP-conjugated secondary antibodies diluted in blocking buffer (anti-goat-HRP [Novus] at 1:14,000; anti-rabbit-HRP and anti-mouse-HRP [GE Healthcare] at 1:3,000) for 1 h at room temperature. Membranes were rinsed in a final series of TBST washes before detection of HRP-conjugated secondary antibody by chemiluminescence using ECL detection reagents (GE Healthcare). Blots were exposed to film and developed.

**Immunoprecipitation**

For each immunoprecipitation sample, lysate was combined from 2 confluent wells of a 6 well tissue culture plate at 36-48 h post-transfection. Monolayers were rinsed once with PBS and each well was lysed with 200µl of 1% NP-40 lysis buffer (170 mM NaCl, 50 mM Tris, 15 mM EDTA, 1% NP-40 plus complete protease inhibitor cocktail tablets [Roche]). After 5 min incubation on ice, lysates were collected in microfuge tubes, and cleared by centrifugation for 5 minutes at 4°C at 12,000 × g. Immunoprecipitation against FLAG was performed using EZView™ Red Protein A anti-FLAG® affinity gel (Sigma) according to the
manufacturer’s directions. Briefly, 400 µL of clarified lysate was incubated with 40 µL of equilibrated beads overnight at 4°C with agitation. Beads were then washed three times in TBS (50 mM Tris-HCl [pH 7.4], 150 mM NaCl) and eluted under acidic conditions (0.1 M glycine HCl at pH 3.5). The dissociated protein eluates were neutralized with buffer (0.5 M Tris HCl pH 7.4, 1.5 M NaCl) before the addition of protein gel loading buffer for immunoblot.

Microscopy

Confocal fluorescence images were acquired with a Carl Zeiss LSM5 Pascal Confocal Laser Scanning microscope equipped with a 40X objective and a 63X oil immersion objective and processed with imaging software. Pinholes were set to acquire optical sections of <0.6 µm and images were averaged four times. After collecting signal from EGFP and Alexa Fluor 594 samples, the fluorescent signals were mapped to green and red, respectively. Fluorescent microscope images (containing DAPI staining) were acquired on a Leica Microsystems fluorescent microscope equipped with a 40X objective using Spot software. Unless otherwise indicated, images shown were acquired using a 63X objective and 2X magnification.

Leptomycin B (LMB) treatment

At 24 h post transfection, cells were treated with LMB (Sigma) to a final concentration of 20 ng/mL or left untreated in the appropriate growth medium and incubated for 6 h. Cells were then fixed and the subcellular distributions of EGFP fusion proteins were examined by confocal fluorescence microscopy.
3.4 RESULTS

**VEE nsP2 Localizes to the Nucleus and Cytoplasm of Infected Mammalian Cells**

VEE nsP2 is a multifunctional protein that is likely to have yet-to-be-defined activities. As one step in its characterization, its intracellular localization during infection was determined. BHK-21 cells were infected with VEE virus (V3000), stained for nsP2, and examined by indirect immunofluorescence and confocal fluorescence microscopy. At 8 hpi VEE nsP2 accumulation was visible in both the cytoplasm and the nucleus of infected cells (Figure 3.1A). VRP are nonpropagating viral vectors that contain a genome encoding the four nonstructural proteins but express a heterologous gene from the subgenomic promoter in place of the structural proteins. We confirmed the nuclear and cytoplasmic localization of nsP2 in VRP infection in multiple cell lines, including HEK-293 cells (Figure 3.1B).

To confirm the subcellular localization pattern of nsP2 during infection, nuclear and cytoplasmic fractions of infected cells were examined for nsP2. VRP-infected BHK-21 cells were harvested at 8 hpi, and subsequently fractionated to obtain cytoplasmic and nuclear fractions for analysis by immunoblot for nsP2. VEE nsP2 was detected in both the cytoplasmic and nuclear extracts (Figure 3.1C). The purity of the nuclear fraction was confirmed by the absence of GRP-78, an abundant cytoplasmic protein that is indicative of cytoplasmic contamination.

The localization of nsP2 when expressed alone also was investigated, as the presence of other viral proteins could affect the localization of nsP2. Full-length nsP2 was expressed from the pCI-neo mammalian expression vector, and 48 h after being transfected into cells, the distribution of nsP2 was assessed by indirect immunofluorescence staining against nsP2.
When expressed alone, nsP2 was distributed throughout the cytoplasm and the nucleus in a manner indistinguishable from VEE or VRP infected cells (Figure 3.2). Therefore, the localization pattern of nsP2 was not dependent upon the presence of other viral proteins.

*Analysis of nsP2 Localization Using EGFP Fusion Proteins*

To identify intracellular transport signals contained within nsP2, a series of EGFP fusion proteins were employed. Sequential thirds of nsP2 were constructed so that EGFP was fused in frame to their C-termini to form the fusion proteins EGFP-1-265-nsP2, EGFP-266-530nsP2, and EGFP-531-794nsP2 (Figure 3.3A). The fusion constructs were individually transfected into cells, and their subcellular localization was analyzed by fluorescence microscopy using a confocal fluorescent microscope. Interestingly, each third of nsP2 displayed dramatically different subcellular localization. The N-terminal third, EGFP-1-265-nsP2, localized to the nucleus and the cytoplasm; the middle third, EGFP-266-530nsP2, was entirely localized to the cytoplasm; while the C-terminal third, EGFP-531-794nsP2, was localized entirely to the nucleus (Figure 3.3B). The distinct localization pattern of each fusion protein suggested that nsP2 contains multiple signals directing its transport within the cell.

*Identification of a C-terminal Nuclear Localization Signal in VEE nsP2*

EGFP-531-794nsP2, which was entirely confined to the nucleus, spanned a region of nsP2 that contained sequence homologous to the SFV NLS. As the work by Rikkonen and colleagues demonstrated the central positively charged residue of the signal (PRRRV) was crucial for its function, the equivalent VEE signal (PGKVMV) was disrupted in the mutation
K646D, where the positively charged lysine was replaced with a negatively charged aspartic acid (Figure 3.4A) (49). When the K646D substitution was present in EGFP-531-794nsP2, the completely nuclear localization pattern was disrupted, and the product was present in both the cytoplasm and the nucleus, suggesting that the mutation disrupted function of a nuclear localization signal (Figure 3.4B).

The K646D mutation was also placed in the context of the full-length nsP2 in the replicon genome. BHK-21 cells were infected with the K646D mutant VRP and the localization of nsP2 was examined using indirect immunofluorescence staining. Although the cytoplasm and nucleus stained positive for K646D mutant nsP2, there appeared to be a diminished amount of nsP2 present in the nucleus in comparison to cells infected with VRP expressing wildtype nsP2 (Figure 3.4C). Interestingly, the K646D mutation reproducibly reduced titers of VRP by 10,000-fold.

Comparison of the Nuclear Localization Signal of SFV with VEE

We sought to directly compare the PRRRV motif in SFV with the PGKMOV motif in VEE. For this comparison, we constructed two EGFP fusion proteins. In the first, EGFP-VEECterm–nsP2, a region spanning the C-terminal half of VEE nsP2 (from N399 to C794) was fused to the C-terminus of EGFP. In the second fusion protein, EGFP-SFVCterm–nsP2, a similar region of SFV nsP2 was fused to the C-terminus of EGFP (SFV4 sequence from N400 to C798). When these constructs were individually transfected into cells, we observed that the C-terminal half of VEE nsP2 distributed to the cytoplasm and diffusely throughout the nucleus, whereas the C-terminal half of SFV nsP2 was nearly entirely contained within the nucleus and only diffusely present in the cytoplasm, similar to what has previously been
reported for SFV nsP2 (Figure 3.5A; (49)). To determine how the PGK MV motif in VEE and the PRRRV motif in SFV contributed to the subcellular distribution of the proteins, we constructed two substitution constructs. First, to obtain a VEE construct with the PGK MV motif modified to PRRRV, a substitution in which RRR replaced GKM was made in the VEE C-terminus (EGFP-VEE CtermRRR–nsP2). Second, the reciprocal SFV construct with the PRRRV motif modified to PGK MV was made by substituting GKM for RRR in the SFV C-terminus (EGFP-SFV CtermGKM-nsP2). Interestingly, the substitution of the SFV NLS in place of the endogenous VEE sequence led to a more homogeneous distribution of the fusion protein throughout the cytoplasm and the nucleus (Figure 3.5B) than for the homologous VEE construct (Figure 3.5A), but it did not cause a predominantly nuclear localization as observed with the natural SFV nsP2 C-terminus (Figure 3.5A). Substituting the VEE motif PGK MV in place of the SFV motif PRRRV (EGFP-SFV CtermGKM-nsP2) produced a distribution of the fusion protein that was identical to that of the VEE C-terminus (EGFP-VEE Cterm-nsP2; Figure 3.5A); the fusion protein was found throughout the cytoplasm and diffusely throughout the nucleus (Figure 3.5B). Thus, substituting PGK MV into SFV nsP2 led to a distribution that was similar to that of VEE nsP2, whereas substituting PRRRV into VEE nsP2 led to a distribution that, although appearing to increase the amount of nuclear nsP2, was not predominantly nuclear like that observed in SFV.

The Nuclear Import Protein Karyopherin-α 1 Binds nsP2.

Given the ability of nsP2 to accumulate in the nucleus, we asked whether nsP2 was able to interact with karyopherin-α (also known as importin-α), a part of the nuclear import machinery that is responsible for recognizing cargo bearing a nuclear localization signal.
Plasmids expressing amino-terminal FLAG-tagged versions of each of four individual karyopherin homologues (karyopherin-α1, -α2, -α3, or -α4) were co-transfected with plasmids expressing each of the nsP2 thirds. At 24 h post-transfection, cell lysates were prepared and immunoprecipitations were performed using anti-FLAG monoclonal antibody and subsequently analyzed by Western blot for EGFP. EGFP-531-794nsP2, which displays an entirely nuclear distribution, co-immunoprecipitated exclusively with karyopherin-α1 (Figure 3.6A).

In order to examine the interaction between nsP2 and karyopherin-α1 in living cells, we employed a YFP fragment complement assay (also called “split YFP” or “bimolecular fluorescence complementation assay” [BiFC]) (38). This approach is based on the reconstitution of a functional fluorescent complex when the two halves of YFP, each fused to a different protein, are brought into close proximity by the interaction of those proteins. Thus, the technique allows for the visualization of protein-protein interactions within a living cell. We constructed fusion proteins such that the C-terminal portion of YFP was fused to the N-terminal end of karyopherin-α1 (KPNA1-YFP2) and the N-terminal portion of YFP was fused to the C-terminus of either 531-794nsP2 or full length nsP2 (YFP1-531-794nsP2 or YFP1-nsP2, respectively). Complementary fusion plasmids were co-transfected into cells, and fluorescence was examined 36 hours later by confocal microscopy. Yellow fluorescence was observed in the nucleus for 531-794nsP2 and throughout both the cytoplasm and the nucleus for full length nsp2 (Figure 3.6B). No fluorescent signal was observed when KPNA1-YFP2 was co-expressed with the leucine zipper-containing control YFP1-Zipper or when either YFP1-531-794nsP2 or YFP1-nsP2 was co-expressed with Zipper-YFP2. This direct visualization demonstrates that karyopherin-α1 interacts with nsP2 in living cells.
Moreover, the 531-794 fragment of nsP2 interacted with karyopherin-α 1 exclusively in the nucleus, whereas the interaction of full length nsP2 with karyopherin-α 1 was detected throughout the nucleus and cytoplasm.

**VEE nsP2 Contains a CRM1-Dependent Nuclear Export Signal**

The results described above demonstrate that different regions of nsP2 vary in their subcellular localization when expressed separately, suggesting that VEE nsP2 contains multiple signals directing the localization of the protein during infection. While mapping the C-terminal nuclear localization signal, we observed that simply shifting the EGFP-531-794nsP2 toward the N-terminus by 25 residues to form EGFP-506-769nsP2 caused a complete loss of nuclear localization (Figure 3.7A). That is, addition of the endogenous 25 residues just upstream of residue 531 coupled with the deletion of twenty-five C-terminal residues yielded a protein predominantly localized to the cytoplasm rather than the nucleus. This suggested that there was either a nuclear localization signal in the deleted 25 residues at the C-terminus or that a nuclear export signal was present in the N-terminal addition.

To test for the presence of an NLS in the C-terminal 25 residues, we deleted the C-terminal 25 amino acids from the original construct to form EGFP-531-769nsP2. Removal of the C-terminal 25 residues did not alter the ability of the protein to localize to the nucleus, demonstrating that there was not a required NLS at the extreme C-terminus (Figure 3.7B). To determine the possible presence of an NES in the upstream 25 residues, a protein was constructed such that the natural 25 residues just upstream of the original N-terminus of the C-terminal third of nsP2 were added to form EGFP-506-794nsP2. The 25 residue addition rendered the fusion protein completely cytoplasmic, thus indicating that the 25 residues 506
to 531 contained an NES, as it directed an otherwise nuclear protein fragment to the cytoplasm (Figure 3.7B). To more carefully map the region within the 25 aa addition that conferred cytoplasmic localization, the residues within that region were sequentially added to the original nsP2 sequence in 5aa increments. The addition of at least 10aa was required to render the protein entirely cytoplasmic (Figure 3.7C).

The canonical NES is composed of a short leucine-rich region, but other hydrophobic amino acids, such as valine, can be substituted for leucine residues (25, 40). Therefore, alanine residues were substituted for the valine residue and each of the leucine residues (V521A, L526A, and L528A) that were contained within the 10aa segment 521-531. Examination of these mutants revealed that L526 and L528 were required for function of the NES, while V521 was not necessary.

The export of a variety of proteins that contain a leucine-rich NES is mediated by the recognition of the NES by the transport protein CRM1 (also called Exportin1) (40). Leptomycin B (LMB) is a small molecule that is able to directly bind CRM1 and consequently block its ability to export proteins (24). To determine if nsP2 export was mediated by CRM1, cells were treated with LMB at 24 h post-transfection with EGFP-521-794nsP2, and the localization of the fluorescent protein was examined by confocal microscopy. Cytoplasmic localization of EGFP-521-794nsP2 was sensitive to LMB, as LMB treatment resulted in the protein being entirely confined to the nucleus (Figure 3.8). Hence, we have identified an NES within nsP2 that functions via the CRM1 transport pathway.
Although the alphavirus protein nsP2 already has multiple critical functions ascribed to it, some of its functions remain elusive. We sought to determine the subcellular localization of the New World alphavirus VEE nsP2 during infection as a first step in elucidating some of these functions. Similar to reports of nsP2 from Old World alphaviruses (1, 19, 34, 41, 49, 50), VEE nsP2 localizes to both the cytoplasm and the nucleus of mammalian cells. Interestingly, VEE nsP2 contains signals that direct both its entry into and its exit from the nucleus. We found that VEE nsP2 associates with karyopherinα-1, a cellular transport protein capable of translocating VEE nsP2 into the nucleus. Additionally, we found that VEE residues L526 and L528 are necessary for the function of an NES. Blocking CRM1 mediated nuclear export prevented the export of a fragment of nsP2 containing the NES, suggesting that nsP2 export relies on CRM1. Taken together, these data suggest that during the course of infection, the localization of nsP2 is dynamic, meaning that nsP2 cycles in and out of the nucleus.

To elucidate signals that governed the subcellular localization of nsP2, we fused sequential thirds of nsP2 to the C-terminus of EGFP. The distribution of each fusion protein fragment was dramatically different, which suggested that the intact nsP2 contained multiple signals directing its nuclear transport. We have been unable to identify any such transport signal in the N-terminal third of nsP2, which led to a cytoplasmic and nuclear distribution of the fusion protein indistinguishable from the distribution of EGFP alone. This region was further investigated by fusing smaller fragments of the N-terminal third to a double EGFP (to prevent simple diffusion of the protein into the nucleus), but none of the fragments were able
to alter the expected distribution of EGFP suggesting that the N-terminal third of nsP2 does not contain an NLS or NES. Studies with SFV nsP2 found that deletion of 85 residues contained in the N-terminal third precluded SFV nsP2 from localizing to the nucleus; however, this region was not sufficient for localization of SFV nsP2, as a point mutation in the C-terminus renders the protein cytoplasmic. Thus, the N-terminus of nsP2 does not seem to be required for nuclear localization of nsP2, although many proteins that localize to both the cytoplasm and the nucleus contain redundant signals directing their subcellular localization, which in turn complicates their identification.

The C-terminal third of VEE nsP2 was able to completely confine EGFP to the nucleus, demonstrating that this region contained an NLS. This VEE nsP2 fragment contains the region homologous to the defined NLS in SFV nsP2. The SFV nsP2 is considered to be P648RRRV, a monopartite NLS which contains three positively charged arginine residues and in which mutation of the central arginine residue renders the protein cytoplasmic (49). Whereas other alphaviruses conserve the positive charge of at least two residues in the canonical NLS, VEE has only a single positively charged residue, the central lysine in the VEE sequence PGKMV. Mutation of the positively charged lysine to a negatively charged aspartic acid diminished, but did not obliterate, the ability of nsP2 to localize to the nucleus, suggesting that the sequence contributes to the nuclear localization of nsP2 but does not account for it entirely. Although the NLS in the alphavirus nsP2 has some features of a classic NLS, the environment in which it is contained appears to be critical to its function, as fusing the SFV NLS to cytoplasmic proteins fails to deliver them to the nucleus (49).

We directly compared the NLS-containing region of SFV and VEE by fusing the C-terminal half of either nsP2 to EGFP. Interestingly, the C-terminal half of SFV nsP2
completely localized EGFP to the nucleus, whereas the construct using VEE nsP2 was found only diffusely in the nucleus. This difference could have resulted from SFV having a stronger NLS with three positively charged residues, in contrast to VEE, having only a single positively charged residue. We substituted the SFV NLS in place of the VEE sequence to determine if we could more strongly promote the nuclear localization of VEE nsP2. There did appear to be a slight increase in the amount of nuclear nsP2 when the SFV NLS was substituted into VEE nsP2, but it was not entirely nuclear as it was for wildtype SFV nsP2. Since the C-terminal half of VEE nsP2 contains an NES which may or may not be functional in SFV, it is possible the VEE fusion protein was also being exported from the nucleus. However, substituting the VEE sequence in place of the SFV NLS led to a phenotype identical to that of wildtype VEE nsP2.

Fusing the middle third of nsP2 to EGFP rendered the protein completely cytoplasmic. This nsP2 fragment contained residues L526 and L528, which we identified as residues critical for the function of an NES. The addition of the ten amino acid sequence VRFFGL$^{526}$DL$^{528}$DS to the completely nuclear localizing EGFP-531-794nsP2 rendered the protein cytoplasmic, and we identified the leucine residues at VEE nsP2 positions 526 and 528 as necessary for function of the NES. The VEE nsP2 NES appears to be potent, as it was able to completely localize an otherwise entirely nuclear protein fragment to the cytoplasm. An examination of the analogous sequence in other alphaviruses reveals that L528 is well-conserved but L526 is not; however, at position 526 of other alphaviruses, a hydrophobic residue is conserved (M, F, or V), which is tolerated within NESs (Figure 3.9) (25, 40). At VEE nsP2 position 532 there is a leucine residue that is conserved amongst the alphaviruses and likely functions as part of the NES. However, L532 was not included in the EGFP-266-
530nsP2, an entirely cytoplasmic protein fragment, suggesting that L532 is not required for NES function, or that there is NES redundancy in that portion of nsP2.

We found that nsP2 interacts in vivo with karyopherin-α 1, a transport protein that recognizes and directly binds to an NLS. Karyopherin-α concurrently binds importin-β1 which allows the complex to traverse the nuclear membrane. Once inside the nucleus, the NLS-bearing cargo protein is released. Moreover, we found that the function of the VEE nsP2 NES is blocked by LMB, an inhibitor of the export transport protein CRM1. Analogous to nuclear import, CRM1 recognizes and directly binds a leucine-rich NES, and transports cargo across the nuclear membrane where it is released into the cytoplasm. Taken together, our data suggest a model in which VEE nsP2 cycles between the nucleus and cytoplasm, being transported into the nucleus via karyopherin-α 1, released, and later exported out of the nucleus via CRM1, where it is released again into the cytoplasm.

The biological significance of the nuclear population of nsP2 remains unclear. However, the presence of nsP2 in the nucleus and the cytoplasm as a result of active nuclear transport suggests that its subcellular localization is vital. Though enucleation studies with SIN have suggested that the nucleus is not essential for the alphavirus life cycle (11), the K646D mutation in VEE nsP2 not only disrupted the nuclear localization of nsP2, but it also dramatically diminished the VRP titer. In addition, the neurovirulence of SFV is compromised in a mutant in which nsP2 nuclear localization is inhibited (13). These observations are compatible if disruption of nuclear function, either by enucleation or an activity of nsP2, is necessary for efficient alphavirus replication.

Evidence of the export of nsP2 from the nucleus suggests that the transport of nsP2 into the nucleus is not simply a mechanism to sequester a subpopulation of nsP2, as has been
postulated for certain positive strand plant viruses, such as tobacco etch virus (28, 61).
Rather, the activity of the NES suggests that it is the cycling of nsP2 between nucleus and cytoplasm that is a key element of nsP2 function.

Hypothetically, the cycling of nsP2 through the nucleus may act at several levels, which are not mutually exclusive, to influence viral replication and host cell response. First, aside from being an essential component of the replication complex in the cytoplasm, nsP2 may also have a specific nuclear function. For example, nuclear nsP2 could impede a normal host activity that occurs in the nucleus, such as blocking the function of transcription factors or interfering with ribosomal assembly (35). Second, the bidirectional nuclear transport of nsP2 may establish an optimal distribution of nsP2 in both the cytoplasm and the nucleus for it to efficiently function in both compartments. While nuclear nsP2 may affect the function of the host cell, the export of nsP2 from the nucleus presumably delivers it back to the compartment in which it functions in the replication complex. Third, the transport of nsP2 into and out of the nucleus could impede the routine movement of essential cellular components through the nuclear pore. For example, the transport of the Ebola virus protein VP24 to the nucleus by karyopherin-α 1 precludes STAT1, which also utilizes karyopherin-α 1, from translocating to the nucleus, which in turn likely blocks the host interferon response (45). Alternatively, because all of the karyopherin-α transport proteins must bind an importin-β1 protein to transport cargo to the nucleus, it is possible that the use of karyopherin-α 1 by nsP2 could clog the import pathway and affect the nuclear transport of proteins that rely on other karyopherin-α proteins. Likewise, nsP2 may occupy sufficient CRM1 to inhibit normal nuclear export. Indeed, the demonstration of the active transport of
nsP2 into and out of the mammalian cell nucleus will stimulate further studies examining the function of nsP2 nuclear cycling in the virus life cycle and its affect on the host cell.
3.6 ACKNOWLEDGMENTS

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FIGURE 3.1

A

Brightfield

nsP2

B

Brightfield

nsP2

C

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FIGURE 3.1: Cytoplasmic and nuclear distribution of nsP2 during infection. (A) During VEE infection, nsP2 is present in both the cytoplasm and nucleus. BHK-21 cells were infected with VEE virus and examined by immunofluorescence staining using α-nsP2 antibody (red) at 8 hpi. Confocal images were acquired using a 40X objective lens. (B) Similar to VEE infection, nsP2 localizes to both the nucleus and the cytoplasm during VRP infection. HEK-293 cells were infected with VRP and the localization of nsP2 was examined at 8 hpi by immunofluorescence staining using α-nsP2 antibody (red). Confocal images were acquired using a 63X objective lens. (C) Confirmation of the intracellular distribution of nsP2 using subcellular fractions. At 8 hpi, VRP-infected BHK cells were fractionated into cytoplasmic and nuclear lysates and examined by immunoblot for nsP2 (upper panel). The cytoplasmic marker GRP-78 (lower panel) is used to confirm purity of the nuclear fraction.
FIGURE 3.2
FIGURE 3.2: Cytoplasmic and nuclear distribution of nsP2 when expressed alone.

Vero (upper panel) or HEK-293 (lower panel) cells were transfected with pCI-neo-nsP2, a mammalian expression vector expressing VEE nsP2. Cells were stained for nsP2 at 24 h post-transfection. DAPI (blue) or histone H1 (red) staining is used to visualize the nucleus of cells. Fluorescent images were obtained using a 40X objective (upper panel) and confocal images (lower panel) were obtained using a 63X objective.
FIGURE 3.3
FIGURE 3.3: Construction and subcellular distribution of EGFP-nsP2 fusion proteins that span nsP2. (A) Illustration of the EGFP-nsP2 fusion constructs, EGFP-1-264nsP2, EGFP-265-529nsP2, and EGFP-530-794nsP2. Dotted lines represent a deleted region of nsP2. There is a 5 aa residue linker between EGFP and any fused portion of nsP2. (B) Distribution of EGFP-nsP2 fusion proteins, as well as EGFP expressed alone, in HEK-293 cells by fluorescence confocal microscopy.
FIGURE 3.4
FIGURE 3.4: Examination of an NLS within the C-terminus of VEE nsP2. (A) The K646D mutation disrupts the putative NLS sequence PGKMV. (B) When the K646D mutation is present in EGFP-530-794nsP2, the protein is no longer entirely contained within the nucleus, as it is also localized in the cytoplasm, as shown by confocal microscopy. (C) When cells infected with a VRP containing the K646D mutation in nsP2 are examined by indirect immunofluorescence staining for nsP2, there is a diminished amount of nuclear nsP2, as shown by confocal microscopy.
FIGURE 3.5
FIGURE 3.5: Direct comparison of the nuclear localization of VEE and SFV nsP2. (A) As illustrated, the C-terminal halves of either VEE nsP2 or SFV nsP2 were fused to the C-terminus of EGFP and used to compare the nuclear localization of VEE and SFV nsP2. The C-terminus of SFV nsP2 resulted in more intense green fluorescence in the nucleus than that of VEE nsP2. (B) Substitutions were made in the NLS region of each of the SFV and VEE fusion proteins such that each protein contained the NLS of the other. Substitution of the VEE NLS in the C-terminal half of SFV nsP2 reduced its ability to localize to the nucleus while substitution of the SFV NLS in the C-terminal half of VEE nsP2 did not alter its localization pattern, as shown by confocal microscopy.
FIGURE 3.6

A

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B

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FIGURE 3.6: VEE nsP2 interacts with karyopherin-α 1. (A) As indicated, 293 cells were co-transfected with two plasmids: one expressing a portion of nsP2 fused to EGFP, and a second expressing one of the FLAG-tagged karyopherin-αs (KPNA) 1, 2, 3, or 4. After 24 h, lysates were made and protein complexes were immunoprecipitated with anti-FLAG antibody. Immunoprecipitated samples (IP) were subsequently examined by immunoblot for EGFP (upper panel). The nuclear localizing portion of nsP2 exclusively co-immunoprecipitated with karyopherin-α 1. Expression of all constructs was confirmed in whole cell lysates (WC) by immunoblot for FLAG (middle panel) and EGFP (lower panel). (B) The interaction between nsP2 and karyopherin-α 1 (KPNA1) was directly investigated in living cells using a YFP complementation reporter approach. 531-794nsP2 or full-length nsP2 was fused to the N-terminus of YFP and karyopherin-α 1 was fused to the C-terminus of YFP. HEK-293 cells were co-transfected with either of the nsP2-YFP1 fusion constructs and KPNA1-YFP2 and fluorescence was examined 36 hours later by confocal microscopy. While the C-terminal third interacts with karyopherin-α 1 exclusively in the nucleus, full length nsP2 interacts with karyopherin-α 1 throughout the cytoplasm and the nucleus. There is no yellow fluorescence detected when either nsP2-YFP1 fusion construct was co-transfected with YFP1-Zipper.
FIGURE 3.7
FIGURE 3.7: Identification of a nuclear export signal in nsP2. (A) Confocal microscopy images show that shifting the C-terminal third by 25aa towards the N-terminus resulted in an entirely cytoplasmic distribution of the fusion protein. (B) To test for the presence of an NLS in the C-terminal 25aa, a fusion protein was constructed that had the original N-terminal end of the C-terminal third of nsP2 but had a 25aa deletion at the C-terminus. To test for the presence of an NES in the N-terminal 25aa addition, an EGFP fusion protein was constructed in which the endogenous upstream 25aa of nsP2 were added to the original C-terminal third of nsP2. The C-terminal 25aa deletion did not alter the ability of the fusion protein to localize entirely to the nucleus whereas the addition of the upstream 25aa rendered the fusion protein completely cytoplasmic. (C) To map the domain required for cytoplasmic localization, the 25 residues present in the N-terminal addition were added in 5aa increments. The residues in nsP2 position 521-794, a 10aa addition to the original EGFP-531-769nsP2 construct, were required to confer cytoplasmic localization. (D) The functionally critical residues of the NES were identified by mutating the indicated residues to alanine residues. Mutation of L528 and L528 disrupted function of the NES.
FIGURE 3.8
FIGURE 3.8: Function of the nsP2 NES is dependent on the CRM1 pathway. The sensitivity of the identified NES to LMB was examined. 24 h after transfection with EGFP-520-794nsP2, cultures were treated with 20 ng/mL of LMB. At 4 h post treatment, cells were fixed, stained for histone H1, and examined by confocal microscopy. The nuclear accumulation illustrates that the nuclear export of the EGFP-520-794nsP2 was sensitive to LMB treatment.
FIGURE 3.9

VBB: VRFFGDLDDLGL
AUR: TKMFMDLSSLGL
BFV: TKMYGFDLLTGL
CHK: TRMYGVDLDSL
EEB: TRFFGVLDLSGL
MAY: TKIYGVDLDSL
ONN: TRIYGFDLVTGL
RRV: TKYYGVDLDSL
SFV: TRFFGVDLDSL
SIN: IKFFGVDLDSL
WEB: TRFFGVDLDSL
FIGURE 3.9: Sequence alignment of NES region from representative alphaviruses. A hydrophobic residue at VEE position 526 is conserved, and the leucine residue at VEE position 528 is well-conserved across alphaviruses. Sequences shown are: VEE (Venezuelan equine encephalitis virus (7)), AUR (Aura virus), BFV (Barmah Forest virus(29)), CHK (Chikungunya virus (56)), EEE (Eastern equine encephalitis virus (64)), MAY (Mayaro virus NCBI sequence accession NP 740688), ONN (O’nyong-nyong virus (26)), RRV (Ross River virus (12)), SFV (Semliki Forest virus (53)), SIN (Sindbis virus (36)), and WEE (Western equine encephalitis virus (62)).
3.7 REFERENCES


CHAPTER FOUR

SUMMARY AND FUTURE DIRECTIONS
In recent years, the interplay of alphaviruses with components of the host cell has begun to be examined. However, many critical details of the interaction of alphaviruses, especially of those concerning the important alphavirus pathogen VEE, with host cells, remain poorly understood. Four particularly important questions are: 1) what host proteins are recruited and utilized by the virus during infection, 2) how do viral nonstructural proteins function outside of their direct role in the replication complex, 3) how does the virus promote the synthesis of its own proteins while inhibiting the synthesis of host proteins, and 4) how do viral proteins co-opt host cellular pathways. The data presented in this body of work begin to address these questions, while also defining critical future required experiments. These topics are considered in detail below.

4.1 HOST PROTEINS UTILIZED BY ALPHAVIRUSES

Alphaviruses, like all viruses, are obligate intracellular parasites, meaning that once they are within the host cell they rely on cellular components for their replication. Often, the host factors that a virus employs for its own needs are also essential components of the host cell. Thus, when a virus uses host proteins for viral functions, there are consequences to normal functions of the host cell.

It has long been postulated that specific host factors participate in the replication of alphaviruses (1, 2, 16, 19, 32). However, few such proteins have been identified to date. The capsid protein transiently associates with ribosomes in an infected host cell, and this interaction with the host is believed to promote uncoating of the virus (34, 38). Recently, nsP3 was found to interact with several cellular proteins, including G3BP2, 14-3-3 proteins,
cytoskeleton proteins, chaperones, elongation factor 1A, heterogeneous nuclear ribonucleoproteins, and some of the ribosomal proteins (3, 9). All of these host proteins, which are crucial to the host, could also be required for successful alphavirus infection.

Aside from protein-protein interactions, alphavirus RNAs also are predicted to specifically interact with host cellular proteins and be crucial to the viral life cycle. 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 (18, 23-26).

In the work presented in Chapter 2, ribosomal protein S6 (RpS6), the major phosphoprotein of the ribosome (10, 17, 28), was demonstrated to specifically interact with nsP2 from both New and Old World alphaviruses (22). Although nsP1 could also be detected in association with nsP2 and RpS6, none of the nonstructural proteins were required for nsP2 to interact with RpS6. These results are noteworthy, as viral replicase proteins have seldom been described as directly associated with components of the ribosome. Furthermore, RpS6 phosphorylation was dramatically diminished within hours after alphavirus infection, suggesting that alphaviruses are able to alter the status of the ribosome during infection (22). Interestingly, since this work was originally published, SIN nsP3 was found in association with other components of the ribosome (9). Thus, the interaction of alphavirus nonstructural proteins with components of the ribosome is becoming an emerging theme.

Further characterization of these and other virus-host interactions will lead to a better understanding of how alphaviruses co-opt cellular factors for their own use during replication. Mapping the interaction domains of RpS6 and nsP2 will provide a better understanding of the interaction, as this will reveal what domain of each protein is crucial for the interaction, and potentially it will allow mutations to be made to disrupt the interaction. Since other
alphavirus nonstructural proteins appear to interact with other ribosomal proteins, it is possible that alphaviruses target multiple components of the translation machinery in order to affect multiple aspects of ribosome function or as a redundancy strategy. Aside from continuing to identify host proteins with which the alphavirus nsPs interact, it will be interesting to determine what host proteins are targeted by nsP precursors and complexes containing combinations of the individual nsPs. Indeed, understanding the interaction of alphavirus nonstructural proteins with the host at the molecular level will advance the understanding of the pathogenesis of alphaviruses.

4.2 ALPHAVIRUS NONSTRUCTURAL PROTEINS FUNCTIONING OUTSIDE OF THE REPLICATION COMPLEX

Over years of alphavirus research, it has become evident that nsP2 carries out critical functions during infection that are indirectly associated with its functions in the replication complex. While other alphavirus proteins may similarly possess auxiliary functions, currently there is less evidence for these proteins having such functions, and accordingly this discussion will focus on the auxiliary functions of nsP2.

Numerous studies have identified nsP2 as a critical determinant of the outcome of infection (4, 7, 27, 36). That is, while alphaviruses characteristically cause lytic infection in mammalian host cells, specific mutations to nsP2 confer the ability of alphaviruses to establish persistent, non-cytopathic infections. Interestingly, recent work suggested a difference in the auxiliary function of nsP2 between Old World and New World alphaviruses, as the mature nsP2 of the Old World alphaviruses SIN and SFV, but not that of VEE, cause
transcription shutoff of the host (11, 12). More recently, the proteinase activity of nsP2 is implicated in cleavage of host proteins associated with an antiviral response (33). Collectively, such observations indicate that nsP2 possesses functions in addition to its direct role in the replication complex.

Even though the alphavirus replication cycle does not require the mammalian cell nucleus (5), nsP2 was identified as the only alphavirus nonstructural protein that is present in both the cytoplasm and the nucleus of infected cells (2, 30, 31). The work presented in Chapter 3 extends the concept of nuclear localization of nsP2 in mammalian cells to the New World alphavirus VEE. This localization pattern was not dependent on the presence of other viral proteins, as when nsP2 is expressed in the absence of other viral proteins, its distribution is similar to that found during infection. Not only does nsP2 localize to the nucleus, but it also accumulates in the nucleolus, the site of ribosome assembly (30; Montgomery and Johnston, unpublished data). Though the role of nuclear nsP2 remains unclear, in SFV the disruption of nsP2 nuclear localization diminished the ability of the virus to inhibit DNA synthesis and also compromised its ability to spread in the brain of an infected mouse (6). Thus, the ability of nsP2 to localize to the nucleus contributes to the pathogenesis of alphaviruses.

Although nsP2 localizes to the nucleus of mammalian cells, it does not localize to the nucleus of mosquito cells that display low CPE upon alphavirus infection, while it does localize to the nucleus of mosquito cells which display high CPE (20, 21). Thus, the correlation of alphavirus CPE and the nuclear localization of nsP2 may be the result of a function of nsP2 that contributes to alphavirus pathogenesis. Interestingly, whereas the
mammalian cell nucleus is not required for efficient alphavirus infection, the mosquito cell nucleus is required (5).

While it is clear that nsP2 possesses additional activities that are important in the outcome of infection and are outside of or indirectly associated with its function in the replication complex, the mechanism(s) by which nsP2 performs such functions remains undefined. The fact that nsP2 is a multifunctional protein poses a challenge to defining such auxiliary functions, as a single mutation to nsP2 may disrupt multiple functions. Thus, to ideally define auxiliary functions of nsP2, targets will be identified that allow for disruption of the nsP2 auxiliary function without disrupting its essential function in the replication complex. For instance, to determine the function of nuclear nsP2, the nuclear localization of nsP2 could be blocked by inhibiting a factor that delivers nsP2 it to the nucleus. While it remains a difficult task to define auxiliary functions of nsP2, elucidation of these functions will greatly advance the field of alphavirus pathogenesis.

4.3 VIRAL PROTEIN SYNTHESIS IN THE WAKE OF INHIBITION OF HOST PROTEIN SYNTHESIS

Not only do alphaviruses initiate translation of their message-sense RNA genome upon its release into a host cell, but they also rapidly inhibit host protein synthesis in the mammalian host cell (37). While the mechanism of alphavirus translation shutoff in mammalian cells is poorly understood, it is independent of both alphavirus host cell transcription shutoff and the PKR-induced shut off of protein synthesis that is a generalized innate cellular response to infection (13, 14).
It is possible that nsP2 plays a role in both inhibiting host protein synthesis and also in promoting the translation of viral proteins. Not only are the structural proteins dispensable for host protein synthesis inhibition ((8); Appendix A.1; Appendix A.2), but in nsP2 mutants that allow a persistent infection to be established in mammalian cells, host translation is not inhibited (4, 7, 27, 36). These data suggest that nsP2 is the viral protein responsible for translation shutoff. The work presented in Chapter 2 suggests that nsP2 might also play a role in promoting viral translation. First, throughout the course of infection, nsP2 specifically associated with a ribosomal protein that is associated with translation initiation (15, 22). Moreover, when RpS6 levels were diminished, though it did not affect the number of cells that became infected, it did diminish the synthesis of virally-encoded protein by greater than 10-fold (22). However, cellular translation was only mildly affected by diminished levels of RpS6. Taken together, this suggests that expression of alphavirus genes depends on RpS6. Thus, RpS6 may serve as a factor to promote the selective expression of alphavirus messages, and this may function together with host shutoff mechanisms to allow alphaviruses to efficiently translate viral proteins in the host cell.

While there are clues to how alphaviruses are able to promote translation of viral proteins while simultaneously inhibiting host protein synthesis, the mechanisms by which these processes occur still need to be fully elucidated. Further analysis of the ribosome and factors which regulate its function is needed in the context of alphavirus infection. Additional characterization of nsP2 mutants which are unable to inhibit host translation, as well as the identification of nsP2 mutants unable to associate with RpS6, will aid in defining the role of nsP2 in mediating host shutoff and promoting viral protein synthesis. Such analysis of the interaction of nsP2 with ribosome and host factors should also be paralleled in
the mosquito cell, which naturally becomes persistently infected by alphaviruses. The
conundrum of synthesizing viral proteins while inhibiting host translation is a fundamental
issue of viral infection, and until the mechanism by which this occurs is defined, an
understanding of alphavirus pathogenesis will remain incomplete.

4.4 ALPHAVIRUS PROTEINS AND HOST CELLULAR PATHWAYS

Since alphaviruses usurp host factors to utilize them for viral functions, alphavirus
infection consequently disrupts the normal function of host cellular pathways. Sometimes
viruses target such pathways to obstruct the host response, whereas at other times the
disruption of such pathways is the indirect effect of changes that are induced by the infection.

The studies presented in Chapter 2 demonstrated that RpS6, the major phosphoprotein
of the ribosome, rapidly becomes dephosphorylated after alphavirus infection. Similarly,
S6K1 (also called p90SK), the primary kinase responsible for phosphorylation of RpS6
becomes rapidly dephosphorylated after alphavirus infection (Appendix A.4; P. Berglund,
personal communication). Currently, it is unclear whether these dephosphorylation events
are the result of the interaction of nsP2 with RpS6 or whether it is an indication that
alphaviruses disrupt the function of a molecule upstream of RpS6. Nonetheless, the pathway
leading to the phosphorylation of RpS6 is required for cell proliferation, and its disruption
during alphavirus infection undoubtedly affects normal cell function (35).

The studies presented in Chapter 3 demonstrated that nsP2 is imported into the
nucleus via karyopherin-α 1 and exported from the nucleus by the CRM1-dependent
pathway. Not only does this suggest a model in which nsP2 is cycled in and out of the
nucleus, but the simple act of nsP2 cycling through the nucleus may impede the normal transport of essential cellular components through the nuclear pore in both directions. For instance, the Ebola virus protein VP24 similarly is transported to the nucleus by karyopherin-α 1 (29). The use of karyopherin-α 1 by VP24 consequently precludes STAT1 translocation to the nucleus, which also utilizes karyopherin-α 1 (29). While this likely serves as a means for Ebola to block the host interferon response, it also demonstrates that a viral protein’s nuclear import impedes the routine transport of host proteins. Preliminary data suggests that STAT1 nuclear translocation is also inhibited during VRP infection, perhaps because the use of karyopherin-α 1 by nsP2 excludes the ability of STAT1 to be imported in to the nucleus (Montgomery, S.A. Simmons, J., Morrison, T.E., Heise, M.T., and Johnston, R.E., unpublished data). While further characterization of this observation is needed, it suggests that the nuclear import of VEE nsP2 disrupts the normal function of nuclear transport in the cell.

Moreover, all of the karyopherin-α transport proteins must bind an importin-β1 protein to transport cargo to the nucleus. Thus, it is possible that the use of karyopherin-α 1 by nsP2 could obstruct the import pathway and affect the nuclear transport of other cellular proteins that utilize other karyopherin-α transport proteins. Likewise, the use of CRM1 by nsP2 may inhibit routine nuclear export in an alphavirus infected cell. Further studies are needed to examine the effect that alphavirus infection has on nuclear transport.

While alphavirus infection likely affects the operation of numerous pathways that are critical for normal cellular function, the effect of alphaviruses on such functions remains largely uncharacterized. As more pathways are examined, more will be revealed on how alphavirus pathogenesis is mediated at the cellular level. Moreover, comparison of the effect
of alphaviruses on such host pathways in mammalian and mosquito cells will be essential for the elucidation of the basic differences responsible for the dramatically different outcomes of infection in these two hosts.

4.5 CONCLUDING REMARKS

While there remains a lack of fundamental knowledge concerning the interaction of VEE with the eukaryotic cell, the work presented here begins to address critical ways in which VEE nsP2 interacts with host factors in the mammalian cell. Clearly, nsP2 is not simply an essential component of the viral replication machinery, but it also mediates crucial interactions with the host. While study of nsP2 mutants in other alphaviruses had revealed that nsP2 functions in determining the outcome of infection, it is now apparent that nsP2 also mediates crucial aspects of infection in the host by interacting with components of the ribosome and shuttling into and out of the nucleus. Analogous studies focusing on the other nonstructural proteins will likely reveal that other, if not all, nonstructural proteins directly interact with components of the host cell in ways that are independent of their roles in the replication complex. While many of the alphaviruses likely interact similarly with cellular components, it is possible that some of the differences in pathogenesis between alphaviruses will be evident at the cellular level. Likewise, it will be interesting to determine how alphavirus-host cell interactions differ amongst various critical cell types targeted by alphaviruses during infection, such as dendritic cells, neurons, and mosquito cells. Indeed, further elucidation of alphavirus-host cell interactions will build a much needed molecular knowledge base that will advance the study of alphavirus pathogenesis both by providing
insight into the virus as well as the interactions of viral and host components that determine susceptibility to infection and disease.
4.6 REFERENCES


33. **Suthar, M., Cruz, C., Simmons, J., Morrison, T.E., and M.T. Heise.** 2007. Antagonism of RIG-I mediated type I IFN induction by the nonstructural proteins of the mouse virulent Sindbis virus, AR86. University of North Carolina - Chapel Hill.


APPENDIX A

ADDITIONAL DATA CHARACTERIZING VRP INFECTION IN THE HOST CELL
A.1 VRP INHIBIT PROTEIN SYNTHESIS IN MAMMALIAN, BUT NOT MOSQUITO, CELLS

Introduction

Many viruses interfere with the synthesis and/or stability of cellular proteins, DNA, and RNA. Though advantageous for the virus, it hinders normal host cell function and accordingly is termed “shutoff”. Host shutoff, coupled with the accumulation of viral products, allows the virus to rapidly take control of cellular machinery and impede cellular biosynthetic pathways. Furthermore, through host shutoff, the virus is able to preempt cellular antiviral defense mechanisms by compromising the synthesis of the necessary machinery to respond to the virus.

An advantage of inhibiting translation, as opposed to earlier steps in the synthesis of a gene product, is that by targeting the final step of the biosynthetic pathway, a virus can rapidly block further production of host proteins, consequently altering total cellular amounts of proteins. RNA viruses encode their own machinery to synthesize viral RNA but rely on host machinery for protein synthesis. Furthermore, for RNA viruses with a message-sense genome (such as alphaviruses), translation is the first step in replication once the genome enters the cell. Thus, the ability to efficiently recruit the cellular ribosome to translate viral, rather than host, proteins is crucial for efficient viral replication. Alphaviruses rapidly inhibit host protein synthesis in the mammalian host cell, but persistently infect mosquito host cells without affecting host translation.

The mechanism by which alphaviruses inhibit translation in mammalian cells is poorly understood. Several mechanisms by which alphaviruses might inhibit host protein synthesis have been proposed, including: a reduction in intracellular ribonucleoside
triphosphate pools as a result of viral RNA synthesis (15); changes in the ionic environment of the cell that favor the translation of viral RNAs over that of host mRNAs (1, 15); and competition between viral and host messages for host factors, such as the ribosome (9, 15). Interestingly, alphavirus infection leads to a termination in the appearance of newly synthesized ribosomes in the cytoplasm of infected cells; specifically, the synthesis of the ribosomal precursor RNA has been found to be preferentially inhibited (15). In SFV, the capsid protein interferes with host mRNAs binding the 80S ribosomal initiation complex (14), and electroporation of high amounts of capsid protein alone into cells inhibits host cell protein synthesis (2). However, the observation that alphavirus capsid protein is responsible for host protein shutoff is disputed by findings that in the SIN replicon system, in which the viral structural proteins are not synthesized, is inhibited (3).

Although the mechanism by which alphaviruses inhibit host translation is not understood, it is presumed to be the result of the virus inhibiting new protein synthesis, as opposed to solely being the result of viral inhibition of transcription or a host antiviral response. Studies in SIN have revealed that the virus independently inhibits both transcription and translation (7). Alphaviruses at least partially inhibit transcription through the downregulation of cellular RNA polymerase I- and II-dependent transcription (5, 6). Moreover, studies with SIN also demonstrated that the alphavirus inhibition of host translation is independent of the PKR-induced shutoff of protein synthesis that is a generalized innate cellular response to infection (8).

In this study, we sought to determine the effect that VRP infection had on host translation in mammalian and mosquito cells, which would also determine if the replication
of the VEE nonstructural proteins was sufficient to cause translation shutoff in mammalian cells.

Materials and Methods

C6/36 cells were incubated at 30°C under 5% CO₂ and maintained in minimal essential medium with Earle’s salts and L-glutamine (Gibco) supplemented with 5% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, 100 U of penicillin per mL and 0.5 mg of streptomycin per mL. Cell culture conditions for BHK-21 cells were previously described (12); however, for the purposes of this experiment, BHK-21 cells were incubated at 30°C so that the kinetics of shutoff could be directly compared in C6/36 and BHK-21 cells.

One day prior to infection, 4 x 10⁵ BHK-21 cells or 4 x 10⁶ C6/36 cells were seeded in a 6 well plate. Monolayers were either mock-infected or infected with 5005-3000 (GFP-VRP) at an moi of 20. Synthesis of VRP has been previously described (12). At time points after infection, the cells were starved of methionine and cysteine by incubation in 1 mL methionine-free/cysteine-free media for 1 h at 37°C. The depleted media was removed, and new protein synthesis was monitored by the addition of starvation media supplemented with 33μCi/mL [³⁵S]-methionine/[³⁵S]-cysteine. After 1 h, each well was washed once with 3 mL of PBS. At the indicated time points, cells were lysed by incubating them at room temperature for 5 minutes in 250 μL of NP-40 lysis buffer. Lysis was monitored under the microscope, and nuclei left adhered to the plastic were seen as the cytoplasmic contents were lysed and released. The lysates were scraped, collected in 1.5mL tubes, and centrifuged for 1
minute at maximum speed in the cold, and the supernatant was saved. From this lysate, 10 µL was separated on a 12% SDS-PAGE gel.

**Results and Discussion**

Within several hours of infection with VRP, host protein synthesis in mammalian, but not mosquito cells, was inhibited (Figure A.1). In further experiments, we found that the kinetics of host shutoff is similar in Vero cells (originating from green African monkey), at the higher moi of 50, and does not depend on the identity of the heterologous gene expressed from the VRP (data not shown).

Thus, the expression of the four nonstructural proteins is sufficient to shut off host protein synthesis in mammalian cells, similar to what has been described for SIN replicons (4). These findings are in contrast to the observation noted with the Old World alphavirus SFV in which capsid alone appeared responsible for inhibition of host protein synthesis (2, 14), as the VRP genome lacks all of the structural protein genes. However, the VRP used to infect the cells contained structural proteins in the particles themselves which could conceivably contribute to the inhibition of protein synthesis in the host cell. This possibility is unlikely, however, as temperature-sensitive SIN mutants, that do not replicate viral RNA, do not shutoff host cell translation (reviewed in (15)). However, interpretations of translation shutoff across alphaviruses may be complicated by recent observations that suggesting that nsP2 is responsible for transcription shutoff in Old World alphaviruses, while capsid is responsible in New World alphaviruses, such as VEE (6).

As expected, VRP infection does not inhibit host protein synthesis in C6/36 mosquito cells. Interestingly, translation of the virally-encoded GFP is suppressed at 26 hpi. Though
alphaviruses are able to establish a persistent infection in mosquito cells, viral replication is blocked by the mosquito cell (10, 11, 13), and thus it is likely that the disappearance of the synthesis of GFP at 26 hpi is the result of the mosquito host inhibiting viral replication.
References


FIGURE A.1

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FIGURE A1: VRP inhibit protein synthesis in mammalian, but not mosquito, cells.

The effect of VRP infection on protein synthesis was examined in mammalian and mosquito cells, BHK-21 and C6/36 cell monolayers were infected at an moi of 20 or mock-infected. At time points post-infection, monolayers were incubated in methionine-free starve medium for 1 h and subsequently incubated with [35S]-methionine/[35S]-cysteine radiolabel medium for 1 h. Immediately after incubation with radiolabel medium, cells were harvested and lysed. Total radiolabeled proteins were examined after SDS-PAGE separation. In mammalian cells (A), host protein synthesis was inhibited nearly completely within 8 h of VRP infection. The synthesis of viral nonstructural proteins was apparent by 2 h and continued throughout the course of infection. In mosquito cells (B), total host protein synthesis was not altered by VRP infection. Although the synthesis of GFP, which was expressed from the viral subgenomic 26S message, was apparent within hours of infection, it was downregulated by 26 hpi.
A.2 INCREASED SYNTHESIS OF THE NONSTRUCTURAL PROTEINS IN THE ABSENCE OF THE STRUCTURAL PROTEINS

Introduction

Though VRP are able to inhibit mammalian host cell protein synthesis within hours of infection (Figure A.1), the kinetics of host shutoff induced by VRP have not been compared to that of VEE virus. It is possible that the production of the structural genes in the full-length VEE virus alters the kinetics of host protein shutoff. Accordingly, translation shutoff induced by VRP and VEE infection was directly compared in a protein synthesis time course.

Materials and Methods

BHK-21 cells were seeded at $1 \times 10^5$ in a 24 well plate 24 h prior to infection. Cells were either mock-infected, or infected with 5005-3000 (GFP-VRP) or V3000 at an moi of 20. Production of VRP and VEE virus has been previously described (2). Time course of protein synthesis was carried out as described in Appendix A.1.

Results and Discussion

Examination of protein synthesis after BHK-21 cells were infected with either GFP-VRP or V3000 virus demonstrated that the rate and extent of translation shutoff was similar between the two (Figure A.2). Although there may be slight variation amongst samples, no consistent differences was seen at any time point across experiments. This observation supports a similar study conducted using SIN, the prototype alphavirus, in which the absence
of the synthesis the structural proteins did not affect the rate of host shutoff in mammalian cells (1). Taken together, these results suggest that during infection, the synthesis of the alphavirus nonstructural proteins is sufficient to induce host shutoff.

During VRP infection, the viral nonstructural proteins were synthesized at a higher level and for a longer duration as compared to VEE virus infection (Figure A.2). Whereas the synthesis of the nonstructural proteins is difficult to visualize by autoradiography during VEE virus infection, they can be easily visualized in VRP infection. Conversely, during SIN replicon infection, the synthesis of the nonstructural proteins is diminished within several hours of infection (1). One explanation for the observation that there is a continued synthesis of the nonstructural proteins during VRP infection but not in VEE infection is that there is an accumulation of replicon genome during VRP infection, because it is not being packaged into progeny particles like it is during a virus infection. Consequently, the accumulation of template allows for a more robust, prolonged synthesis of the nonstructural proteins. Alternatively, it is possible that the presence of the structural proteins actively suppresses the expression of the nonstructural proteins. Regardless, the elevated synthesis of the nonstructural proteins does not alter the kinetics of host shutoff.
References


FIGURE A.2: Increased synthesis of the nonstructural proteins in the absence of the structural proteins. The kinetics of translation shutoff induced by VRP and VEE virus were compared in BHK-21 cells. Monolayers were mock-infected (M) or infected at an moi of 20 with GFP-VRP (VRP) or V3000 (VEE). At time points post-infection, monolayers were incubated in methionine-free starve medium for 1 h, followed by incubation with $[^{35}\text{S}]$-methionine/$[^{35}\text{S}]$-cysteine radiolabel medium for 1 h, and then immediately harvested and lysed (time point is indicated). Total protein synthesis was examined by 8% SDS-PAGE separation. The sizes of the nonstructural proteins are indicated, and specific protein bands to which the arrowheads refer are marked by either closed or open circles (see VRP lane at 6 hpi). In the lane containing the VEE-infected samples at 10 hpi, two plus signs mark the E2 and E1 structural proteins while a single plus sign below marks capsid. The rate of host protein shutoff was similar between VRP infection and VEE. The synthesis of nonstructural proteins was more robust and persists longer during VRP infection that in VEE infection (compare protein band below solid dot across the time course).
A.3 OVER-EXPRESSION OF RPS6 IN INFECTED CELLS ALTERS NEITHER VIRAL PROTEIN SYNTHESIS NOR HOST SHUTOFF

Introduction

Throughout the course of infection, nsP2 is associated with the critical host factor ribosomal protein S6 (RpS6) (1). Moreover, nsP2 is found in association with the whole ribosome (1, 2), suggesting that nsP2 could associate with RpS6 in the context of an active translation complex. This finding is noteworthy, as few viral replicase proteins have been described as being directly associated with components of the ribosome. Not only is nsP2 associated with the ribosome, but VEE infection apparently alters the state of ribosomal proteins, as RpS6 becomes dephosphorylated after VRP infection (1). Interestingly, alphavirus infection leads to a loss in the appearance of newly synthesized ribosomes in the cytoplasm of infected cells (3). Moreover, similar to other alphaviruses, VEE rapidly shuts off host protein synthesis in mammalian cells (Appendix A.1 and A.2). Thus, nsP2 interacts with RpS6, an essential component of the protein synthesis machinery, VEE alters ribosomes of infected cells, and VEE infection affects cellular protein synthesis. Taken together, this suggests that the interaction of nsP2 with RpS6 could function to inhibit host protein synthesis and/or promote the synthesis of viral proteins. In order to investigate the possibility that the complex containing RpS6 and nsP2 alters protein synthesis in an infected cell, the effect of over-expression of RpS6 on host and viral protein was investigated.

Materials and Methods
Protocols describing immunoprecipitation and immunoblot methods, as well as the construction of a VRP expressing human RpS6 (RpS6-VRP) have been previously described (1). For examination of protein-synthesis, \(1.7 \times 10^5\) HEK-293 cells were seeded in 24 well plates one day prior to infection. Cells were either mock-infected or infected with RpS6-VRP or 5005-3000 (GFP-VRP) at an moi of 10. A translation time course post-infection was performed as described in Appendix A.1.

Results and Discussion

A replicon genome was engineered that expressed human RpS6 as the heterologous gene (RpS6-VRP). Thus, when RpS6-VRP was used to infect human cells, all cells containing nsP2 would also express excess RpS6. To confirm that RpS6 was over-expressed in cells infected with RpS6-VRP, a Western blot for total RpS6 was performed on lysates from mock-infected, RpS6-VRP-infected, or GFP-VRP-infected cells at 8 hpi (Figure A.3A). For each of the three samples (mock, RpS6-VRP, and GFP-VRP), an equal number of cells were lysed in an equal volume of NP-40 lysis buffer, and a dilution series of each sample allowed for analysis of the total amount of RpS6 by Western blot. RpS6 was more abundant in RpS6-VRP infected lysates than in either mock or GFP-VRP-infected lysates. While there is no difference in the amount of actin in these samples, there is less RpS6 in GFP-VRP infected lysates than in mock lysates, suggesting that VRP infection results in a diminished amount of total RpS6 in the cell. Furthermore, the RpS6 in GFP-VRP lysates migrates as a smaller protein than in either mock or VRP-RpS6. Because RpS6 is a phosphoprotein that migrates as a larger protein when phosphorylated, it is possible that this difference is due to the lack of phosphorylated RpS6 during GFP-VRP infection. While it has been reported that
RpS6 is not phosphorylated during alphavirus infection (1), this suggests that over-expression of RpS6 during infection could overcome the dephosphorylation of RpS6.

Next, to determine whether more complexes containing RpS6 and nsP2 were formed when RpS6 was over-expressed (as compared to cells infected with GFP-VRP), an immunoprecipitation for nsP2 was performed from equal volumes of infected lysates, and the entire immunoprecipitate sample was analyzed by Western blot for RpS6 (Figure A.3B). Indeed, more RpS6 co-immunoprecipitated with nsP2 when RpS6 was over-expressed (compare second and third lanes, GFP-VRP and RpS6-VRP, in left panel of Figure A.3B). A Western blot was performed against nsP2 on the samples immunoprecipitated for nsP2 in order to determine the relative amount of nsP2 that was immunoprecipitated from each lysate (right panel in Figure A.3B). Although more RpS6 co-immunoprecipitated in RpS6-VRP lysates than in GFP-VRP lysates, the amount of nsP2 was consistent in each sample, suggesting that RpS6 could be a limiting factor in the formation of the complex containing RpS6 and nsP2.

When RpS6-VRP and GFP-VRP are used to infect cells, no difference was observed between the rate and degree of translation shutoff induced by either VRP (Figure A.3C). Although it appeared that over-expression of RpS6 might lead to a faster inhibition of protein synthesis at the 8 h time point, when individual protein bands corresponding to cellular proteins were quantified, there were no significant differences (examine the shutoff of the host protein denoted by arrow; quantification not shown). Similarly, analysis of the protein bands corresponding to the nonstructural proteins revealed that over-expression of RpS6 during infection had no effect on the synthesis of the viral nonstructural proteins (protein bands corresponding to nsPs marked by arrowheads). Although over-expression of RpS6
increased the amount of nsP2-RpS6-containing complexes formed, it did not alter host shutoff or translation of the viral nonstructural proteins. Thus, whereas the diminution of RpS6 during infection inhibits expression of viral proteins more than that of host proteins (1), the simple over-expression of RpS6 in infected cells did not promote viral-induced host shutoff or expression of viral proteins.
References


FIGURE A.3

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IB: RpS6

IB: actin

B

IP: nsP2

M  GFP  RpS6

IB: RpS6

IP: nsP2

M  GFP  RpS6

IB: nsP2

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KDa

250
160
105
75
50
35
25
15
10

nspPs

RP56

GFP breakdown product

181
FIGURE A.3: Over-expression of RpS6 in infected cells alters neither viral protein synthesis nor host shutoff. (A) Upper panel, RpS6 is over-expressed in cells infected with RpS6-VRP. Lower panel, examination of actin as a loading control. (B) Left panel, more RpS6 co-immunoprecipitates with nsP2 from cells infected with RpS6-VRP than with GFP-VRP. M, mock-infected; RpS6, RpS6-VRP; GFP, GFP-VRP. Right panel, examination of the amount of nsP2 present in immunoprecipitated samples and whole cell lysates of mock-infected, GFP-VRP infected, and RpS6-infected cells. The samples immunoprecipitated for nsP2 (shown in the right and left panels) are the same samples. (C) Examination of protein synthesis after infection with RpS6-VRP. Samples are all shown in triplicate. The kinetics of host shutoff (exemplified by host protein denoted by full arrow) and synthesis of viral nonstructural proteins (marked by arrowheads) after infection with RpS6-VRP do not differ from that induced by infection with GFP-VRP. Similarity among corresponding time points between the two samples was confirmed by quantifying the marked protein bands by ImageQuant™.
A.4 DIMINISHED PHOSPHORYLATION OF S6 KINASE DURING ALPHAVIRUS INFECTION

Introduction

Although alphavirus infection leads to the rapid dephosphorylation of RpS6 (1), the phosphorylation status of S6K1 (also called p90<sup>SK</sup>), the primary kinase responsible for phosphorylation of RpS6, remains unknown. S6K1 is a mitogen-activated kinase that is required for cell growth and contains four phosphorylation sites (2). Of these sites, phosphorylation of Thr-389 is the most critical for S6K1 kinase function and is the best predictor of S6K1 kinase activity in vitro (2, 3). In this study, we sought to examine the steady state level of S6K1 and the phosphorylation status of S6K1 at Thr-389 was examined after VRP infection.

Materials and Methods

HEK-293 cells were cultured and infected, lysates were prepared, and immunoblots were performed as previously described (1). Briefly, monolayers were infected with GFP-VRP at an moi of 10 in a minimal volume of phosphate-buffered saline (PBS) supplemented with 1% DCS and Ca<sup>+</sup>/Mg<sup>+</sup>. VRP were allowed to adsorb for 1 h at 37°C before the addition of complete growth medium. At the indicated time points after addition of growth medium, cells were lysed in NP-40 lysis buffer. Protein preparations were separated by 10% SDS-PAGE, transferred to Immun-blot PVDF (Bio-Rad), and the membrane was blocked for 1 h at room temperature in 5% dry milk. Primary antibodies (total S6K1 and p-S6K1-Thr 389, Cell Signalling) were diluted according to manufacturer’s recommendations in 5% bovine
serum albumin (BSA) in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl and 0.1% Tween-20), and membranes were probed overnight at 4°C. Subsequently, membranes were probed with appropriate HRP-conjugated secondary antibodies (anti-rabbit-HRP [GE Life Sciences],) for 1 h at room temperature. Blots were washed three times in TBST followed by detection of HRP-conjugated secondary antibody by chemiluminescence using ECL detection reagents (Amersham Pharmacia).

**Results and Discussion**

To examine the status of S6K1 after VRP infection, monolayers of HEK-293 cells were infected with GFP-VRP at an moi of 10. After infection, lysates were prepared over a time course and subjected to Western blot analysis for total S6K1 and S6K1 phosphorylated at Thr-389 (p-S6K1 [Thr-389]). Over this time course after VRP infection, the total amount of S6K1 protein was not altered; however, the amount of S6K1 phosphorylated at Thr-389 was dramatically diminished as compared to mock (Figure A.4). Note that there are two isoforms of S6K1, a smaller cytoplasmic form (p70) that utilizes RpS6 in ribosomes as it substrate, as well as a larger nuclear isoform (p85) that is believed to phosphorylate RpS6 during ribosome biogenesis in the nucleolus. Also, note that S6K1 was dephosphorylated in both mock- and VRP-infected samples at the 0 h timepoint. S6K1 became dephosphorylated during the VRP adsorption period, an incubation in which a low serum diluent was employed. Whereas S6K1 is rapidly phosphorylated in mock lysates once complete medium was replaced, S6K1 never became phosphorylated after VRP infection. Although it is possible that VRP infection only prevented S6K1 from becoming re-phosphorylated, when high serum media was used during SFV infection, S6K1 became rapidly dephosphorylated (P.
Berglund, unpublished data), suggesting that VRP infection may be capable of S6K1 dephosphorylation in addition to or instead of simply preventing its phosphorylation once it is dephosphorylated. Taken together, these data suggest that alphavirus infection causes the dephosphorylation of S6K1.

Although S6K1 and its substrate, RpS6, both are rapidly dephosphorylated after VRP infection, it remains undefined whether the dephosphorylation of S6K1 is responsible for RpS6 dephosphorylation. While the lack of phosphorylated S6K1 after infection could lead to the dephosphorylation of RpS6, it is possible that VEE targets multiple steps of this pathway and that the resulting dephosphorylation of the two proteins are independent. Additionally, although S6K1 is the predominant kinase responsible for RpS6 phosphorylation, RpS6 can be phosphorylated by other kinases, and consequently RpS6 may be dephosphorylated after infection because a different kinase is targeted during VEE infection. Finally, although alphavirus infection alters the ribosome and nsP2 interacts with RpS6 (1), it remains unknown whether diminished RpS6 and S6K1 phosphorylation are involved in this interaction.
References


FIGURE A.4

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IB: Total S6K1

IB: Phospho-S6K1 (Thr 389)
FIGURE A.4: Diminished phosphorylation of S6 kinase during alphavirus infection.

HEK-293 monolayers were infected with GFP-VRP at an moi of 10. After 1 h VRP adsorption period, complete medium was added to cells (t=0), and lysates were prepared at the indicated time points. Equal volumes of lysates were analyzed by Western blot for total S6K1 and phospho-S6K1(Thr 389). The cytoplasmic isoform of S6K1(70 KDa) is indicated by a large arrowhead, and the nuclear isoform of S6K1 (85 KDa) is indicated by a small arrowhead. While there is no dramatic change in the total amount of S6K1 after VRP infection, there is a lack of phosphorylated S6K1.
A.5 CYTOPLASMIC LOCALIZATION OF VEE NSP2 IN MOSQUITO CELLS

Introduction

While alphavirus infection in mammals can lead to a wide range of disease symptoms, alphaviruses persistently infect mosquitoes without shortening their lifespan. Some of the fundamental differences of alphavirus pathogenesis in the mammalian and mosquito hosts can be modeled in cell culture. Alphavirus infection of mammalian cells causes a highly cytopathic infection and ultimately leads to cell death. In mosquito cells, alphaviruses cause less cytopathogenicity, albeit that varies somewhat depending on cell line (5, 6, 8), and ultimately a persistent infection is established. The virus-host interactions leading to the dramatically different biological outcomes of mammalian and mosquito cell infection are poorly understood.

Although alphaviruses naturally establish persistent infections only in mosquito cells, it is possible to establish persistent alphavirus infections in mammalian cells by mutating nsP2. Two independent approaches found that mutation of the proline residue at position 726 in nsP2 in SIN renders the virus able to establish a persistent infection in BHK-21 cells (2, 4, 12). More recently, studies with both SIN and SFV have revealed that the precise location of these amino acid changes is not critical, as mutation to the amino terminus or a small region of the carboxy terminus of nsP2 can establish a persistent infection (10, 11).

The subcellular localization of nsP2 is an additional difference between alphavirus infection in mammalian and mosquito cells. Whereas SFV and SIN nsP2 is found in the nucleus during viral infection of mammalian cells (1, 9), SIN nsP2 is exclusively cytoplasmic in *Aedes albopictus* C6/36 mosquito cells (7). The identification of several mutations in nsP2
that confer persistence in mammalian cells, as well as the nuclear localization of nsP2 in mammalian but not mosquito cells suggests that there are undefined roles and interactions of nsP2 that affect host cell survival. Accordingly, we sought to determine the localization of VEE nsP2 in *Aedes albopictus* C6/36 mosquito cells.

*Materials and Methods*

C6/36 cells were cultured as described in Appendix A.1. At 8 hpi with GFP-VRP, cells were fixed in 2% PFA for 35 minutes at room temperature, washed three times with PBS, and permeabilized. Cells were permeabilized in acetone at -20°C for 20 minutes, rehydrated in three PBS washes, and blocked for 1 h in 7.5% bovine serum albumin (BSA) in PBS. After excess blocking solution was removed in three PBS washes, monolayers were stained with rabbit anti-SIN nsP2 antibody (gift of C. M. Rice) at a 1:200 dilution in 0.1% BSA in PBS for 1h. After PBS washes, cells were stained in 0.1% BSA in PBS for 1h with the appropriate anti-IgG secondary antibody directly conjugated to Alexa Fluor 594® (Invitrogen) diluted at 1:400. After a final series of PBS washes, cells were mounted under a glass coverslip using a polyvinyl alcohol/glycerol mounting medium. For DAPI (4’,6’-diamidino-2-phenylindole) staining, coverslips were mounted using Vectashield containing DAPI (Vector Labs). Cells were examined by confocal or deconvolution microscopy.

*Results and Discussion*

Indirect immunofluorescence staining for VEE nsP2 of VRP-infected C6/36 mosquito cells revealed that VEE nsP2 is predominantly cytoplasmic in mosquito cells. This observation is in contrast with indirect immunofluorescence staining of mammalian cells.
(BHK-21, Vero, and HEK-293) infected with VRP, which showed that nsP2 localizes to both the cytoplasm and the nucleus of mammalian cells. These data are consistent with the nsP2 localization of SIN nsP2 in mosquito and mammalian cells (7).

The difference in subcellular localization of nsP2 in mammalian and mosquito cells is intriguing, as there are numerous poorly understood differences between the two hosts. SIN nsP2 does not target the nucleus of Aedes albopictus C6/36 mosquito cells, although it does target the nucleus of A. albopictus C7-10 mosquito cells, which display cytopathology that resembles that of vertebrate cells (8). Thus, in two hosts in which alphaviruses are highly cytopathic, mammalian cells and C7-10 cells, nsP2 localizes to the nucleus. Interestingly, the alphavirus life cycle does not require the mammalian nucleus but does require the mosquito nucleus (3). Since nsP2 has undefined functions in determining the outcome of infection and there are differences in its subcellular localization in mammalian and mosquito cells, understanding the role of nuclear nsP2 may reveal how alphaviruses establish either a persistent or cytopathic infection in a particular cell type.
References


FIGURE A.5: Cytoplasmic localization of VEE nsP2 in mosquito cells. The subcellular localization of VEE nsP2 during VRP infection was examined by indirect immunofluorescence staining in C6/36 mosquito cells at 8 hpi. Confocal images were obtained using a 40X objective (upper panel) and deconvolution images were obtained using a 100X magnification (lower panel). GFP fluorescence shown in green, nsP2 stained in red, and DAPI staining shown in blue. In contrast to the localization of VEE nsP2 in mammalian cells, nsP2 predominantly localizes to the cytoplasm of mosquito cells.
A.6 OVER-EXPRESSION OF NSP2 IS MORE TOXIC TO MOSQUITO CELLS THAN OVER-EXPRESSION OF NUCLEAR LOCALIZING NSP2

Introduction

Since VEE nsP2 localizes to both the cytoplasm and nucleus of mammalian cells, but only the cytoplasm of mosquito cells, it is possible that the differential compartmentalization of nsP2 in these cell types contributes to differences in infection, such as cytopathic effects (CPE). Hence, perhaps directing a subpopulation of nsP2 to the nucleus of Aedes albopictus mosquito cells would alter the outcome of infection. In this study, the effect of directing a subpopulation of nsP2 to the nucleus of mosquito cells on CPE was examined.

Materials and Methods

Using PCR amplification of nsP2 from pVR21, a second copy of nsP2 was inserted into pVR21 under the 26S subgenomic promoter between the Apa I and Pac I sites. Then, to insert a strong, exogenous NLS (DPKKKRKV) in triplicate repeat to the C-terminus of nsP2, the DNA sequence was synthesized by BioBasic, Inc. and subcloned in-frame at the 3’ end of the nsP2 expressed from the subgenomic promoter using standard molecular biology techniques. Once the identity of each was confirmed by DNA sequencing, VRP were packaged in glycoprotein 3000 coat as previously described (nsP2-VRP or nsP2NLS-VRP, respectively) (7). Titers of nsP2-VRP and nsP2NLS-VRP preparations were determined on BHK-21 cells by indirect immunofluorescence staining using sera against the nsPs. BHK-21 (hamster) or C6/36 (mosquito) cells were cultured as previously described ((7) and Appendix A.1, respectively) and infected with GFP-VRP, nsP2-VRP, or nsP2NLS-VRP at an moi of 10.
Cells were examined at 10 and 24 hpi. Images were obtained with a Nikon Eclipse TS100 inverted microscope a 20X objective and 10X eyepiece.

Results and Discussion

In order to force nsP2 to localize to the nucleus of mosquito cells, an exogenous NLS (DPKKKRRKV in triplicate repeat), which functions in both BHK-21 and Aedes albopictus C6/36 cells (data not shown), was fused to the C-terminus of nsP2. A VRP was constructed that expresses a second copy of nsP2 from the 26S promoter with the NLS (nsP2NLS) fused to it. Since expressing nsP2NLS from the 26S subgenomic promoter would result in an excess amount of nsP2, as a control a VRP was constructed that expresses a second copy of nsP2 but lacks the NLS (Figure A.6A).

In order to examine the effect of excess nsP2 or nsP2NLS on CPE, BHK-21 and C6/36 cells were mock-infected or infected with GFP-VRP, nsP2-VRP, or nsP2NLS-VRP at an moi of 10. It is important to note that all of the VRP preparations used were titered using BHK-21 cells, and there is variability amongst titers of VRP as assayed on BHK-21 and C6/36 cells. Specifically, VRP titers are approximately one log lower on C6/36 cells than on BHK-21 cells; thus, while BHK-21 cells were infected at an moi of 10, C6/36 cells were more likely infected at an moi of 1. Cell monolayers were examined at 10 and 24 hpi by light microscopy. While no difference was apparent in any of the BHK monolayers (Figure A.6B), differences in CPE were present in the C6/36 cell monolayers. In particular, nsP2-VRP is highly cytopathic in C6/36 cells whereas nsP2NLS-VRP causes moderate CPE (Figure A.6C). Both nsP2-VRP and nsP2NLS-VRP caused more CPE in mosquito cells than
GFP-VRP, which does not over-express any form of nsP2. The differences observed in CPE were apparent and consistent at both 10 hpi and 24 hpi.

Recently, it has been demonstrated that SIN nsP2 is cytotoxic to mammalian cells, and that effect is at least partially determined by the ability of SIN nsP2 to inhibit transcription in mammalian cells (3). However, it was also found that whereas SIN nsP2 and nsP2 from other Old World alphaviruses cause host transcription shutoff, in New World alphaviruses, such as VEE, it is capsid, rather than nsP2, that is responsible for host transcription shutoff (4). The studies presented here support the finding that nsP2 from VEE, a New World alphavirus, is not cytotoxic to mammalian cells. Conversely, in this study the New World alphavirus nsP2 is cytotoxic to mosquito cells, as the over-expression of VEE nsP2 caused marked CPE in C6/36 cells. Interestingly, expression of a version of nsP2 that localized to the nucleus of mosquito cells caused less CPE than over-expression of unaltered, cytoplasmic nsP2. However, the over-expression of nsP2NLS caused increased CPE as compared to the amount of nsP2 present in a normal VRP infection. Taken together, these data suggest that cytoplasmic VEE nsP2 is more toxic than nuclear nsP2 in mosquito cells. Unfortunately, the role of nuclear nsP2 in mammalian cells (2, 8), as well as the requirement of the mosquito nucleus for productive alphavirus infection, remain unclear (1). To further characterize the CPE caused by nsP2-VRP and nsP2NLS-VRP, additional Aedes albopictus cell lines which display varying degrees of CPE after alphavirus infection should be employed (5, 6).
References


FIGURE A.6

A

B

C

mock

GFP-VRP

nsP2-VRP

nsP2NLS-VRP

mock

GFP-VRP

nsP2-VRP

nsP2NLS-VRP
FIGURE A.6: Over-expression nsP2 is more toxic to mosquito cells than over-expression of nuclear localizing nsP2. (A) Design of nsP2-VRP and nsP2NLS-VRP. The subgenomic 26S promoter (indicated by arrow) was used to express a second copy of nsP2 (nsP2-VRP), or nsP2 with a C-terminal fusion of the NLS DPKKKRKV in triplicate. Not drawn to scale. (B) At 24 hpi, neither nsP2-VRP nor nsP2NLS-VRP altered CPE in BHK-21 cells as compared to GFP-VRP. (C) At 24 hpi, nsP2-VRP caused vast CPE in C6/36 cells, and nsP2NLS-VRP caused more CPE than GFP-VRP.
APPENDIX B

DETAILED PROTOCOLS
MEDIA FOR CELL CULTURE

**BHK-21** (*Mesocricetus auratus*; fibroblast)

- Alpha minimal essential medium (Gibco)
- 10% DCS
- 10% tryptose phosphate broth
- 0.29 mg/mL L-glutamine
- 100 U/mL penicillin
- 0.5 mg/mL streptomycin

**BHK-21 (electroporation)** (*Mesocricetus auratus*; fibroblast)

- Alpha minimal essential medium (Gibco)
- 10% FBS
- 10% tryptose phosphate broth
- 0.29 mg/mL L-glutamine
- 100 U/mL penicillin
- 0.5 mg/mL streptomycin

**HEK-293** (*Homo sapiens*; epithelial)

- Dulbecco’s modified Eagle medium (DMEM) (Gibco)
- 10% FBS
- 100 U/mL penicillin
- 0.5 mg/mL streptomycin
**HeLa** *(Homo sapiens; epithelial)*

- Dulbecco’s modified Eagle medium (DMEM) (Gibco)
- 10% FBS
- 100 U/mL penicillin
- 0.5 mg/mL streptomycin

**Vero** *(Cercopithecus aethiops; epithelial)*

- Dulbecco’s modified Eagle medium (DMEM)-F12 (Gibco)
- 10% FBS
- 0.29 mg/mL L-glutamine
- 100 U/mL penicillin
- 0.5 mg/mL streptomycin

**C6/36** *(Aedes albopictus)*

- Minimum essential medium containing Earle’s salts and 2 mM L-glutamine (Gibco)
- 5% FBS
- 0.1 mM non-essential amino acids
- 100 U/mL penicillin
- 0.5 mg/mL streptomycin
CELL LYSIS BUFFERS

NP-40 Lysis Buffer

170 mM NaCl
50 mM Tris
15 mM EDTA
0.2% NP-40

Add Mini Complete protease inhibitor cocktail (Roche) and, if necessary, phosphatase inhibitor cocktail (Sigma) immediately before use.

RIPA Lysis Buffer

50 mM Tris-HCl
150 mM NaCl
0.001 M EDTA
1% Triton X-100
1% Sodium deoxycholate

Add 1% BSA if samples are for immunoprecipitation.

Add Mini Complete protease inhibitor cocktail (Roche) and, if necessary, phosphatase inhibitor cocktail (Sigma) immediately before use.
COOMASSIE STAINING
(FOR SUBMISSION FOR MALDI-TOF/MS SEQUENCING)

Supplies

- Gel fixative (25% isopropanol, 10% acetic acid in Milli-Q water)
- Coomassie stain solution (0.01% R-250 Coomassie (Bio-Rad) in 10% acetic acid)
- Destain solution (10% acetic acid)
- Storage solution (3% acetic acid)
- Anti-Fade mounting solution
- No. 1 ½ glass coverslips (Corning)

Protocol

Day 1:

1. To fix SDS-PAGE gel, soak in gel fixative for 20 minutes (based on 1-mm thick gels; increase incubation equivalently for thicker gels).

2. To fix SDS-PAGE gel, soak in gel fixative for 20 minutes (based on 1-mm thick gels; increase incubation equivalently for thicker gels).

3. To stain gel, soak in Coomassie stain solution overnight on an orbital shaker at 40 rpm.

Day 2:

4. Pour off Coomassie stain solution and replace with destain solution.
5. As destain solution turns blue, replace with fresh destain solution until background of gel becomes destained, being careful not to remove stain from protein bands. When destaining is complete, replace with storage solution.
EGFP FUSION PROTEIN ASSAY: TRANSFECTION INTO CELLS AND MOUNTING FOR MICROSCOPY

Supplies

- HEK-293 cells and growth media
- Lab-Tek II CC²™ 8 well chamber slides (Nunc)
- DNA preparations of EGFP fusion protein plasmids
- OptiMEM I (Gibco)
- FuGENE 6 Transfection Reagent (Roche)
- 2% PFA (Electron Microscopy Sciences)
- PBS
- Anti-Fade mounting solution
- No. 1 ½ glass coverslips (Corning)

Protocol

Day 1:

1. Seed HEK-293 cells at 1-2×10⁴ cells in 0.2 mL of media per well of each chamber slide. Monolayers should be approximately 50% confluent at transfection.

Day 2:

2. Mix 100 μL of OptiMEM I with 6 μL of FuGENE. Allow to incubate for 5 min at RT.

3. Add 1 μg of plasmid DNA to the OptiMEM I/FuGENE mixture. Mix well and incubate for 15 min at RT.
4. Add 7 µL of OptiMEM I/FuGENE/DNA mixture to each well of a slide. Incubate at 37°C for 24-48 h.

5. Fix HEK-293s in 2% PFA overnight at 4°C.

6. If desired, monolayers can be used in indirect immunofluorescence assay (i.e., stain for histone H1). Otherwise, hydrate cells by washing 3 times for 5 min in PBS. Carefully remove plastic gasket and mount glass coverslip using pre-cleared Anti-Fade.
IMMUNOPRECIPITATION

Supplies

- Protein A Sepharose beads (Sigma).
- PBS
- Chilled lysis buffer (either NP-40 or RIPA containing BSA)
- Chilled IP bead wash (50mM Tris-HCl, pH 7.6)
- Primary antibody and isotype-matched normal sera

Protocol

Day 1:

1. Resuspend protein A beads by adding 12 mL PBS to 1 g of dry beads. Store at 4°C.

2. As follows, prepare washed bead using 40µL of sepharose bead slurry per IP sample.

   Batch preparations of beads can be done for multiple samples.

3. In a microfuge tube, add 1 mL of PBS per 1X (40µL) of bead aliquot needed, thoroughly mixing to resuspend beads.

4. Spin beads at no more than 1000 rpm at 4°C for 5-8 min to pellet the slurry.

5. Aspirate off the wash.

6. Repeat PBS wash for a total of 2 PBS washes.

7. Add 1 mL of lysis buffer per 1X (40µL) of bead aliquot (use the same lysis buffer that the lysates are made in).

8. Spin beads at no more than 1000 rpm at 4°C for 5-8 min.


10. Resuspend beads at 1 mL per 1X reaction in lysis buffer.
11. Before the slurry mixture settles out, aliquot 1 mL into microfuge tubes.

12. Allow beads to settle for at least 30 min at room temperature.

13. Spin washed beads in tabletop microcentrifuge at 1000 rpm for 2 min.


15. Add lysate (~150-200 µL) to bead aliquot. *Keep lysates on ice (or 4°C) throughout the protocol!

16. Optional step: If there is concern about having nonspecific binding to the Protein A Sepharose beads, add an incubation of beads alone with lysate. Incubate lysate with 40µL beads per sample with rotation at 4°C for at least 2 hours and then continue on using this “pre-blocked” lysate.

17. To block, add 1µL isotype-matched normal sera to each lysate and bead mixture.

18. Incubate with rotation at 4°C for at least 2 hours (can be overnight if necessary).

19. Spin out beads in tabletop microcentrifuge 1000 rpm for 2 min.

20. Move blocked lysate into fresh tube (with out beads).

21. Add 2 µL of primary antibody to each lysate.

22. Incubate with rotation at 4°C at least 2 hours (generally done overnight).

Day 2:

23. Do a quick spin of lysate and primary antibody mixture and transfer it to a new tube containing a fresh batch of washed beads.

24. Incubate with rotation at 4°C for 2 hours (DO NOT do overnight incubation).

25. Spin out beads in tabletop microcentrifuge 1000 rpm for 2 min, aspirate off liquid.

26. Wash beads 3 times with 1 mL of lysis buffer at 4°C.

27. Wash beads once with 1 mL of IP bead wash (50mM Tris-HCl, pH 7.6).
28. Add 40µL 2x protein loading buffer. (If needed for smaller wells, can reduce volume).

29. Boil beads 3-5 min.

30. Spin out beads for 2 min at top speed. Load onto gel.
INDIRECT IMMUNOFLUORESCENCE STAINING

Supplies

- 8 well glass chamber slides (Nunc): Lab-Tek™ or Lab-Tek II CC²™ (for HEK-293s)
- PBS
- 2% PFA (Electron Microscopy Sciences)
- Blocking solution (7.5% BSA in PBS)
- Acetone pre-chilled at –20°C
- Destain solution: 10% acetic acid
- Wash solution (0.1% BSA in PBS)

Protocol

Day 1:

1. Seed 8 well glass titer slides (BHK and HEK-293 cells at $1 \times 10^4$ cells/well)
2. Chill acetone at -80°C for tomorrow.

Day 2

3. Infect cells with VRP.

Day 3

4. At desired time post infection, fix BHK or Vero cells in 2% PFA for 35 minutes at room temperature; fix HEK-293s in 2% PFA overnight at 4°C.
5. Pull off plastic wells and wash slide 3 times for 5 min in PBS with agitation.
6. Permeabilize cells by placing slides in pre-chilled acetone at -20°C for 20 minutes.
7. Re-hydrate cells by washing 3 times for 5 min in PBS.
8. Block for 1 h in 7.5% BSA at RT.
9. Wash cells with PBS three times for 5 min.

10. Block for 1 h in 7.5% BSA at RT.

11. Wash 3 times for 5 min in PBS 0.1% BSA.

12. Stain with primary antibody 4°C overnight in 0.1% BSA. Use ~100µL per well and carefully lay lid on to prevent evaporation.

13. Wash 3 times for 5 min in PBS 0.1% BSA.

14. Stain with Alexa-Fluor directly conjugated-secondary antibody at room temperature for 1 h and carefully lay lid on to prevent evaporation.

15. Wash 3 times for 5 min in PBS 0.1% BSA.

16. Carefully remove plastic gasket and mount glass coverslip using pre-cleared Anti-Fade (or DAPI containg anti-fading mounting medium).
MEASURING PROTEIN SYNTHESIS BY TCA PROTEIN PRECIPITATION TO EXAMINE $^{35}$S-INCORPORATION

Supplies

- Appropriate cells and media
- 12 well tissue culture plates
- Starvation media (for all cell types use Eagle’s Minimum Essential Medium, cysteine/methionine-free [Sigma])
- Pro-mix $[^{35}S]$-methionine/$[^{35}S]$-cysteine labeling mix (GE Life Sciences)
- NP-40 lysis buffer
- Trypsin
- Chilled 10% TCA
- Glass test tubes
- Glass filters and appropriately sized vacuum
- Chilled Precipitation Buffer (0.01mM Tris-HCl [pH 7.4], 0.2M NaCl, 0.001 M EDTA, 1% SDS)
- Chilled 95% ethanol

Protocol

Day 1:

1. Seed cells in 12 well plate so that monolayer will be subconfluent tomorrow (HeLa, $7 \times 10^4$ cells/well).

Day 2:
2. Infect cells at an moi of 10-20 in 200 µL for 1 hour at 37°C, rotating plate during infection.

3. After 1 hour incubation, add 1 mL of warmed media to each well. This is the beginning of the timecourse (t=0).

4. Two hours before the time point, remove the media and add 300 µL of starvation media (lacking methionine and cysteine but containing DCS at a final concentration of 1% and 1% L-glutamine if needed). Incubate at 37°C for 1 hour.

5. Replace starvation media with pre-made mix of starvation media plus \[^{35}\text{S}^\]-methionine/\[^{35}\text{S}^\]-cysteine at a final concentration of 33µCi/mL (add 1µL to 300 µl starve media). Incubate for 1 hour at 37°C.

6. Before performing TCA precipitation, trypsinize cells and count in order to adjust cpm per number of cells.

7. Lyse sample of cells from each well in 125 µl NP-40 lysis buffer.

8. In a glass test tube, mix 25 µl of lysate with 175 µl of Precipitation Buffer.

9. On ice, add 200 µl of ice cold 10% TCA (final concentration 5% TCA). Incubate 30-60 minutes on ice.

10. Pour mixture onto glass filter mounted to vacuum. (Include two extra filter samples as the first and last filters to check for background and contamination.)

11. Wash filter 3 times by pouring 1 mL of ice cold 10% TCA over filter.

12. Wash filter 3 times by pouring 1 mL of ice cold 95% ethanol over filter.

13. Allow filter to air dry.

14. Use scintillation counter to get cpm counts for each filter.
MEASURING PROTEIN SYNTHESIS BY EXAMINING $^{35}$S-INCORPORATION OVER A TIME COURSE ON AN SDS-PAGE GEL

**Supplies**

- Appropriate cells and media
- 24 well tissue culture plates
- Starvation media (for all cell types use Eagle’s Minimum Essential Medium, cysteine/methionine-free [Sigma])
- Pro-mix $[^{35}\text{S}]-$methionine/$[^{35}\text{S}]-$cysteine labeling mix (GE Life Sciences)
- NP-40 lysis buffer
- Gel fixative (40% methanol, 10% acetic acid)

**Protocol**

*Day 1:*

1. Seed cells in 24 well plate so that monolayer will be nearly confluent tomorrow
   
   (BHK, $1 \times 10^5$ cells/well; C6/36, $1 \times 10^6$ cells/well;).

*Day 2:*

2. Infect cells at an moi of 10-20 in 165 $\mu$L for 1 hour at 37$^\circ$C, rotating plate during infection.

3. After 1 hour incubation, add 1 mL of warmed media to each well. This is the beginning of the timecourse (t=0).
4. Two hours before the time point, remove the media and add 300 µL of starvation media (lacking methionine and cysteine but containing DCS at a final concentration of 1% and 1% L-glutamine if needed). Incubate at 37°C for 1 hour.

5. Replace starvation media with pre-made mix of starvation media plus $[^{35}\text{S}]$-methionine/$[^{35}\text{S}]$-cysteine at a final concentration of 33µCi/mL (add 1µL to 300 µl starve media). Incubate for 1 hour at 37°C.

6. To lyse cells, remove radiolabel-containing media and wash with PBS. Lyse cells with 105 µl NP-40 lysis buffer. Allow lysis buffer to incubate for ~3 minutes. Then, scrape bottom of well with pipette tip. Microfuge lysate for 1 minute at top speed in the cold, move cytoplasmic lysate to a new tube. Freeze at -80°C until load onto gels.

Day 3:

7. Pour 10% SDS-PAGE gels.

8. For each sample, add 14 µL of 2X loading buffer and 14 µL of cell lysate. Load 20µL (10µL of each sample) of boiled samples.

9. After the gel has been run, incubate in gel fixative for 30 min.

10. Lay fixed gel on Whatman filter paper and dry.

11. Expose to film or PhosphorImager screen O/N.
NUCLEAR PURIFICATION

Supplies

- PBS
- Chilled Buffer A (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT) with mini complete proteinase inhibitor cocktail (Roche)
- Chilled Homogenization Medium (0.25 M sucrose, 25 mM KCl, 5 mM MgCl$_2$, 20 mM Tris-HCl [pH 7.8])
- Chilled Diluent (150 mM KCl, 30 mM MgCl$_2$, 120 mM Tris-HCl [pH 7.8])
- 10% NP-40 (diluted in water)
- OptiPrep density gradient medium (Sigma)
- NE Buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 25% glycerol)
- 5 M NaCl

Protocol

Day 1:

1. Harvest approximately 2-5×10$^7$ cells by scraping.
2. Rinse cells in 20 mL PBS and pellet by centrifugation.
3. Save one fifth of cells to make whole cell lysates.
4. **Nuclear purification**: Resuspend cells in 350 µL cold Buffer A freshly spiked with protease inhibitors, DTT, and phosphatase inhibitors, if needed.
5. Incubate on ice for 15 min.
6. Add 40 µL of 10% NP-40 and vortex for 1 min.

7. Pellet crude nuclei by centrifugation at 1,000-1,500 rpm in microcentrifuge for 10 min at 4°C.

8. Save the supernatant as the cytosolic fraction.

9. Rinse nuclei in 300 µL Homogenization Medium.

10. Pellet nuclei by centrifugation at 1,000-1,500 rpm in microcentrifuge for 10 min at 4°C. Discard supernatant.

11. Resuspend crude nuclear pellet in Homogenization Medium, bringing volume up to 400 µL.

12. Dilute in 400 µL OptiPrep Working Solution (5 mL OptiPrep + 1 mL Diluent).

13. Transfer to 2 mL microfuge tube.

14. Underlay with 600 µL 30% OptiPrep (2.4 mL OptiPrep Working Solution + 0.6 mL Homogenization Medium).

15. Use a Pasteur pipette to underlay with 35% OptiPrep (2.4 mL OptiPrep Working Solution + 0.6 mL Homogenization Medium) until tube is full (approximately 400 µL).

16. Spin in swinging bucket microfuge rotor at 7,000 rpm (10,000×g) for 20 min at 4°C.

17. Extract band of nuclei (from interface between 30% and 35% layers) to a fresh 1.5 mL microfuge tube.

18. Mix in 500 µL of Homogenization Medium and pellet at 7,000 rpm (10,000×g) for 10 min at 4°C. Discard supernatant.

19. Without mixing, add 1 pellet volume NE Buffer, freshly spiked with protease inhibitors, DTT, and phosphatase inhibitors, if needed.
20. Without mixing, add 5 M NaCl to adjust final salt concentration to 400 mM. (volume needed = total volume/31.25)

21. Add another pellet volume of NE buffer and vortex to resuspend.

22. Incubate on ice for 10 min, vortexing periodically.

23. Spin nuclear fraction for 10 min at top speed in microcentrifuge.

24. Save supernatant as nuclear fraction.

25. **Whole cell lysates:** Resuspend cells in 350 µL RIPA, freshly spiked with protease inhibitors, DTT, and phosphatase inhibitors, if needed.

26. Nutate for 15 min at 4°C.

27. Clarify by centrifugation for 10 min at top speed in microcentrifuge at 4°C.

28. Save supernatant as whole cell lysates.
POLYSOME PROFILES

Supplies

- One confluent T-175 flask of cells per sample
- Cell scrapers
- 10% RNase-free sucrose (Ambion) in HSB (0.5 M NaCl, 50 mM MgCl$_2$, 10 mM Tris-HCl [pH 7.4])
- 40% RNase-free sucrose (Ambion) in HSB
- 5X HSB (2.5 M NaCl, 250 mM MgCl$_2$, 50 mM Tris-HCl [pH 7.4])
- RSB (10 mM NaCl, 3 mM MgCl$_2$, and 10 mM Tris-Cl, pH 7.4)
- RSB detergent solution (1% deoxyxholic acid and 2% Tween-40 in RSB)
- Vanadyl adenosine ribonucleoside complex
- Cyclohexamide (Sigma; CHX)
- Chilled RNase-free PBS-CHX (0.1 mg/mL CHX in PBS)

Protocol

Day 1:

1. Infect a nearly confluent T-175 with VRP.
2. Pour 10-40% sucrose/HSB continuous gradients. Store at 4°C O/N.

Day 2:

3. At 18 hpi and several minutes prior to harvesting cells, add CHX to a final concentration of 0.1 mg/mL.
4. Use cell scrapers to harvest cells into cold PBS-CHX.
5. Pellet cells, remove supernatant, and resuspend in cold PBS-CHX

6. Pellet cells, remove supernatant, and resuspend in 200 µL RSB containing 20% vanadyl adenosine ribonucleoside complex and 0.1 mg/ML CHX.

7. Incubate on ice for 5 min to allow cells to swell.

8. While vortexing, add 0.2 mL RSB detergent solution.

9. Incubate on ice for 5 min.

10. Vortex briefly and pellet nuclei at 2000 × g for 15 min at 4°C.

11. Transfer cytoplasm to a new tube. While mixing, add 0.1 mL of 5X HSB to adjust to high salt condition.

12. Load 0.5 mL of cytoplasmic extract onto preformed, chilled sucrose gradients.

13. Centrifuge gradients at 37,000 rpm for 1 h 45 min at 4°C.

14. After centrifugation, polysome profiles are analyzed by pumping off the top of the gradient using an AUTO DENSIFLOW (LABCONCO) gradient pump through an EM-1 Econo UV monitor (Bio-Rad). Record absorbance at 254 nm using a Rec-111 (GE Healthcare) recorder. Fractions are collected from each gradient using a Frac-100 (Pharmacia) fraction collector.
siRNA TRANSFECTION, VRP INFECTION, AND HARVESTING CELLS FOR FACS ANALYSIS OF GFP EXPRESSION

Supplies

- 12 well cell culture plates
- HeLa cells and appropriate media
- Lipofectamine 2000 (Invitrogen)
- OptiMEM I (Gibco)
- 20 µM stocks of desired siRNAs (Dharmacon)
- PBS
- Trypsin
- 5 mL polystyrene round bottom tubes (Falcon)
- FACS Buffer (PBS containing 2% FBS)
- 1% FACS Fix Solution (1:10 dilution of EM grade 10% formaldehyde in PBS)

Protocol

Day 1:
1. Seed wells of a 12 well plate with 7 × 10⁴ HeLa cells per well in 2 mL media.

Day 2:
2. For each well, mix 1.2 µL Lipofectamine 2000 and 148.8 µL of OptiMEM I. For each well, mix 5 µL of stock siRNA and 145 µL of OptiMEM I. Allow each mixture to incubate at RT for 15 min.
3. Combine two mixtures (total of 300 µL). Allow mixture to incubate at RT for 15 min.
4. Remove media from monolayer. Add 300 µL of the lipofectamine/OptiMEM I/siRNA mixture. Incubate at 37°C for 2 h.

5. Add 1 mL media to each well. Allow transfection to incubate as long as needed (48 h).

*Day 4:*

6. Infect cells with GFP-VRP at an moi of 1 and/or 5. Replace with complete medium. Allow infection to proceed as long as needed.

*Day 5:*

7. At 18 hpi, aspirate media from wells. Wash with 1-2 mL PBS and aspirate washes off. Add 0.5 mL trypsin and incubate at 37°C for 2-3 min.

8. Add 1 mL media to quench the trypsin and transfer the cells to 5 mL polystyrene round bottom tubes.

9. Rinse the well with FACS Buffer and add to the cells. Fill the tube with 4-5 mL FACS Buffer (total of 4-5 mL) and pellet cells at 4°C.

10. Remove supernatant from cell pellet by aspiration. Resuspend cells in residual volume (approximately 0.1 mL) by finger-flicking the tube. Wash again with 4-5 mL FACS Buffer as above.

11. Remove supernatant and resuspend residual volume by finger-flicking the tube. Add 0.5 mL of 1% FACS Fix Solution. Mix thoroughly by gently vortexing. Store at 4°C until ready for FACS analysis.
WESTERN BLOT DETECTION

Supplies

- 1X TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Tween-20)
- 1X TBS (50 mM Tris-HCl [pH 7.4], 150 mM NaCl)
- Dry milk (Food Lion)
- Antibodies: primary and HRP-conjugated secondary
- ECL or ECL Plus (for quantitative western blot) detection reagents (Amersham)

Protocol

Day 1:

1. Block PVDF membrane in 5% dry milk in TBST (“blocking solution”) at RT for 1.
2. Dilute primary antibody in blocking solution and incubate the membrane in this mixture overnight at 4°C with gentle rotation.

Day 2:

3. Rinse membrane in 1X TBST.
4. Wash membrane three times in 1X TBST for at least 5 min with vigorous agitation.
5. Dilute secondary antibody in blocking solution and incubate the membrane in this mixture for 1 h at RT with gentle rotation.
6. Wash membrane three times in 1X TBST for at least 5 min with vigorous agitation.
7. Soak membrane in 1X TBS for 5 min with gentle agitation.
8. Develop using ECL (ECL Plus for quantitative Western) detection reagent according to manufacturer’s instructions.