The Dissertation Committee for Raghavendran Kulasegaran Shylini certifies that this is the approved version of the following dissertation

STRUCTURE-FUNCTION STUDIES OF THE VENEZUELAN EQUINE ENCEPHALITIS VIRUS 5'UTR PROMOTER ELEMENT AND ITS ROLE IN ATTENUATION OF THE VIRUS

Committee:

David Gorenstein, Ph. D., Supervisor

Ilya Frolov, Ph. D., Co-Supervisor

Vincent Hilser, Ph. D.

Edward Nikonowicz, Ph. D.

Krishna Rajarathnam, Ph. D.

Stanley Watowich, Ph. D.

_____

Dean, Graduate School

STRUCTURE-FUNCTION STUDIES OF THE VENEZUELAN EQUINE ENCEPHALITIS VIRUS 5'UTR PROMOTER ELEMENT AND ITS ROLE IN ATTENUATION OF THE VIRUS

by

Raghavendran Kulasegaran Shylini, B. Tech.

Dissertation

Presented to the Faculty of the Graduate School of
The University of Texas Medical Branch
in Partial Fulfillment of the Requirements
for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch
May 2009

To my father, Kulasegaran, and my mother, Shylini, for being my biggest source of inspiration and for all their love, sacrifices and support

Acknowledgements

My heartfelt gratitude goes to several people who made my years in graduate school here at the University of Texas Medical Branch, a productive and rewarding experience.

I am deeply indebted to Drs. David Gorenstein and Ilya Frolov for serving as my mentors during my graduate study. Thank you for your constant support and encouragement, for believing in my potential and your endearing guidance and advice that helped mold me as a scientist that I am today.

I thank all members of my supervisory committee Drs. Vincent Hilser, Edward Nikonowicz, Krishna Rajarathnam and Stanley Watowich for your guidance and involvement during my training, as well as your constructive criticism.

I especially thank Dr. Edward Nikonowicz for his guidance and for generously providing access to his research facility for my initial NMR related experiments.

My sincerest thanks go to Dr. Lillian Chan, Program Director of the graduate program in Biochemistry and Molecular Biology for her guidance, constant encouragement, and support.

I would like to express my gratitude to all members of the Gorenstein and Frolov laboratories, for their help and support, for providing a pleasant work environment all these years, for your suggestions, discussions and comments. I thank Drs. Dave Volk and Varatharasa Thiviyanathan for being my NMR gurus, for numerous discussions, suggestions and guidance in experiments and data analysis, and for your sage advice.

I thank the McLaughlin Endowment for the Pre-doctoral Fellowship awarded to me in 2007.

I thank all the administrative support staff of BMB and Gorenstein Laboratory, with special thanks to Debora Botting, Lori Blackwell, and Angelina

Johnson for your administrative support and interest in my well being over the years.

I sincerely thank all my friends, fellow colleagues who have provided continuous support, encouragement throughout my graduate school experience.

Finally, I wish to express my heartfelt gratitude to my father, Kulasegaran, who is not with us today, but who would have had the greatest pride in seeing me grow into the individual that I am today. I am deeply indebted and express my heartfelt gratitude to my mother, Shylini, for believing in me all my life and encouraging me to come all this way to pursue a career of my choice. I thank my sister, Gayathrie for her unconditional love, constant support, and encouragement in all my activities to this day. My special thanks to my friend Lakxmi for always being there to encourage me through good and tough times. Thank you!

STRUCTURE-FUNCTION STUDIES OF THE VENEZUELAN EQUINE ENCEPHALITIS VIRUS 5'UTR PROMOTER ELEMENT AND ITS ROLE IN ATTENUATION OF THE VIRUS

Publication No._____

Raghavendran Kulasegaran Shylini, Ph. D.

The University of Texas Medical Branch, 2009

Supervisor: David Gorenstein
Co-Supervisor: Ilya Frolov

RNA structural elements play critical roles in several viral processes. An attempt to elucidate the role of one such RNA structural element encoded by the 5' Untranslated Region (5'UTR), in regulating virus replication and attenuation of Venezuelan equine encephalitis virus (VEEV) is described. VEEV is one of the pathogenic members of the *Alphavirus* genus in the *Togaviridae* family. VEEV infection causes debilitating illness complicated by neurological manifestations. The only available vaccine for VEE infection, the attenuated strain VEEV TC-83 provides minimal protection against virulent strains, but the molecular basis for its attenuation remains poorly understood. Interestingly however, the attenuation of TC-83 was shown to strongly depend on two point mutations, one of which, the G3A mutation, was found in the 5'UTR of the viral genome.

Results from my biochemical and biophysical studies demonstrate that the G3A mutation strongly affects the structure of the VEEV 5'UTR. Further functional analysis revealed that this change in 5'UTR RNA structure affects various processes in virus replication. The G3A mutation moderately enhanced translation of the downstream polyprotein, and strongly increased replication of the viral genome, but led to a significant decrease in the synthesis of subgenomic RNA (sgRNA). Based on my findings and those of others, I propose a model for attenuation of the vaccine strain TC-83. The enhanced functionality of the TC-83 5'UTR in viral processes

prompted further investigation into the structural requirements within the VEEV 5'UTR for efficient virus replication.

Results from these studies revealed that the sequence, secondary structure and stability of the stem-loop in this region are critical for virus replication. Mutations affecting any of the above resulted in pseudorevertants that either acquired compensatory AU or AUG repeat sequences in the 5'UTR, or accumulated mutations in the VEEV non-structural proteins. Results from my mutational analyses thus provide evidence that during the replication of the viral genome, the ends of the dsRNA replication intermediate stay single stranded and fold into individual stem-loops that are critical for virus replication, and the sequence and folding determines the efficiency of the promoter in this region for genomic RNA synthesis.

Table of Contents

Chapter 1 Introduction 1

Alphaviruses 2

Venezuelan Equine Encephalitis Virus (VEEV) 5

VEEV Infection and Pathology 5

Diagnosis and Prevention 6

Molecular Biology of Venezuelan Equine Encephalitis Virus 7

VEEV Virion 7

Genome Organization 8

5' Untranslated Region (5'UTR) 11

Non-structural Proteins (nsPs) 12

Structural Proteins 15

3' Untranslated Region (3'UTR) 17

Alphavirus Replication 18

Virus entry 18

Genome replication and translation 20

Virus assembly and budding 22

Overview of the Dissertation 23

Chapter 2 Structural Characterization of VEEV TRD and TC-83 5'UTR RNA 27

Introduction 27

Materials and methods 29

Enzymatic secondary structure analysis 29

RNA sample preparation 30

UV melting studies 30

NMR spectroscopy 31
Results 31
Predicted secondary structures of VEEV TRD and TC-83 5'UTR RNA 31
Enzymatic analysis of 5'UTR RNA secondary structure 33
Absorbance melting curves of RNA 36
NMR studies confirm that G3A mutation affects RNA structure and stability 37
Discussion 41

Chapter 3 Functional analysis of the effect of G3A mutation on translation of viral proteins, viral RNA synthesis and virus replication 47
Introduction 47
Materials and methods 49
Cell cultures 49
Plasmid constructs 49
RNA transcription 50
RNA transfection 51
In vitro translation assay 52
Analysis of virus replication 52
Analysis of viral RNA synthesis 52
Results 53
G3A mutation moderately enhances RNA translation efficiency 53
Effect of G3A mutation on virus specific RNA synthesis 57
Effect of G3A mutation on virus replication 63
Discussion 64

Chapter 4 Structural requirements within the VEEV 5'UTR encoded promoter for efficient virus replication 69

Introduction 69

Materials and methods 71

Cell culture 71

Plasmid constructs 72

RNA transcription 73

RNA transfection 73

Infectious center assay 74

Analysis of virus replication 74

Selection of pseudorevertants and sequencing of viral genomes 75

Analysis of viral RNA synthesis 75

Results 76

A critical role for the 5' terminal nucleotide sequence in VEEV replication 76

Modifications to the loop sequence do not affect virus replication 82

Stability of the 5' stem-loop affects virus replication 84

5' adaptations observed in the pseudorevertants enhance RNA synthesis and virus replication 90

5' modifications affect RNA replication but do not affect translation of the encoded polyprotein 94

Discussion 98

Chapter 5 Summary and future directions 105

Summary 105

Model for interaction of the VEEV 5'UTR with the viral replicase and its role in viral RNA synthesis 108

Future directions..... 108

Appendix 111

Reference.....115

Vita

List of Tables

Table 4.1. Sequences of 5' ends in the genomes of (G2C25)VEE/SINV pseudorevertants isolated from the plaques 78

Table 4.2. 5'sequence insertions due to increased stability of the stem 87

Table 4.3. 5'sequence insertions due to decreased stability of the stem 89

List of Figures

Figure 1.1. Phylogenetic classification of alphavirus species 4

Figure 1.2. Structure of VEEV virion 9

Figure 1.3. Organization of the VEEV genome indicating the encoded viral non-structural and structural proteins 11

Figure 1.4. Overview of the alphavirus replication cycle in host cells 19

Figure 1.5. Sequence comparison of VEEV TRD and TC-83 genomes 24

Figure 2.1. Mfold predicted secondary structure of VEEV TRD and TC-83 5' UTR stem-loops 33

Figure 2.2. Enzymatic probing of TRD and TC-83 5'UTR RNA structure using RNase A, T1 and V1 35

Figure 2.3. dA/dT plot of thermal melting curves for TRD and TC-83 5'UTR RNA observed by UV spectroscopy 38

Figure 2.4. NMR thermal melting analysis of TRD and TC-83 31nt RNA fragments 39

Figure 2.5. Portion of the 2D NOESY spectra for unlabeled TRD and TC-83 31nt RNA molecules collected at 5 °C 41

Figure 2.6. Comparison of the H1'- H6/H8 regions of the 2D ^{1}H-^{1}H NOESY spectra for TRD and TC-83 31nt RNA fragments 44

Figure 3.1. Effect of G3A mutation on RNA stability in cells 55

Figure 3.2. Effect of G3A mutation on RNA translation efficiency 56

Figure 3.3. Effect of G3A mutation on virus-specific RNA synthesis 58

Figure 3.4. Replication of the DI RNAs encoding VEEV TRD- and VEEV TC-83-specific 5'UTRs 61

Figure 3.5. Expression of genes encoded by subgenomic RNA of VEEV replicons harboring either VEEV TRD- or VEEV TC-83-specific 5'UTRs 62

Figure 3.6. Analysis of chimeric virus replication in BHK-21 (A) and NIH-3T3 (B) cells 63

Figure 4.1. 5' terminal nucleotide mutations 77

Figure 4.2. Analysis of the effects of nt3-specific mutations on VEE/SINV viability and replication 80

Figure 4.3. Sequence requirements within the stem for efficient VEE/SINV replication 81

Figure 4.4. Modifications to the loop do not affect RNA synthesis and chimeric virus replication 83

Figure 4.5. Effect of mutations affecting the stability of the 5'-terminal stem on VEE/SINV viability 86

Figure 4.6. Replication of (C19C20)VEE/SINV variants having additional AUG repeats at the 5' terminus of the genome 91

Figure 4.7. Analysis of (Del)VEE/SINV variant 93

Figure 4.8. Effects of 5'UTR mutations on RNA replication 95

Figure 4.9. Effects of 5'UTR mutations on translation 97

Figure 4.10. Model for interaction of the genomic ends in the dsRNA intermediate with VEEV replication complex (RC) during genomic RNA replication 102

Figure 5.1. A model for attenuation of VEEV TC-83 107

Figure 5.2. A model for interaction of the 5'UTR with the viral replicase and its role in viral RNA synthesis 109

CHAPTER 1

INTRODUCTION

RNA is perhaps the only biomolecule that is capable of enzymatic activity as well as propagation of genetic information. Recent advances in RNA biology have also implicated small RNAs in regulation of key biological processes such as gene expression across all living organisms, ranging from humans to plants and metazoans (Bartel, 2009; Grimson et al., 2008), highlighting the importance of RNA in the evolution of biological species. The role of RNA in evolution is perhaps best studied in viruses that encode RNA genomes. In these viruses, the RNA genome serves as the template for translation of viral proteins that are involved in virus replication, and for synthesis of progeny viral genomes. Viral RNA genomes have been shown to possess various complex structural motifs, ranging from simple secondary structures like hairpin loops and pseudoknots (Brierley, Pennell, and Gilbert, 2007) to complex structural motifs like IRES (Kieft, 2008), that are critical to viral processes. Analyses of such structural motifs across various virus families have shown that though the RNAs do not possess identical sequences, they do contain conserved secondary and tertiary structure motifs that are key to their functions (Baird et al., 2006). Earlier research has shown such motifs to have high levels of specificity that are prerequisite for biomolecular interactions in vivo and that subtle changes completely disrupt key interactions and affect virus viability. Mutational studies of several viral RNA motifs aided by computer prediction of their secondary structures have highlighted the importance of RNA structure in viral life processes. However, comprehensive structural studies of RNA molecules remain a challenge.

The experiments described in this thesis involve one such attempt to elucidate the role of RNA structural elements in regulating virus replication and attenuation of Venezuelan equine encephalitis virus (VEEV), specifically mediated by a 5' untranslated region (5'UTR) mutation. This chapter will briefly outline the characteristics of the genus *Alphavirus* as well as the origin of VEEV, its epidemiology, and clinical features of VEEV infections. A major part of this chapter has been devoted to reviewing the current knowledge on the molecular characteristics of VEEV including its virion structure and genome organization, the molecular determinants of VEEV virulence, and the mechanism of alphavirus replication, with special emphasis on regulation of genome replication by non-structural proteins and cis-acting promoter elements.

ALPHAVIRUSES

Venezuelan equine encephalitis virus (VEEV) belongs to the genus *Alphavirus*, classified in the family Togaviridae. Togaviruses are enveloped, plus strand RNA viruses that are spherical and are classified into two distinct genera: alphaviruses and rubiviruses. The alphaviruses, the larger of the two genera, include about 37 recognized member viruses that are predominantly transmitted by arthropods. These include several known pathogenic members such as Chikungunya, VEEV, Eastern equine encephalitis virus (EEEV), and Western equine encephalitis virus (WEEV). Alphaviruses have been found on all continents including Antarctica, and classically, are described as either Old World or New World viruses depending on their distribution (Powers et al., 2001). Phylogenetically, alphaviruses are classified into seven antigenically related groups that cycle between invertebrate insect vectors and vertebrate reservoir hosts (Fig. 1.1).

The earliest records of diseases caused by alphaviruses date back to the 18th and 19th centuries (Griffin, 2007). Incidence of human disease caused by alphavirus infection is rare; yet, the severity of disease caused by some alphaviruses is significant. It is worth noting that some of the pathogenic members of the *Alphavirus* genus, including VEEV, have been categorized as bio-warfare agents that had previously been weaponized by both the USA and the former Soviet Union. However, the pathogenesis of only a few alphaviruses has been studied.

Alphaviruses replicate in, and are transmitted by a wide range of arthropods, primarily mosquitoes. They cause persistent and life-long infection in mosquitoes characterized by virus accumulation in salivary glands. The viruses are then transmitted to vertebrate hosts during the blood meal. Most alphaviruses can infect a variety of vertebrates including birds, mammals, and fish that serve as their primary amplifying and reservoir hosts. Once inside a vertebrate host, alphaviruses develop a high titer viremia, and induce a variety of illness (Griffin, 2007). In humans, the symptoms of alphavirus infection include encephalitis, arthritis, fever, rash, and arthralgia. For many alphaviruses however, no human or veterinary disease has been recognized.

The molecular biology of many pathogenic members of the *Alphavirus* genus including VEEV has not been elucidated. In spite of limited specific knowledge about individual viruses, it is well established that these viruses share common features in their genome organization and replication mechanisms.

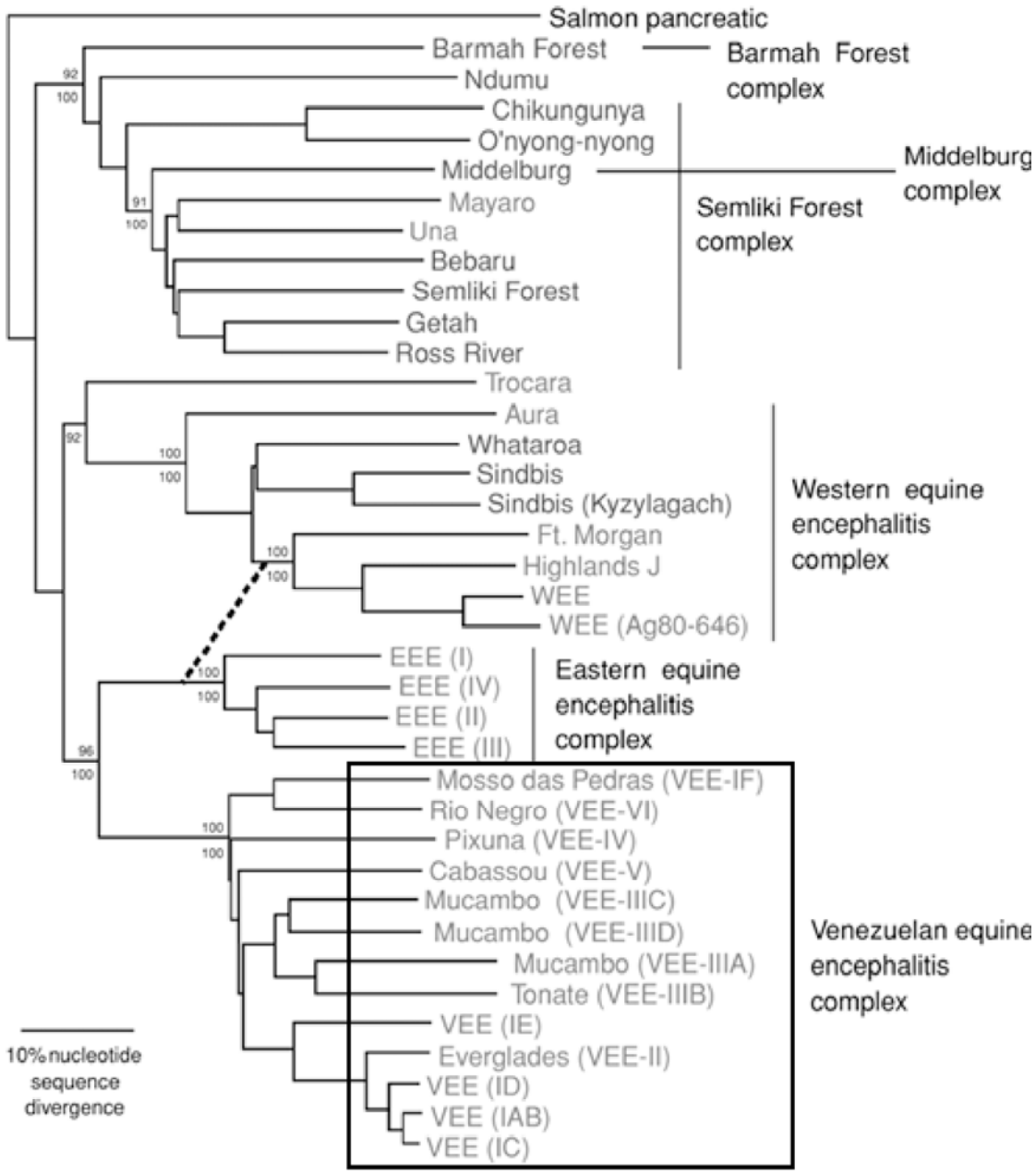


Fig 1.1: Phylogenetic classification of alphavirus species. Phylogenetic tree of alphavirus species generated using partial E1 envelope glycoprotein nucleotide sequences and the neighbor-joining method as described by Powers et al.

VENEZUELAN EQUINE ENCEPHALITIS (VEEV)

VEEV was first isolated in 1936 in Venezuela during an equine encephalitis outbreak. It was the third encephalitic alphavirus identified in the Americas, after EEEV and WEEV (Beck and Wyckoff, 1938). Several VEE-related viruses have been isolated from outbreaks in both South and North America. Viruses belonging to the VEE complex have been classified into six subtypes, I through VI, with VEEV classified as subtype I. The VEE subtype I viruses have been further subdivided serologically into IAB, IC, ID, IE, and IF (Weaver et al., 2004), of which subtypes IAB, IC, and IE have been associated with various VEE-related epizootics, while subtypes ID, IE, and IF are predominantly enzootic viruses.

VEEV Infection and Pathology

As described earlier, VEE was initially identified as an equine infection. Later it was identified to also cause human disease (Sudia and Newhouse, 1975). Studies indicate that clinical infection can occur with both enzootic and epizootic VEEV strains (Weaver et al., 2004), with the latter infection being more severe. The members of subtypes I-AB and IC have been associated with major human epidemics and equine epizootics. Generally in humans, VEEV infection causes febrile illness with the abrupt onset of chills, headache, myalgia, somnolence, vomiting, diarrhea, pharyngitis, and a high incidence of permanent neurological sequelae in certain cases (Rivas et al., 1997). Infection in pregnant females causes fetal abnormalities, spontaneous abortions, and stillbirths (Weaver et al., 1996). During VEEV epizootics, equine mortality rates can reach up to 83%, and in humans, neurological diseases can be detected in up to 14% of all infected individuals, especially children. While the incidence of encephalitis in humans is generally low, all ages and both sexes are equally susceptible to infection; however, disease manifestations vary with age (Rivas et al., 1997). The

mortality rate of VEEV infection is less than 1%, with deaths primarily occurring in children, the elderly and immuno-compromised individuals. Pathology in fatal cases revealed disease conditions including myocarditis, hepatic necrosis, inflammation, generalized lymphoid depletion, and severe neurologic damage, with widespread necrosis, hemorrhage and hypolasia particularly in congenitally infected infants (Griffin, 2007).

Diagnosis and Prevention

Diagnosis of VEEV infection can be made by virus isolation from blood (Bowen and Calisher, 1976; Ehrenkranz and Ventura, 1974). Primary infection may be detected by rising IgG antibody titer and detection of anti-VEEV IgM. Further, viral RNA in the serological isolates can be confirmed by virus specific nucleic acid amplification or other methods like complement fixation (CF), enzyme immunoassay (EIA), or neutralization tests.

A number of methods have been used to prevent and control VEEV outbreaks. Formalin inactivated preparations were the first vaccines developed for horses and laboratory workers (Randall, Maurer, and Smadel, 1949). Later, live attenuated vaccine TC-83, developed by serial passage of the wildtype Trinidad donkey (TRD) strain in guinea pig heart tissue cell cultures, was used for immunization (Cole, May, and Eddy, 1974; McKinney et al., 1963). Although vaccination with TC-83 causes fever and mild infection in 15 – 30 % of the recipients, it is still the best available vaccine against VEEV (Burke, Ramsburg, and Edelman, 1977; Engler et al., 1992; Jahrling and Stephenson, 1984).

MOLECULAR BIOLOGY OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

VEEV differs significantly from most alphaviruses, in its ability to cause debilitating disease complicated by neurological manifestations. In spite of its serious threat to public health, VEEV has been studied less intensively than other alphaviruses, and very little is known about the molecular biology of the virus, especially the mechanism of virus replication and the role of viral proteins in virus-host interactions.

VEEV Virion

VEEV is a spherical virus, like all alphaviruses, with a diameter of 70 nm. The virion is composed of repeating units of the E1 and E2 transmembrane glycoproteins, the capsid or nucleocapsid protein (C), a host-derived lipid bilayer, and a single molecule of the RNA genome (Fig. 1.2) (Paredes et al., 2001). The virion is organized as shells of molecules that protect and deliver the viral RNA to susceptible host cells. The outer shell of the virion is made up of envelope protein trimers, arranged as hexameric or pentameric capsomeres, on a T = 4 icosahedral lattice. Each envelope trimer is made up of E1 and E2 glycoproteins that exist as heterodimers, with E1 lying parallel to the surface, and E2 forming petal-like spikes rising to about 50 Å from the surface (Paredes et al., 2001). The icosahedral nature of the virion results in an ordered distribution of these spikes. Small openings present at the twofold and fivefold axes and around the base of each spike reveal the underlying lipid bilayer. The lipid bilayer is derived from the host plasma membrane and surrounds the nucleocapsid. The spherical nucleocapsid shell is made up of repeating capsid protein units that are also arranged as both pentameric and hexameric capsomeres on a T = 4 icosahedral lattice that complements the organization of the envelope glycoproteins (Paredes et al., 2001; Paredes et al., 2003). The nucleocapsid is about 40 nm in diameter

and protects a single copy of the viral RNA genome. The envelope glycoprotein and the capsid subunits in both the outer and the inner shells, interact with one another to form a rigid structure across the membrane in a one-to-one relationship.

Genome Organization

VEEV has a genomic organization similar to that of other alphaviruses. The virus encodes a non-segmented, single-stranded, positive-sense RNA genome that is 11.4 kb in length containing a 5'-methylguanylate cap and a 3'-polyadenylate tail (Strauss and Strauss, 1994). The 5’ two thirds of the genome codes for the nonstructural proteins (nsP1-4), and the 3’ one third codes for the structural proteins (capsid, E1 and E2). The junction between the nonstructural and the structural proteins encodes the subgenomic RNA (sg) promoter, used for the synthesis of sgRNA (Fig. 1.3). The 5’UTR in VEEV is 44 nt long, having a short stem-loop at the very 5’ terminus. The 3’UTR is 117 nt in length and is polyadenylated. The non-structural proteins are translated directly from the viral RNA that acts as a messenger RNA to produce two polyproteins that are processed by the virus-encoded protease in nsP2 to form the mature viral replicase. The sgRNA serves as the template for synthesis of the structural polyprotein, which are then processed to produce individual structural proteins that form the virion.

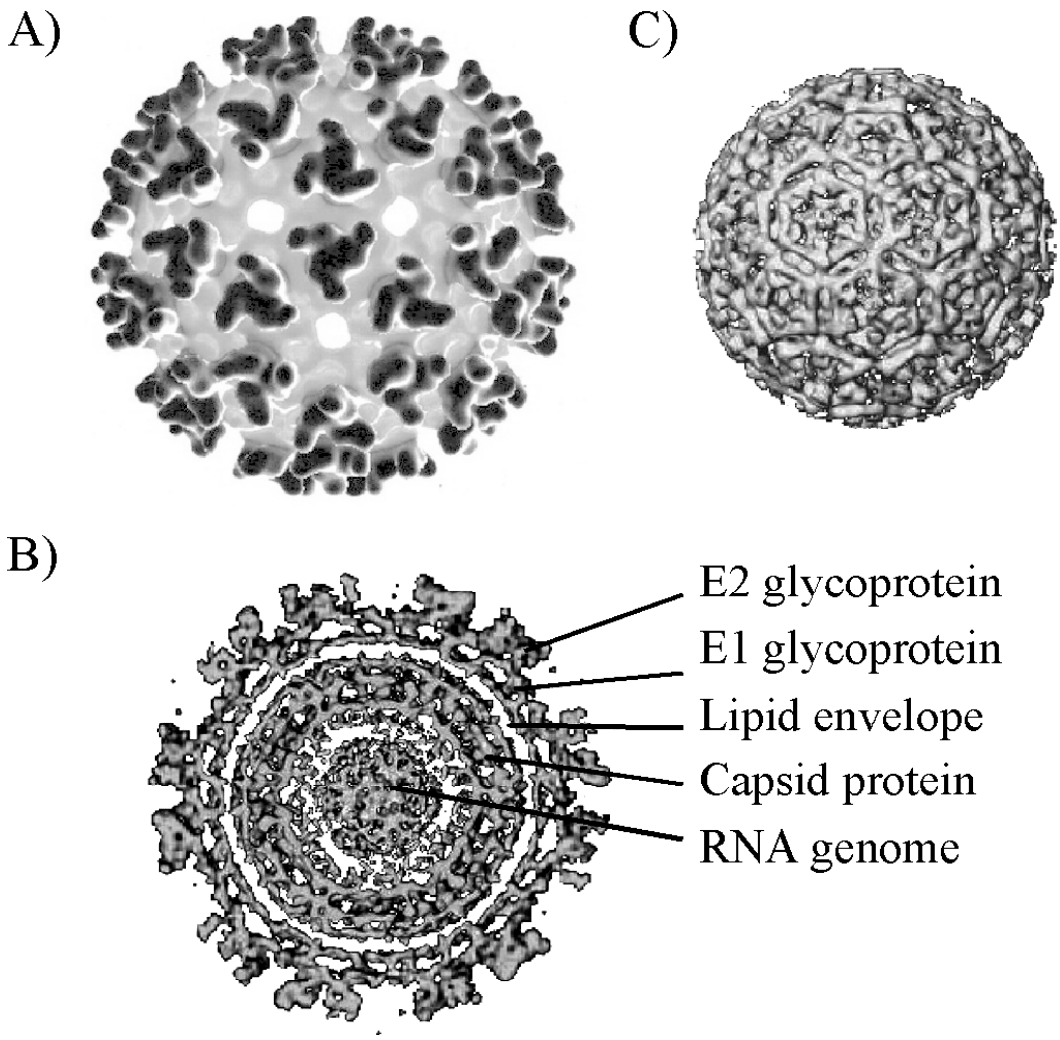


Fig. 1.2: Structure of VEEV virion (modified from Paredes et al., 2001). (A) Envelope structure of VEEV virion showing surface spikes. (B) Cross section of the virion showing the inner components of the virus. (C) Surface structure of the nucleocapsid.

Comparison of alphavirus genomes has identified important cis-acting sequences that play crucial roles in viral genome replication and sgRNA transcription (Strauss and Strauss, 1994). One such conserved sequence element (CSE) is found at the 3′ end immediately preceding the poly-(A) (Ou, Strauss, and Strauss, 1981), while another is found in the junction region between nonstructural and structural genes (Ou et al., 1982). The CSE in the 3'UTR has been shown to be important for initiation of minus strand RNA synthesis. Mutations to this region affect both RNA synthesis and virus replication (Hardy and Rice, 2005; Thal et al., 2007). The CSE found in the junction region, on the other hand, is critical for the synthesis of sgRNA and constitutes the sgRNA promoter. Two other conserved regions are found near the 5′ end of the genome: the 5' UTR, and the 51-nt conserved sequence element (CSE) (Ou, Strauss, and Strauss, 1983). The 51-nt CSE has been shown to enhance virus replication and is not crucial for virus replication (Fayzulin and Frolov, 2004; Michel et al., 2007), while the CSE in the 5'UTR and its complement in the 3'-end of the negative strand have been shown to be important for plus-strand and minus-strand RNA synthesis (Frolov, Hardy, and Rice, 2001).

Comparative studies of the virulent Trinidad donkey (TRD) and avirulent TC-83 vaccine strains have led to identification of the 5′ UTR and the E2 glycoprotein as important determinants of VEEV virulence in mice (Kinney et al., 1993). Recent studies indicate that E2 mutations positively enhance VEEV infections (Brault et al., 2002; Brault et al., 2004; Greene et al., 2005), but the role of the 5'UTR in attenuation is not clearly understood.

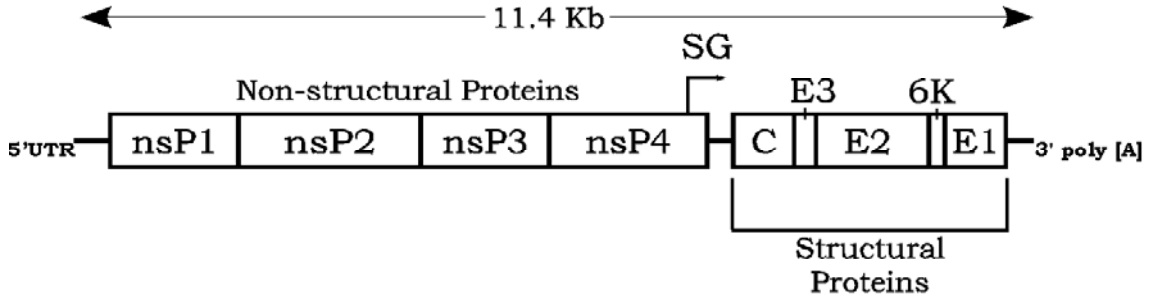


Fig. 1.3: Organization of the VEEV genome indicating the encoded viral non-structural and structural proteins

5'Untranslated Region (5'UTR)

The **5'UTR** of the VEEV genome is 44 nucleotides long and has a 5' terminal stem-loop. Sequence comparison of VEEV isolates indicates that the 5'UTR sequence is highly conserved among VEEV members (Fig. A1). However, comparison of the 5'UTRs of several alphaviruses suggests that the 5'UTR sequence is not conserved. In spite of the lack of sequence identity, all alphavirus genomes begin with a 5' AU and have a stem-loop secondary structure motif at the 5' terminus. The conserved sequence element in the 5'UTR is found in almost all of the alphaviral genomes, and it has been implicated to play different roles in replication. In the plus strand, this element is believed to modulate the initiation of nonstructural protein translation. Deletion of specific nucleotides in the Sindbis virus (SINV) 5'UTR (Niesters and Strauss, 1990) gave rise to a viable virus, but the replication rates were well below that of the wildtype virus, suggesting that this element is critical for virus replication. The complement of the 5' UTR serves as a promoter at the 3' end of the minus strand, for initiation of synthesis of plus-strand RNA from a minus-strand template (Frolov, Hardy, and Rice, 2001; Gorchakov et al., 2004) and has been shown to bind host

proteins (Pardigon, Lenches, and Strauss, 1993; Pardigon and Strauss, 1992). Further, in addition to being important for minus-strand RNA synthesis, the 5' UTR is involved in plus-strand RNA synthesis (Frolov, Hardy, and Rice, 2001).

It has been suggested that the stem loop structure at the 5' end of the genome, in addition to the RNA sequence, is a key regulator of one or all of the above mentioned processes (Niesters and Strauss, 1990; Ou, Strauss, and Strauss, 1983). Point mutations in the 5'UTR region of SINV have been shown to affect the virulence of the virus in mice (Kobiler et al., 1999; McKnight et al., 1996), thus clearly highlighting the importance of the sequence and the RNA secondary structure in this region.

Non-structural proteins (nsPs)

The nonstructural proteins are translated from the mRNA like viral RNA genome as either one or two polyproteins: P123 and/or P1234. The P123 polyprotein is produced when translation is terminated at the opal codon region between nsP3 and nsP4, while a read-through of this codon results in the synthesis of the P1234 polyprotein. The viral non-structural polyproteins are processed by the virus-encoded protease in nsP2. The entire polyprotein as well as the individual nsPs are part of the viral replicase that replicates the viral genome (Shirako and Strauss, 1994). Differential processing of the full-length non-structural polyprotein to produce individual nsPs is believed to regulate the process of viral RNA replication (Lemm and Rice, 1993b; Strauss and Strauss, 1994). In addition to their independent enzymatic activities in RNA replication, the individual nsPs are believed to possess additional unknown functions that might be involved in regulating virus-host interactions.

nsP1 is the first of the non-structural proteins and is about 540 amino acids long. Alphavirus nsP1 has been known to possess guanine-7-methyltransferase and guanylyltransferase activities necessary for mRNA and

genomic RNA capping (Ahola et al., 1997; Mi et al., 1989). nsP1 has also been shown to be important for minus-strand synthesis, and is thought to interact directly with nsP4 in the replication complex (Fata, Sawicki, and Sawicki, 2002). Furthermore, nsP1 has been shown to be associated with intracellular smooth membranes (Peranen et al., 1995) mediated by palmitoylation of cysteine residues (Ahola et al., 2000; Laakkonen, Ahola, and Kaariainen, 1996). This membrane anchoring mediated by nsP1 is suggested to be required for efficient activity of the virus replicase (Ahola et al., 1999; Salonen, Ahola, and Kaariainen, 2005).

nsP2 is the largest of the non-structural proteins, with a length of about 800 amino acids and is multifunctional. As part of the viral replication complex, nsP2 is involved in viral RNA replication and transcription of the sgRNA. The N-terminal domain of nsP2 has helicase, RNA triphosphatase and nucleoside triphosphatase activities (Mayuri et al., 2008; Rikkonen, Peranen, and Kaariainen, 1994; Vasiljeva et al., 2000). The C-terminal domain of nsP2 encodes a papain like serine protease (Ding and Schlesinger, 1989; Hardy and Strauss, 1989; Merits et al., 2001), which regulates the processing of the non-structural polyproteins to generate non-structural protein precursors followed by mature nsPs that form the viral replicase. In addition to its protease activity, the VEEV nsP2 C-terminus has been shown to contain a methyltransferase domain (Russo, White, and Watowich, 2006) and is proposed to be involved in regulation of protease activity, and regulation of RNA replication (Mayuri et al., 2008; Russo, White, and Watowich, 2006). nsP2 has also been identified to play a role in induction of cytopathic effects, establishment of persistent infections and transcriptional and translational shutoff (Frolov et al., 1999; Frolova et al., 2002; Garmashova et al., 2006; Mayuri et al., 2008).

nsP3 is about 550 amino acids long. Its functions in virus replication are not well defined although *in vitro* studies and genetic analyses indicate that it

plays a role in RNA synthesis (LaStarza, Lemm, and Rice, 1994; Li et al., 1990; Wang, Sawicki, and Sawicki, 1994). The N-terminal domain, also called the X-domain, is highly conserved among alphaviruses. On the other hand, the C-terminal domain is not very well conserved, and is rich in phosphorylated serine and threonine residues (Lastarza, Grakoui, and Rice, 1994; Vihinen et al., 2001; Vihinen and Saarinen, 2000). Studies (in SFV) have shown nsP3 to have an affinity for vesicle- or vacuole-like structures (Peranen and Kaariainen, 1991). This observation has been confirmed by studies in other alphaviruses (in SINV) (Gorchakov et al., 2008). Interestingly, several host proteins have been shown to associate with nsP3-containing complexes, clearly highlighting the role of nsP3 in virus replication and virus-host interaction (Gorchakov et al., 2008).

nsP4, the RNA-dependent RNA polymerase (RdRP), with a length of about 600 amino acids, forms the core of the virus replication complex (Lemm et al., 1990; Lemm and Rice, 1993b). The RdRp core domain is encoded in the C-terminus of the protein and has two distinct functions: terminal transferase activity and *de novo* minus strand synthesis activity. While the RdRp core domain can function as a terminal transferase on its own (Tomar et al., 2006), its *de novo* minus strand synthesis activity requires the presence of the N-terminal domain and the P123 polyprotein (Lemm et al., 1994; Rubach et al., 2009; Tomar et al., 2006). The N-terminus of nsP4 is not conserved among viral RdRps, and it has been suggested that it may be important for protein-protein interactions with other non-structural proteins or host factors. nsP4 also contains a conserved N-terminal tyrosine residue, which results in rapid degradation of the protein by the N-end rule pathway (de Groot et al., 1991; Shirako and Strauss, 1998). Interestingly, it has been suggested that the N-end rule pathway only applies to free nsP4, and not to nsP4 that is part of the viral replicase complex (de Groot et al., 1991). In cells, nsP4 levels are slightly less than that of other nsPs, as it is only translated as part of the P1234 polyprotein complex. Studies have shown

that the levels of nsP4 in cells are optimal for efficient viral RNA synthesis, as efforts to increase nsP4 synthesis by replacing the opal termination codon with a sense codon resulted in reduced virus replication in cells (Li and Rice, 1989). This is supported by *in vitro* studies showing that increasing molar concentrations of nsP4 inhibits RNA synthesis by full-length nsP4 and the P123 complex (Rubach et al., 2009), and also by the observation that excess nsP4 is rapidly degraded in cells (de Groot et al., 1991).

Structural proteins

The structural proteins are translated from the subgenomic messenger RNA as a single polyprotein. Host and viral proteases then process the polyprotein during and post translation to generate individual structural proteins that form the virion. Studies have shown that the translation of the structural polyprotein is enhanced by a hairpin secondary structure in the subgenomic mRNA downstream of the initiating AUG (Frolov and Schlesinger, 1996).

The **capsid** protein is the first structural protein to be translated, as the order of translation is capsid-PE2(E3+E2)-6K-E1. The capsid has an intrinsic autoprotease activity that enables the proteolysis of the structural polyprotein immediately after it exits the ribosome to release individual capsid proteins (Nicola, Chen, and Helenius, 1999). The proteolysed capsid protein has been shown to have a strong affinity for ribosomes (Soderlund and Ulmanen, 1977; Ulmanen, Soderlund, and Kaariainen, 1976). The mature capsid forms the viral nucleocapsid that encloses the viral genomic RNA. The capsid is about 250 amino acids long, and forms two distinct domains. The N-terminal domain, not conserved among alphaviruses and rich in lysine and arginine, is a positively charged RNA-binding domain. This domain is hypothesized to play a critical role in the packaging of virus-specific RNA (Warrier et al., 2008). Comparative studies of New World and Old World alphaviruses have shown that the N-terminus of the

capsid in VEEV and EEV function in transcriptional shutoff and inhibit nuclear import in cells (Garmashova et al., 2007a; Garmashova et al., 2007b). The C-terminal domain, on the other hand, is highly conserved among alphaviruses and functions as a protease. It is also believed to contain regions that promote capsid-capsid interactions critical for rapid and efficient nucleocapsid assembly (Perera et al., 2001). The C-terminus of the capsid protein also interacts with the cytoplasmic domains of the envelope glycoproteins, and this interaction is essential for virus assembly and budding (Lopez et al., 1994; Wilkinson et al., 2005)

The **Envelope glycoproteins (E1 and E2)** are proteolytically processed from the structural polyprotein. E2 is initially translated as a PE2 polyprotein which includes both E3 and E2, but which is subsequently subject to proteolytic cleavage. Processing of the PE2 polyprotein into individual E2 and E3 envelope proteins is critical, as incorporation of uncleaved PE2 into the virus envelope has been shown to significantly affect virus entry into cells (Carleton et al., 1997; Ryman, Klimstra, and Johnston, 2004). During translation, the envelope proteins are translocated into the endoplasmic reticulum, where they are processed and undergo post-translational modifications. E1 and E2 together form the viral envelope, and interaction between these proteins has been shown to be important for virus budding and entry into cells (Mukhopadhyay et al., 2006; Navaratnarajah and Kuhn, 2007; Sjoberg and Garoff, 2003). In the viral envelope, these proteins exist as E1-E2 heterodimers, with E1 lying on the surface parallel to the lipid envelope and E2 protruding from the surface forming spikes (Mukhopadhyay et al., 2006; Paredes et al., 2001). The envelope proteins are essential for attachment and viral entry into host cells. The E2 glycoprotein contains multiple receptor-binding sites and functions in attachment of the virus to the cell surface (Cutler and Garoff, 1986; Navaratnarajah and Kuhn, 2007). E1, on the other hand, functions in fusion of the viral and host membranes inside

the endocytic vesicle, resulting in the release of the viral nucleocapsid into the host cytoplasm (Barth et al., 1992; Wahlberg and Garoff, 1992). Mutations in either E1 or E2 have been shown to disrupt the interactions between the envelope and the nucleocapsid affecting virus assembly and virus entry into cells (Brault et al., 2002; Dubuisson and Rice, 1993; Levine et al., 1996; Liu et al., 1996).

The **6K** protein, about 55 amino acids long, has been shown to be important for virus budding and formation of virions (Loewy et al., 1995; Schlesinger, London, and Ryan, 1993). During translation, the C-terminal domain of the 6K polypeptide acts as a signal sequence for the translocation of El to the ER. Along with E1, 6K is also proposed to have ion channel properties (Melton et al., 2002) that function to reduce the pH in the endosome during virus entry. Mutations in 6K have been shown to affect glycoprotein trafficking and virion assembly (Ivanova, Le, and Schlesinger, 1995; Sanz and Carrasco, 2001; Schlesinger, London, and Ryan, 1993).

3'Untranslated Region (3'UTR)

The **3'UTR** of the VEEV genome is 117 nucleotides long, and is polyadenylated. The 19nt CSE in the 3' UTR is highly conserved among alphaviruses (Pfeffer, Kinney, and Kaaden, 1998) and serves as the promoter for the initiation of minus-strand RNA synthesis (Hardy, 2006; Kuhn, Hong, and Strauss, 1990). The 3'UTR requires at least 11 adenine residues adjacent to the 19nt CSE for efficient synthesis of the minus-strand RNA (Hardy and Rice, 2005). It also prevents its deadenylation in both mammalian and mosquito cells (Garneau et al., 2008). Mutations in the 3'UTR significantly impact the efficiency of virus and genome replication in cells (Hardy, 2006; Hardy and Rice, 2005; Kuhn, Hong, and Strauss, 1990; Thal et al., 2007). The evolution of viruses encoding mutations in the 3'UTR results in addition of poly(A) or AU rich

sequences in this region (George and Raju, 2000; Hardy and Rice, 2005; Raju et al., 1999).

ALPHAVIRUS REPLICATION

The mechanism of alphavirus replication has been extensively studied in Sindbis, (Strauss and Strauss, 1994). Essentially, alphavirus replication proceeds in three key stages, which include (i) virus entry, (ii) genome replication and translation and (iii) virus assembly and budding.

Virus entry

The process of virus attachment and entry is one of the first steps in virus infection. The virus envelope proteins function in this process; they enable the virus to attach to the surface membrane and then enter host cells. During their lifecycle, alphaviruses infect multiple hosts that serve as reservoirs or dead end hosts and the same envelope proteins are involved in attachment and entry in all hosts. Based on this, it has been hypothesized that the virus might use a ubiquitous receptor that is conserved across species and that E2 glycoprotein contains multiple receptor-binding sites so that it can bind to distinct host cell receptors. SINV-related studies in distinctive virus permissive cells have implicated a wide range of cellular receptors (Marsh, Kielian, and Helenius, 1984; Smith and Tignor, 1980; Strauss and Strauss, 1994) ranging from laminin receptor, DC-SIGN and L-SIGN (Klimstra et al., 2003), and heparan sulfate. Among these, one category of receptors facilitates virus entry into cells while the second serve for high affinity attachment to the cells to enable better interaction with entry receptors.

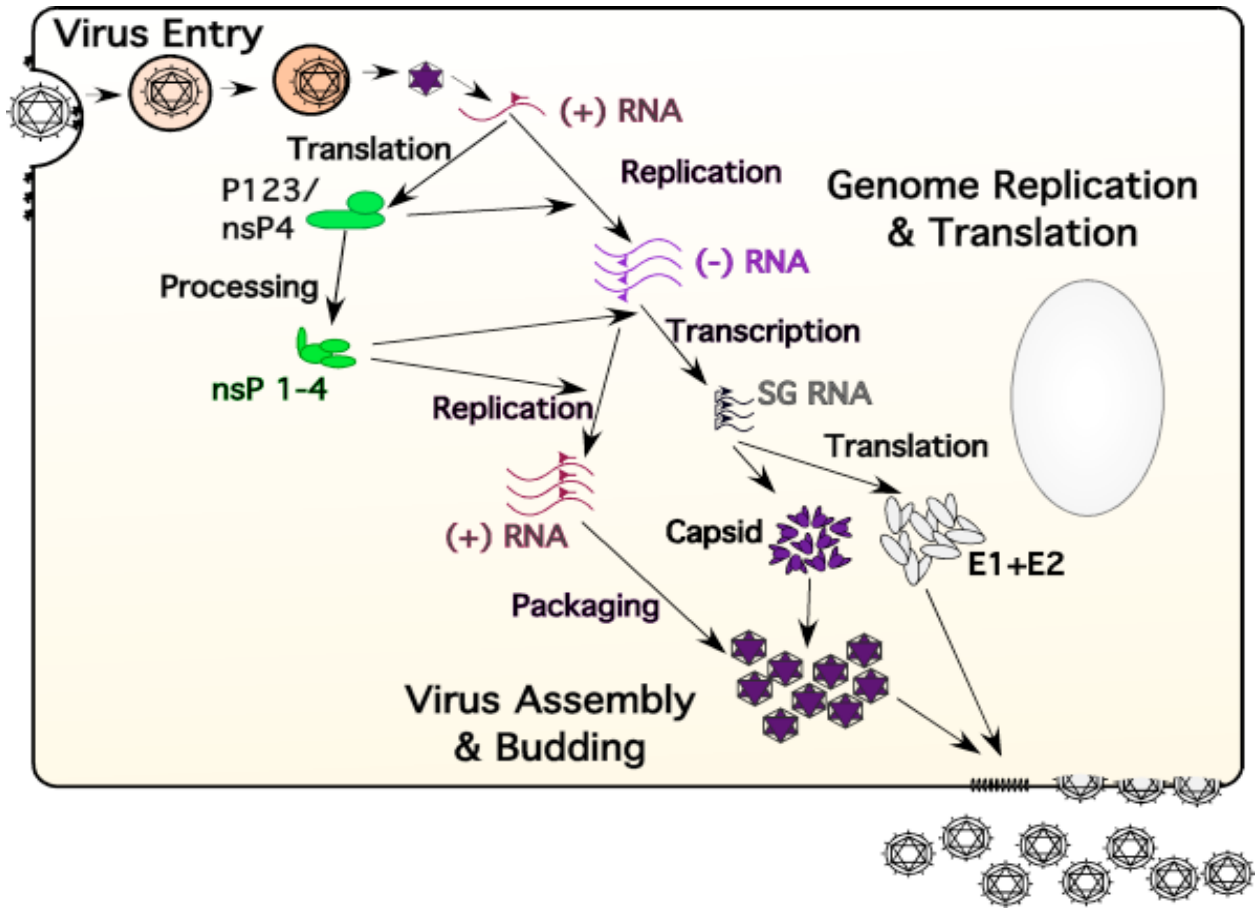


Fig. 1.4: Overview of the alphavirus replication cycle in host cells. The major steps in virus replication: virus entry, genome replication & translation and virus assembly & budding are indicated.

The next step after attachment is virus entry. Virus entry of alphavirus proceeds through an endocytotic clathrin-dependent pathway (DeTulleo and Kirchhausen, 1998; Kolokoltsov, Fleming, and Davey, 2006; Vonderheit and Helenius, 2005). Once endocytosed, the pH change inside the vesicles triggers dissociation and conformational change of the envelope proteins, resulting in membrane fusion (Abell and Brown, 1993; Wahlberg and Garoff, 1992). The membrane fusion results in the formation of a fusion pore, and the nucleocapsid core is released into the cytoplasm. In the cytoplasm, the nucleocapsid disassembles to release the single-stranded messenger RNA genome (Singh and Helenius, 1992). This mRNA genome serves as the template for replication and transcription of the virus genome and for the translation of viral proteins to synthesize progeny viruses.

Genome Replication and translation

Alphavirus genomes encode for all of the viral proteins necessary for replication and packaging of the viral genome. It is well established that alphavirus replication is a highly regulated, multi-step process which includes synthesis of three different RNA species (Fig. 1.4) (Strauss and Strauss, 1994). Replication of the viral genome takes place as a two-step process, where in the first step the viral genomic RNA is used as the template for synthesis of a full length minus strand intermediate. This minus strand intermediate is then used as a template for the production of large quantities of plus-strand genomic and subgenomic RNAs in the second step. The differential processing of the viral nonstructural proteins that constitutes the viral replicase involved in the synthesis of the plus- and minus-strand genome regulates the process of genomic RNA replication (Strauss and Strauss, 1994).

Briefly, as soon as the viral RNA is released into the cytoplasm, translation of the genomic RNA is initiated at the AUG codon near the 5’ terminus of the

RNA, and translation proceeds for two-thirds of the mRNA until it encounters the termination codons upstream of the subgenomic promoter, near the start of the subgenomic (SG) RNA. The nonstructural proteins are translated from one or two polyproteins: P123, and/or P1234. The polyprotein P123 encoding the sequences of nsP1, nsP2, and nsP3 is produced when translation terminates at the stop codon between nsP3 and nsP4. The polyprotein P1234 is produced upon the read-through of this stop codon. nsP4 is then cleaved in cis from the P1234 polyprotein and along with the P123 complex is capable of initiating the minus strand synthesis from the 3' 19nt promoter (Lemm et al., 1994; Shirako and Strauss, 1994; Strauss and Strauss, 1994). The processing of the P123 complex is performed by nsP2-associated protease activity (Hardy and Strauss, 1989), leading to the formation of the mature replicase from the original enzyme and contains fully processed nsP1, nsP2, nsP3, and nsP4 proteins and possibly other host factors. This new mature replicase complex is efficient in positive-sense genomic and subgenomic RNA synthesis and does not synthesize the negative strand intermediate (Shirako and Strauss, 1994). The subgenomic RNA synthesis is controlled by a minimal promoter element that is encoded in the junction between the nsP and structural protein coding regions, and spans from -19 to +5 relative to the start of the sg mRNA (Raju and Huang, 1991; Wielgosz, Raju, and Huang, 2001). The sgRNA serves as the template for synthesis of the structural proteins that form the virion. These proteins are translated as a single polyprotein that is then processed to produce individual structural proteins that assemble to form the virus envelope.

Although the viral protein composition of the replicases is known, the role of any host proteins in the complex is not clear. Several studies of the conserved viral promoter regions have suggested and implicated host proteins that bind to these regions; studies of vesicular membrane associated viral replication complexes, where nsP1 and nsP3 act as membrane anchors (Froshauer,

Kartenbeck, and Helenius, 1988; Peranen et al., 1995), have suggested the presence of certain host factors in these complexes, but their roles in virus replication, if any, has not been clearly identified (Gorchakov et al., 2008). Furthermore, membrane associated replication complexes have been shown to contain viral dsRNAs, suggesting that the negative strand intermediate is likely to exist as double-stranded duplex along with the positive strand genome (Gorchakov et al., 2008), suggesting that replication may occur via a dsRNA intermediate.

Virus assembly and budding

The sgRNA serves as the template for the synthesis of the structural proteins that form the virus envelope. The viral structural polyprotein synthesis begins with the synthesis of the capsid. Once the capsid protein is synthesized during the structural polyprotein synthesis, it is auto-proteolyzed. The capsids cleaved during synthesis associate with the ribosome and eventually assemble into the viral core (Soderlund and Ulmanen, 1977). This assembly requires the interaction of the capsid with viral nucleic acids, but the exact mechanism is not yet clear (Linger et al., 2004; Tellinghuisen and Kuhn, 2000).

The cleavage of the capid from the polyprotein is followed by the translocation of the PE2, E1 and the 6K across the ER (Migliaccio et al., 1989; Mulvey and Brown, 1996). In the ER, the envelope proteins (PE2, E1 and 6K) are then processed and undergo post-translational modifications. The E1 and E2 form heterodimers in the ER (Carleton et al., 1997; Knipfer and Brown, 1989; Mulvey and Brown, 1996) and are then transported to the Golgi-network before being transported to the plasma membrane (Knipfer and Brown, 1989).

Virus budding is the last step in the virus replication cycle. The nucleocapsids are packed with a single copy of progeny genome and are transported to the plasma membrane where they interact with the glycoproteins

to promote budding. Although some studies have shown certain key requirements for budding, such as the expression of capsid and the interaction of capsid motifs with that of E2 glycoproteins, the exact sequence of events and nature of the interactions that promote virus budding are yet to be described.

OVERVIEW OF THE DISSERTATION

Attenuated vaccines have generally been obtained by continuous propagation of the virus in non-native cell lines so that it becomes less pathogenic to its original host as it evolves in the new conditions. Vaccines thus obtained have been very effective for immunization, and they elicit life-long immunity in a cost effective manner. However, the molecular basis of attenuation for such vaccines remains poorly understood, and very little is known about the process by which the attenuating mutations arise and evolve. Hence, an understanding of the molecular basis of vaccine attenuation would enable one to design better vaccines through more focused approaches, rather than by blind passages. The obvious gap in knowledge here is in our understanding of attenuation at a genetic level, as to how these attenuating mutations lead to molecular changes resulting in the shift from virulence to attenuation. TC-83, the vaccine strain of VEEV, was derived by serial passage of the wild-type TRD strain in guinea pig heart cells (Berge, Banks, and Tigertt, 1961). Comparison of the sequences of VEEV wild type (TRD) and vaccine strain (TC-83) genomes revealed a very limited number of nucleotide differences (Fig. 1.5) (Kinney et al., 1993). One of the critical mutations identified in the 5’ UTR was shown to have a very strong effect on viral pathogenicity (Kinney et al., 1993; White et al., 2001). However, the molecular role of the 5’UTR mutation in VEEV replication and its apparent role in the attenuation of the vaccine strain are yet to be evaluated and fully understood.

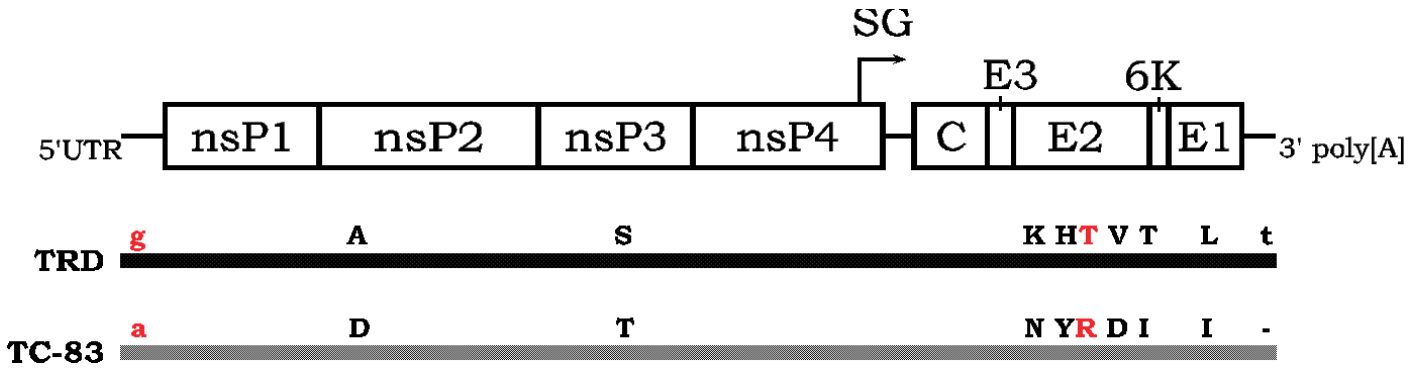


Fig. 1.5: Sequence comparison of VEEV TRD and TC-83 genomes. Comparison of TRD and TC-83 genome sequences indicating the nucleotide and amino acid sequence differences between the two genomes. The nt3 (G→A) mutation and the E2 (T120R) mutation are highlighted

My main objective was to address this gap in knowledge: **To identify the molecular basis for VEEV attenuation mediated by the 5'UTR mutation**.

RNA secondary structures at the 5' and 3' ends of RNA viruses are known to be important for virus replication, and the 5'UTR in alphaviruses is critical for certain viral processes. Hence, to fully understand the role of mutation in virus attenuation, it was essential to identify the changes to the RNA structure in the 5'UTR (Chapter 2). The changes to the 5'UTR structure was mapped using biophysical experiments supported by enzymatic probing with sequence- and strand-specific ribonucleases (RNases). These studies revealed that the mutation affects both the structure and the stability of the stem-loop found in the 5'UTR (Kulasegaran-Shylini et al., 2009b).

We then sought to characterize the effect of this mutation on virus replication (Chapter 3). *In vitro* studies using viral replicons and chimeric viruses encoding the 5'UTR mutation demonstrated that the 5'UTR mutation influences selected steps of virus replication. It moderately enhances translation of the genome-encoded proteins and increases genomic RNA synthesis. Subgenomic RNA synthesis is lowered, resulting in a decrease in structural protein synthesis. Careful analyses of these results and relevant literature reveal a plausible mechanism for the attenuation of the virus mediated by the 5'UTR mutation.

The enhanced translation and increased viral RNA synthesis due to the 5'UTR mutation suggested that the changes to the RNA structure in the 5'UTR enhanced interaction with the translation machinery and the virus replicase. Hence, we sought to identify the significance of RNA sequence and the features of the 5'-UTR stem-loop structure that were critical for virus replication (Chapter 4). The outcome of these studies was that the sequence, structure, and the stability of the stem were important for efficient virus replication. Modifications affecting either of these proved detrimental to both genomic RNA synthesis and virus replication resulting in virus pseudorevertants having additional

compensatory mutations to the 5'UTR or viral non-structural proteins (Kulasegaran-Shylini et al., 2009a).

CHAPTER 2

STRUCTURAL CHARACTERIZATION OF VEEV TRD AND TC-83 5'UTR RNA

INTRODUCTION

RNA molecules can adopt a variety of secondary and tertiary conformations despite the limited number of ribonucleotide residues. This structural diversity is based on the capability of RNA to form networks of stacked nucleobases with several hydrogen bonds in both the nucleobase and the ribose moiety. In addition to their structural variability, RNA molecules encode genetic information; they possess enzymatic activity and are involved in key functions like translation and other gene regulatory functions in several organisms. The dynamic folding properties of RNA molecules play a key role in their functional diversity and minor changes to their sequence can affect their folding and functional capabilities.

VEEV is a pathogenic member of the alphavirus genus. Attenuation of the TC-83 vaccine strain was attributed to two mutations in the genome, one of which was found in the 5'UTR. While the molecular effect of the envelope glycoprotein mutation has been defined to regulate virus entry into cells, the effect of the 5'UTR mutation in virus attenuation remains to be described. The 5'UTR in alphaviruses functions in diverse viral replication processes (Strauss and Strauss, 1994). A conserved sequence element encoded in this region is believed to be the promoter for viral RNA replication (Frolov, Hardy, and Rice, 2001; Niesters and Strauss, 1990; Ou, Strauss, and Strauss, 1983). The complement of the 5'UTR located in the 3' end of the negative strand is essential

for plus strand RNA synthesis (Frolov, Hardy, and Rice, 2001). 5'UTR mutations in SINV and other viruses affect both RNA replication and virus replication in cells. The nt8 and nt5 mutations in SINV were shown to affect the pathogenicity of the virus (Kobiler et al., 1999; McKnight et al., 1996), however, their effect on RNA secondary structure and the molecular mechanism for pathogenicity are unknown. Analysis of 5' ends of various alphaviruses indicated that the 5' terminus in all of the alphaviruses fold into stem-loop structures, which suggests that this structure might play a critical role in virus replication (Ou, Strauss, and Strauss, 1983). It is likely that the RNA structure and the thermodynamic stability of the stem-loops could be critical for interactions with viral and/or host-cellular protein complexes. Hence, a detailed understanding of the RNA folding and the thermodynamic stability of the individual contacts that define the tertiary structure could provide clues to the attenuating effect of such mutations.

This chapter details some of the biochemical and biophysical studies towards understanding the effect of the VEEV nt3 mutation on the structure of the 5' stem-loop. The results from the enzymatic probing experiments indicate that the nt3 mutation does indeed affect the structure of the 5'terminal stem-loop in TC-83 5'UTR. NMR experiments to characterize the tertiary structure of the two elements in TRD and TC-83 revealed that the nt3 mutation lowers the stability of the stem-loop in TC-83 and that the loop regions in both stem-loops are highly dynamic. The results presented here support the hypothesis that the RNA secondary structure along with the thermodynamic stability and the dynamic property play an important role in the functioning of the RNA in virus replication processes.

MATERIALS AND METHODS

Enzymatic secondary structure analyses

Plasmid DNA encoding the 5'terminal 135 nucleotides of both the VEEV TRD and TC-83 genomes was cloned into pRS2 plasmids under the control of the T7 promoter. A BamH1/EcoR1 restriction site was used to linearize the plasmids before transcription. Linearized cDNA templates were purified by phenol-chloroform extraction and ethanol precipitation before being used in transcription reactions. RNA transcripts were obtained by in vitro transcription of the 1-2ug of cDNA templates using the T7 Megascript (Ambion Inc) reaction system. RNA transcripts thus obtained were gel purified by denaturing polyacryamide gel electrophoresis (16% polyacrylamide; 7M urea). Purified transcripts were then 5' end-labeled using T4 polynucleotide kinase (KinaseMax kit, Ambion Inc) and [^{32}P] ATP (GE Healthcare/Amersham Pharmacia Biosciences). Radio-labeled transcripts were further purified using MEGAclear kits (Ambion Inc) or on 12% PAGE gels to remove unincorporated ATPs.

For RNase digestion, labeled RNA transcripts were refolded by incubating at 70 °C in RNA structure buffer containing 10 mM Tris-HCL (pH 7.0), 100 mM KCL, 10 mM Mg^{2+} supplemented with 1 ug of yeast tRNA for 10 minutes followed by slow cooling to room-temperature. Digestions were performed using either RNase T_1 (0.01 or 0.001 U/µl), RNase A (0.01 or 0.001 µg/ml), or RNase V_1 (0.001 or 0.0001 U/µl) at 25 °C for 10 min. RNase T1 and RNase A ladders were generated simultaneously by digesting 5'end-labeled transcripts that were heat denatured at 50 °C for 5 minutes, and digested with RNase T_1 (0.1 or 0.01 U/µl) and RNase A (0.01 or 0.001 µg/ml) at 25 °C for 5 minutes. Samples were precipitated, re-suspended in 10 µl of denaturing loading buffer, and subjected to electrophoresis in 12% or 20% sequencing gels. Alkaline hydrolysis ladders were

obtained by treatment of 5′ end-labeled transcripts in the alkaline buffer (Ambion Inc) at 95 °C for 5 min.

RNA sample preparation

The 31-mer RNA molecule corresponding to residues A1 - A31 in the 5' UTR of TRD and TC-83 genome, were synthesized by run-off *in vitro* transcription of appropriate DNA templates using T7 DNA-dependent RNA polymerase (Milligan et al., 1987). The HPLC-purified DNA templates were obtained from IDT and their purities were tested by gel electrophoresis. In vitro transcription reactions were carried out at 37 °C for 4 – 8 hrs. RNA transcripts were precipitated by ethanol and purified on 20% polyacrylamide gels containing 8M urea. The RNA oligonucleotides were then electro-eluted from the polyacrylamide gels, ethanol precipitated, equilibrated in NMR buffer (10 mM sodium phosphate (pH 6.5), 10 mM KCl and 0.05 mM EDTA), and concentrated using Centricon concentrator devices (Millipore). Concentrated RNA transcripts were lyophilized and dissolved in either 90% H_2O/10% D_2O or 99.96% D_2O for NMR experiments. The RNA samples were heated at 95 °C for three minutes and transferred to ice to facilitate the formation of hairpin loops. NMR samples were transferred to Shigemi tubes, with a volume of 300 ul and RNA concentrations of 0.2 – 0.5 mM.

UV melting studies

The melting of both wt TRD and vaccine strain TC-83 31nt RNA fragments were monitored by UV-absorbance at 260 nm, as a function of temperature on a Cary UV–visible spectrophotometer (Varian) equipped with a Peltier temperature control device. Standard 1 cm path length quartz cuvettes were used for the measurements. The heating rate was set at 0.5 °C/min and the data was recorded every minute. RNA stock solutions at 0.4 mM concentration were diluted (500 dilution factor) in buffers containing either 10 mM NaCl and 10 mM

KCl, or 10mM NaCl,10 mM KCl and 5 mM $MgCl_2$. The diluted samples were heated at 90 °C for 3 min followed by immediate immersion in liquid nitrogen for 3 min to enable the formation of RNA hairpin loops and to prevent formation of RNA duplexes. The melting temperatures of the RNA molecules were calculated from the melting curves. For each molecule, the melting curves were repeated at least twice.

NMR spectroscopy

All NMR spectra were acquired on Varian Direct Drive spectrometers (Varian Associates), operating at either 750 MHz or 800 MHz, equipped with pulsed field gradients and direct-drive architecture. Melting of the RNA molecule was studied by monitoring the imino proton NMR signals as a function of temperature. One-dimensional ^{1}H-NOESY spectra were collected at 5 °C, 10 °C, 15 °C, 20 °C, and 25 °C using spectral widths of 15 ppm and 1024 complex data points. 2D ^{1}H-^{1}H NOESY spectra (with 250 ms or 400 ms mixing times) were acquired at 5 °C and 25 °C. Proton spectral widths of 15 ppm were used in both the F2 and F1 dimensions for both samples in 99.96% D_2O and a total of 2048 complex data points were collected. The HOD signal at 4.76 ppm was used for referencing the spectra. NMR data were processed using VNMRJ software (Varian).

RESULTS

Predicted secondary structure of VEEV TRD and TC-83 5'UTR RNA

Predicted RNA secondary structure for TRD and TC-83 for various lengths of 5' terminal sequences were obtained using Mfold (Zuker, 2003) and Vienna fold (Hofacker, 2003) online webservers (Fig. 2.1). Both servers calculated identical secondary structures for same length RNA, with the 5' terminus

predicted to fold into a stem-loop for both TRD and TC-83. The stem-loop in TRD-specific 5'UTR contained 5 basepairs terminating in a huge loop with a calculated free energy of -8 kcal/mol. The stem-loop in TC-83-specific 5'UTR also had a 5 basepair stem but with an internal bulge resulting in a smaller terminal loop and a higher free energy of folding (-4.2 kcal/mol.) implying a less stable stem-loop structure. Although the stem region was predominantly made of G-C basepairs in both TRD and TC-83, they were quite different from one another, specifically with respect to the third nucleotide. G3 in TRD was predicted to basepair with C25 and was part of the stem, while in TC-83 A3 was part of the unpaired 5' terminal overhang. In TRD, the stem region began with an A-U basepair U2:A25 at the 5' terminus extending to C6:G21 and terminating in a loop constituting the residues G7- A20. In TC-83, on the other hand, the first three nucleotides were single stranded, the five basepair G-C rich stem, was separated into two short stems by a 5 nucleotide internal bulge. G4:C29 and G5:C28 formed the first, two basepair stem, while the second three basepair stem starts with G7:C23 and terminates with the C9:G21 basepair. Nucleotides G10 to A20 form the loop in TC-83.

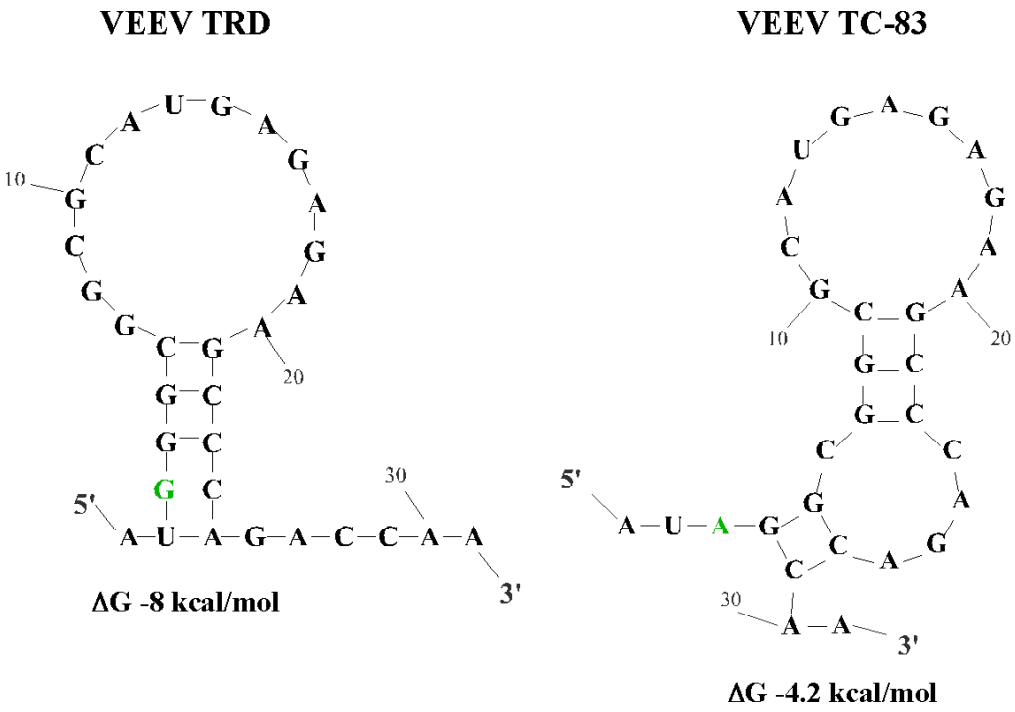


Figure 2.1: Mfold predicted secondary structure of VEEV TRD and TC-83 5' UTR stem-loops. The nt3 mutation and the theoretical free energy for folding for the 31nt RNA molecule calculated by Mfold are indicated.

Enzymatic analysis of TRD and TC-83 specific 5'UTR RNA secondary structure

The secondary structure and the folding of the 5' terminal stem-loop was determined by enzymatic analysis using nucleotide and strand specific RNases (Knapp, 1989) including RNase A (cleaves single-stranded cytosines and uracils), RNase T1 (cleaves single-stranded RNA at guanines), and RNase V1 (cleaves stacked or double stranded nucleotides). The 5' terminal RNA molecules corresponding to the first 135 nucleotides of both TRD and TC-83

were synthesized by in vitro transcription using T7 RNA polymerase, end-labeled and used for RNase probing. The enzymatic probing conditions were optimized such that 80-90% of the labeled RNA remained intact (Vournakis et al., 1981). The cleavage products were analyzed by polyacrylamide gel electrophoresis under denaturing conditions.

Analysis of the enzymatic probing revealed that the two stem-loops in TRD and TC-83 5'UTRs were indeed different (Fig. 2.2). In TRD, RNase V1 cleavage was observed for G3-G5, G21, C23, and C24; while in TC-83, RNase V1 cleaved G7, G8, G21, C24, C27, and C28. Partial T1 cleavage was observed for G21 in both TRD and TC-83, and G7 and G8 in TC-83, suggesting these nucleotides might possess alternate single-stranded conformations. Additionally, nucleotide C23 in TRD and C24 in both TRD and TC-83 were partially cleaved by RNase A, while C27 and C28 in TC-83 were strongly cleaved by RNase A suggesting two different confirmations for these residues. Strong RNase T1 digestion patterns were observed for nucleotides, G7 and G8 in TRD; G14, G16, and G18 in both TRD and TC-83; while weak cleavage pattern was observed for nucleotide G26 in both TRD and TC-83 suggesting these residues were in single-stranded conformation. Nucleotides C8 and C29 in TRD and C9, C11, U13 were strongly cleaved in both TRD and TC-83, while nucleotides C22, and C23 in both TRD and TC-83 were partially cleaved.

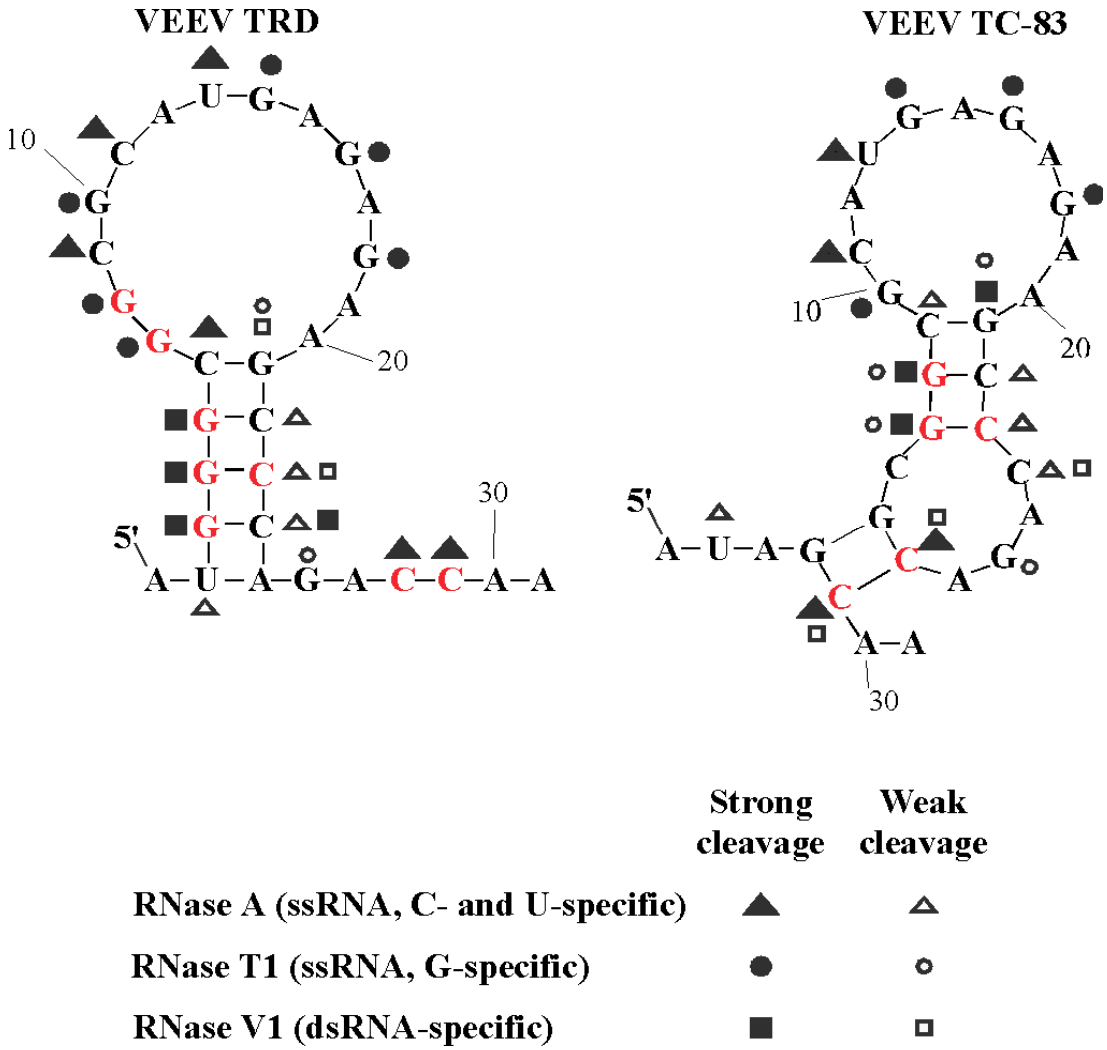


Figure 2.2: Enzymatic probing of TRD and TC-83 5'UTR RNA structure using RNase A, T1 and V1. Results of RNA cleavage patterns obtained from enzymatic digestion are mapped onto the predicted secondary structures. Residues that demonstrated marked differences in RNase cleavage marked in red. Activity of specific RNases is indicated by open and closed symbols

Mapping of the RNase digestion patterns onto the predicted secondary structures revealed marked differences between TRD- and TC-83-specific 5'stem-loop structures. The data clearly showed that nt3, G3 in TRD, was basepaired. Further, nucleotides G4 and G5 in TRD basepair with C23 and C22, while in TC-83 they basepair with C29 and C28 forming the two-basepair stem. However, the G4-C29 and G5-C28 base pairs were likely to be weak as both C28 and C29 in TC-83 were also cleaved strongly by RNase A. Nucleotides G7 and G8 form weak basepairs with C23 and C22 and are part of the three-basepair stem in TC-83 while, they are single-stranded in TRD. RNase A and T1 cleaved the Us, Cs and Gs between residues, G7-A20 in TRD and G10-A20 in TC-83, indicating they were single stranded. Lastly, nt C24 and G26 in TC-83 were cleaved by their respective single-strand specific enzymes supporting the existence of the internal bulge in TC-83-specific stem-loop. The results of enzymatic probing strongly supported the predicted stem-loop structures in the TRD and TC-83-specific 5'UTR.

Absorbance melting curves of RNA

Computer predicted secondary structure suggested a lower free-energy of folding for TRD-5'stem-loop and the results of enzymatic probing indicated that the stem-loop in TC-83 5'UTR is not very stable as multiple nucleotides were cleaved by both single and double-strand specific enzymes. The 31nt RNA oligonucleotides corresponding to the 5'stem-loop in TRD and TC-83 were subject to thermal melting analysis to calculate melting temperatures and to identify the effect of the nt3 mutation on the stability of the RNA secondary structure. RNA stocks diluted into 1 ml buffer solutions were used for thermal melting experiments. RNA samples were heated to 95 °C and slowly cooled to 20 °C at the rate of 0.5 °C/min to measure melting profiles. The A_{260} vs. temperature (dA/dT) profiles were plotted (Fig. 2.3) to calculate the melting temperature (Tm).

The melting temperature of the stem-loop in TRD-specific 5'UTR was 43.3 °C, while the TC-83-specific 5'stem-loop had a T_m of 34.8 °C. The lower melting temperature of TC-83 clearly suggested that the TC-83 stem-loop is less stable due to the nt3 mutation and the subsequent change in secondary structure.

NMR studies confirm that G3A mutation affects RNA structure and stability

Enzymatic probing experiments, while confirming the change in RNA secondary structure due to the nt3 mutation, also suggested that the terminal stem-loop in TRD is more stable than that in TC-83. Further, thermal melting temperatures of the TRD and TC-83 5'-terminal stem-loop RNA fragments, as monitored by UV-absorbance, revealed that the G3A mutation decreased the melting temperature of the indicated ribo-oligonucleotide by 8.5 degrees celcius. We then sought to identify the tertiary structure of the 5'terminal stem-loops using NMR spectroscopy. However, in order to solve the tertiary structure, it was essential to establish the optimal temperature conditions for NMR experiments. Imino proton signals in 1D NMR spectra are a good indication of RNA secondary structure. Sharp, downfield-shifted NMR signals in the imino region (11.5 -14 ppm) indicate that these protons are base-paired and are thus protected from rapid exchange with the solvent. Hence, 1D NMR spectra were collected as a function of temperature and the imino proton signals of the TRD and TC-83 5'UTR fragments were monitored. 1D NMR spectra were collected at 5 °C, 10 °C, 15 °C, 20 °C, and 25 °C with RNA samples in 90% H_2O. Additional 2D NMR spectra were collected at 5 ºC and 25 ºC with the RNA samples in either 90% H_2O or 99.96% D_20.

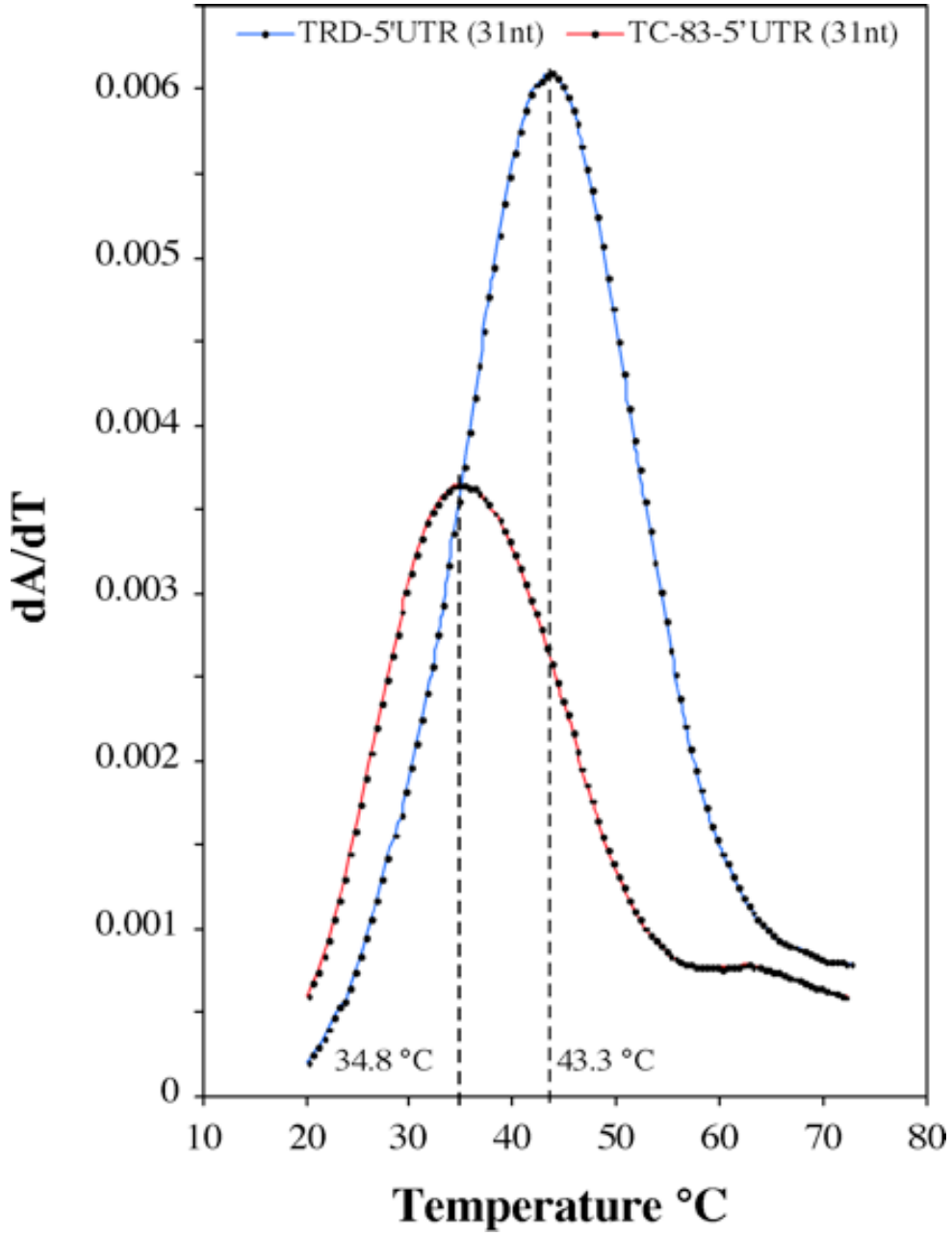


Figure 2.3: dA/dT plot of thermal melting curves for TRD (blue) and TC-83 (red) 5'UTR RNA observed by UV spectroscopy. The melting temperatures obtained from the plot are indicated by dotted lines

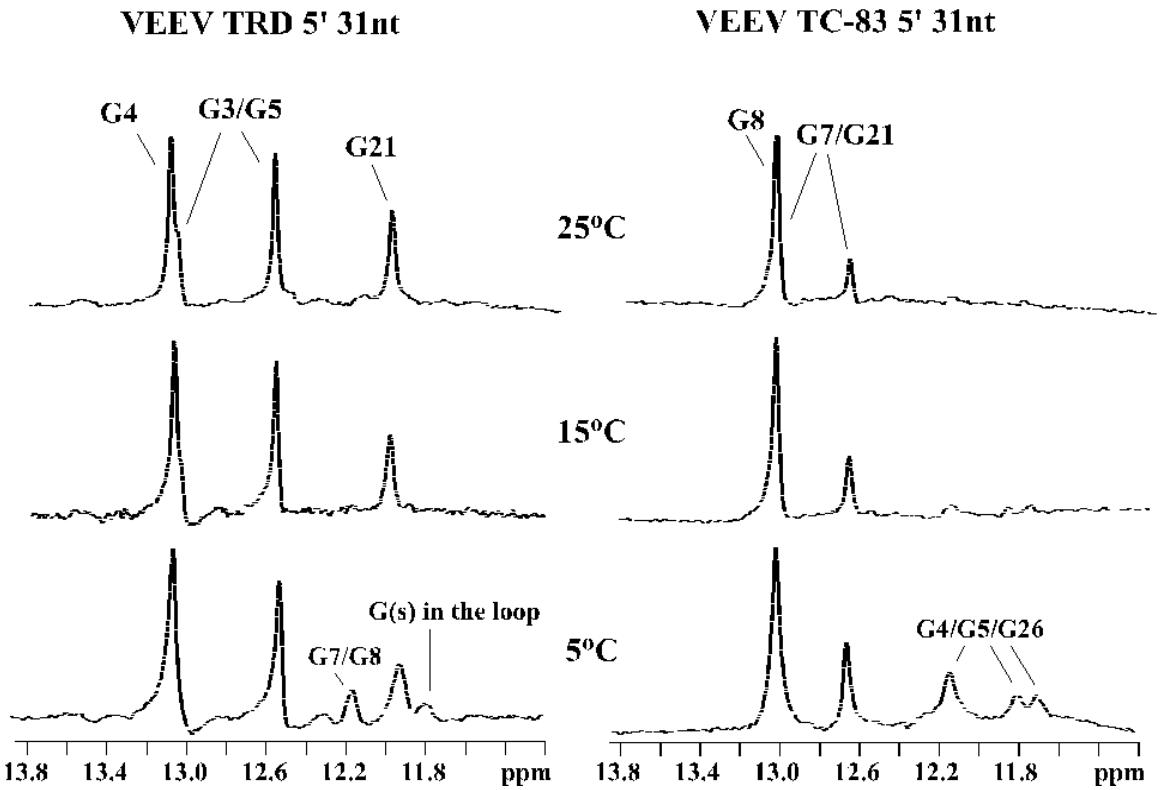


Fig. 2.4: NMR thermal melting analysis of TRD and TC-83 31nt RNA fragments. Comparison of the imino proton region of 1D NMR spectra collected at 5 ºC, 15 ºC and 25 °C. Residues that are base-paired in VEEV TRD and TC-83 31nt RNA fragments are indicated.

At 5 °C, 5 imino proton signals were observed for both TRD and TC-83 specific 5'stem-loops (Fig. 2.4). With increasing temperature, the number of imino signals detected decreases, indicating local unfolding and/or exchange with solvent. At 15 °C and 25 °C, TRD had 4 peaks while TC-83 had only 3 peaks. Exchangeable protons observed in the 1D spectra were assigned from 2D ^{1}H-^{1}H NOESY spectra. The imino and amino protons in base-paired residues are protected from solvent exchange and will give rise to imino-imino cross peaks in the 2D NOESY spectra. The imino protons were assigned from the cross-peak pattern observed in the 2D NOESY spectra for both TRD and TC-83 samples. TRD spectra suggested a 4 base-pair stem with cross-peaks between G4/G3 and G5, while TC-83 spectra suggested a 3 base-pair stem (Fig. 2.5). Assignments for at least two signals were not possible because they were observed only as diagonal peaks without any cross peaks. Disappearance of these cross-peaks with increasing temperature suggested that they probably were from residues in the loop for TRD and from the internal bulge or the two-base-pair stem in TC-83. Hence, in the TRD spectra, these peaks were tentatively assigned to residues G7/G8 or other Gs in the loop; while in TC-83 spectra, they were assigned to residues G26 in the internal bulge, and G4 and G5 in the two-base-pair stem. Generally, the imino-imino signals were broad and their intensities were weak, suggesting that the stem-region was not very stable. Only some of the aromatic hydrogen peaks were observed in the 2D NOESY spectra of which only a few were identified by the sequential H8/H6 to H1' cross peaks in the 2D NOESY spectra. Comparison of the 2D spectra (Fig. 2.6) strongly suggested that the TRD and TC-83 specific 5'terminal stem-loops had different secondary and tertiary structures.

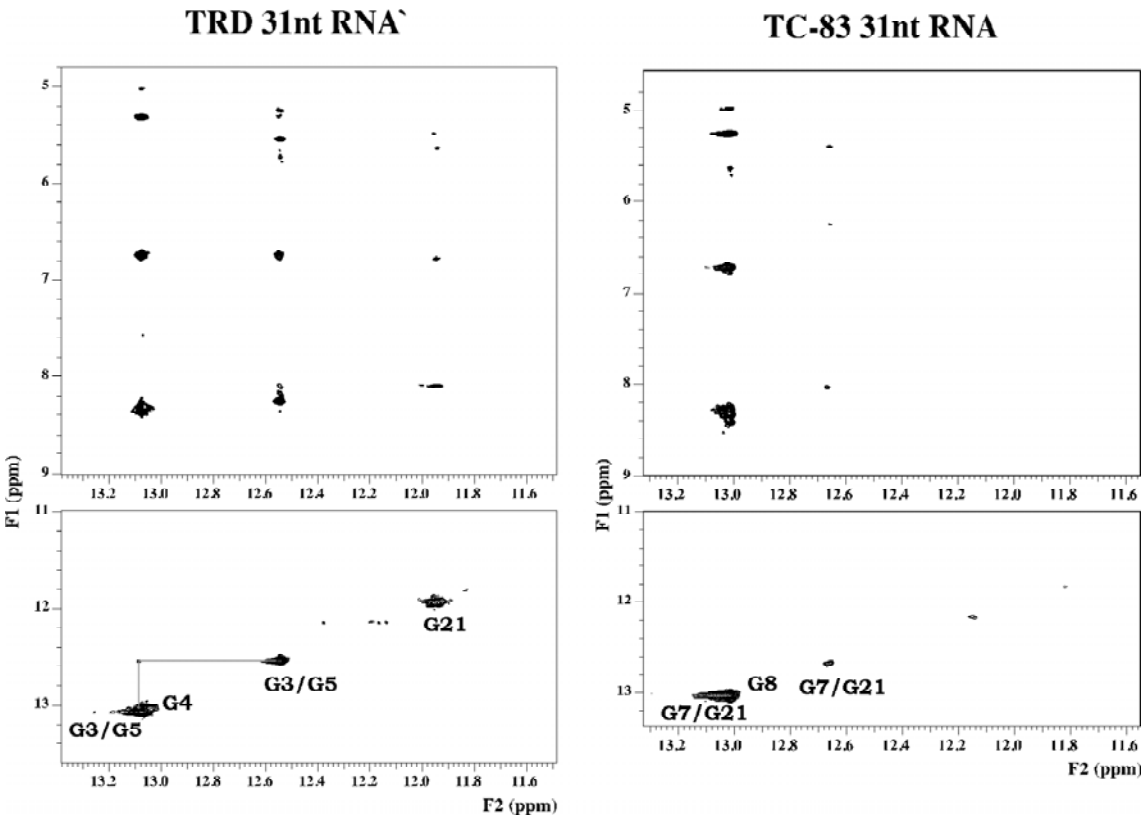


Figure 2.5: Portion of the 2D NOESY spectra for TRD and TC-83 31nt RNA molecules collected at 5 °C. The imino signals could not be assigned unambiguously due to the absence of cross-peaks and sequential NOEs

DISCUSSION

RNA viruses encode RNA elements in the 5'UTR that form secondary structures that play critical roles in several viral processes. These regions are bound by viral and host-protein factors that direct the processes in which these elements function. Mutations to these elements affecting both the sequence and the RNA secondary structure in different RNA viruses have been shown to affect

their functioning in various steps of viral replication. The alphavirus 5'UTR plays multiple roles in virus replication. Mutations to the 5'terminal stem-loop in the viral genome affect the pathogenicity of the virus (Kobiler et al., 1999; McKnight et al., 1996; White et al., 2001). In order to understand the role of these elements in various viral processes, it is essential to characterize the structure and the effects of the structural changes in these elements.

VEEV 5'UTR mutation was shown to be a critical determinant of virus pathogenecity but the molecular effects of the mutation is not clear (Kinney et al., 1993; White et al., 2001). Based on current evidence on the importance of RNA secondary structure of critical conserved elements in viruses, I hypothesized that the nt3 mutation in VEEV TC-83 changes the structure of the RNA element encoded in the 5' UTR, thus affecting its functions in virus replication, ultimately mediating virus attenuation. The studies described in this chapter were directed towards understanding the effect of the mutation on the 5'terminal stem-loop structure that is conserved across several alphavirus species.

RNA secondary structure prediction using Mfold and Vienna fold web servers (Fig. 2.1) suggested that the nt3 mutation changed the structure of the 5' stem-loop. The predicted free energy of folding for the TC-83-specific stem-loop was 4kcal/mol higher than that in TRD, suggesting that the mutation also affected the stability of the stem-loop. Enzymatic probing studies confirmed the change in secondary structures as key residues in TRD and TC-83 were identified to be single-stranded in one and double-stranded in the other. G7, G8, C28, and C29 in TRD were strongly cleaved by single-strand specific RNase T1 and RNase A, while they were cleaved by double-strand specific RNase V1 in TC-83. C28 and C29 were also cleaved by single strand specific RNase A, suggesting they formed weak base-pairs. G21 in both TRD and TC-83 was cleaved by both double- and single-strand specific enzymes, but mapping of the digestion patterns suggested that G21 base-paired with C6 in TRD, while it formed a weak

base-pair with C9 in TC-83. Similarly, G3 in TRD was strongly cleaved by RNase V1, was suggested to base-pair with C24, while A3 in TC-83 was suggested to be unpaired and was flanked by unpaired U2 and weakly base-paired G4.

Residues G7-A20 in TRD and G10-A20 in TC-83 were suggested to form the terminal loop because single-strand specific RNase A and T1 strongly cleaved the Cs, Us, and Gs in this fragment. Mapping of the enzymatic digestion onto the predicted secondary structure revealed a remarkable agreement between the results of enzymatic probing and RNA secondary structure prediction (Fig. 2.2).

Partial or strong digestion of certain key nucleotides in TC-83 by both the single-strand specific RNAses (RNase A & RNase T1) and double-strand specific RNase V1 suggests that the stem-loop structure in TC-83 5'UTR is not very stable compared to that in TRD 5'UTR. Thermal melting experiments using 31nt RNA fragments corresponding to the 5'terminal stem-loops affirmed these findings. TC-83 5' fragment had a melting temperature that was 8.5 °C lower than the TRD 31nt fragment. This low melting temperature of TC-83 was unexpected and so it was critical to identify the optimal temperature for NMR experiments. Hence, imino signals were monitored in 1D NMR experiments at different temperatures.

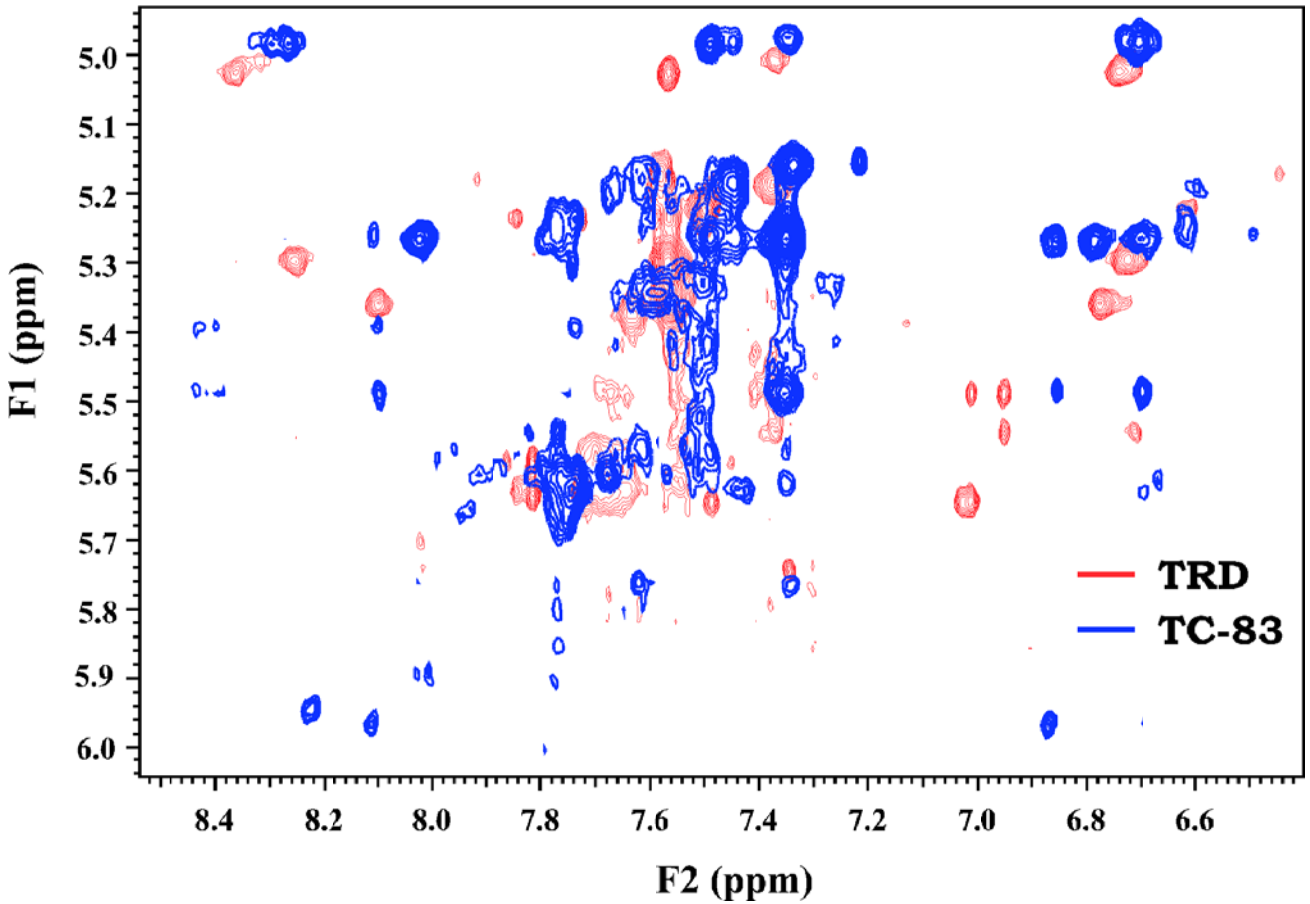


Fig. 2.6: Comparison of the H1'- H6/H8 regions of the 2D ^{1}H-^{1}H NOESY spectra for TRD and TC-83 31nt RNA fragments. Peaks corresponding to TRD are in marked red, while peaks for TC-83 are blue.

1D NMR experiments revealed dramatic differences in the imino hydrogen signals that correspond to the number of base-paired residues. Although NMR spectra obtained at 5 °C for both the TRD and TC-83 31nt fragments had 5 imino signals corresponding to 6 base-paired residues, the chemical shifts and intensities of the signals were different between the two spectra (Fig. 2.4). NOE cross-correlations observed in 2D NOESY experiments were used to assign the 1D imino proton signals. With increasing temperature the number of signals and the corresponding number of base-paired residues declined. Some of the signal assignments were ambiguous as no cross-correlations were observed, even at low temperatures, and the imino-imino cross peaks disappeared at high temperatures, suggesting weak hydrogen base-pairing of the residues. At 25 °C, 1D imino signals in the TRD 31nt fragment's spectra were observed only for residues G3, G4, G5, and G21, while in TC-83 spectra, signals corresponding to only G7, G8 and G21 remained. The residues that form the stem and the observed difference in the number of base-paired residues with increase in temperature clearly imply that the TRD and the TC-83 5'stem-loops have different structures.

In the 2D NOESY spectra for both of the 31nt stem-loop fragments, only a few peaks corresponding to the aromatic hydrogen atoms were observed, and subsequent comparison of the two spectra revealed marked differences (Fig. 2.6). Complete peak assignments in the homonuclear NOESY spectra were not carried out due to a lack of definite cross-correlations. However, both 1D and 2D NOESY spectra clearly indicate that the nt3 mutation changes the structure of the stem-loop in TC-83 5'UTR.

Studies of RNA stem-loops in other plus strand RNA viruses have shown these elements to affect several viral processes. The 5'UTR stem-loop in poliovirus was shown to modulate RNA translation in human cells. Similar studies of terminal stem-loops in BVDV (Becher, Orlich, and Thiel, 2000; Yu et al., 2000),

and Hepatitis C, have demonstrated that mutations to this region affect virus RNA replication and translation (Friebe et al., 2001). The complement of the 5' UTR in the 3'terminus of the alphavirus has been shown to bind host factors in addition to binding viral protein complex to initiate plus strand synthesis (Pardigon, Lenches, and Strauss, 1993; Pardigon and Strauss, 1992). Studies in SINV encoding a nt5 mutation have demonstrated that the mutation affects the negative strand synthesis even though virus replication is enhanced (Nickens and Hardy, 2008). Here, we show that the nt3 mutation affects the RNA structure and stability of the 5' terminal stem-loop encoded in the VEEV genome. However, the functional effects of the structural changes leading to the attenuation of the vaccine strain due to the mutation are still not clear. It is likely that the UTR-protein complex interactions are both sequence and structure specific and the nt3 mutation could affect these interactions ultimately resulting in viral attenuation and this needs to be studied further.

CHAPTER 3

FUNCTIONAL ANALYSIS OF THE EFFECT OF G3A MUTATION ON TRANSLATION OF VIRAL PROTEINS, VIRAL RNA SYNTHESIS, AND VIRUS REPLICATION

INTRODUCTION

Venezuelan equine encephalitis virus (VEEV) is one of the most pathogenic members of the *Alphavirus* genus in the *Togaviridae* family. In nature, VEEV and other serologically related viruses within the *alphavirus* persistently replicate in mosquito vectors, which infect vertebrate hosts during blood meals. In mammals, VEEV infection is characterized by high-titer viremia, rash and fever, followed by severe encephalitis that can result in death or neurological disorders (Dal Canto and Rabinowitz, 1981; Johnston and Peters, 1996; Leon, 1975). Among VEEV isolates, members of subtypes IAB and IC are responsible for VEEV infections in humans. These viruses continue to circulate throughout the Americas, causing several VEEV epidemics and epizootics. In spite of the continuous threat of VEEV epidemics, no safe and efficient vaccine, or therapeutic means have been developed for this pathogen. The only vaccine, VEEV TC-83, was developed more than four decades ago by the serial passage of the wild type Trinidad donkey (TRD) strain of VEEV in guinea pig heart cells (Berge, Banks, and Tigertt, 1961) and is currently used only as an experimental vaccine for at-risk laboratory workers and military personnel.

Comparative analysis of the wild type and the vaccine strain genomes revealed that TC-83 had accumulated 12 genomic mutations, but its attenuated phenotype was shown to rely only on 2 point mutations, an nt3 mutation in the 5' untranslated region (5'UTR) and an E2 envelope glycoprotein mutation (Kinney

et al., 1993). The T120→R mutation, located in the E2 glycoprotein, increases the protein's positive charge. Positively charged amino acid substitutions have been shown to enhance binding of an envelope glycoprotein to heparan sulfate surface receptors (Bernard, Klimstra, and Johnston, 2000). Such positively charged mutations in VEEV E2 were also shown to confer accelerated penetration and attenuated phenotype for the virus (Davis et al., 1991; Johnston and Smith, 1988). However, it was also demonstrated that the attenuating E2 mutations do not reduce the ability of the virus to illicit protective immune response (Davis et al., 1991). The second attenuating mutation, G3→A (G3A), found in the 5'UTR of the viral genome, was shown to affect the virulence of the virus in adult mice, having a fully competent type I IFN system (White et al., 2001). However, the molecular mechanism of the nt3 mutation mediated decrease in virulence and attenuation of VEEV remains to be understood.

The alphavirus 5'UTR plays a role in several processes during virus replication. It is necessary for the translation initiation of the viral nonstructural proteins (nsP1-4) that form the virus replicase required for the replication of viral genome and transcription of the subgenomic RNA (Barton, Sawicki, and Sawicki, 1990; Lemm and Rice, 1993a). In addition to its role in translation initiation, the 5'-terminal sequence is involved in other processes in RNA replication. The 5'UTR is one of the four conserved sequence elements that are involved in viral RNA replication. The complement of this sequence at the 3' end of the negative strand RNA intermediate functions as a promoter for positive strand genome synthesis (Gorchakov et al., 2004; Niesters and Strauss, 1990), and in the case of other alphaviruses, the 5'UTR was also suggested to function as a part of the promoter of negative strand RNA synthesis (Frolov, Hardy, and Rice, 2001; Gorchakov et al., 2004). Given these key roles, it is logical to expect that the G3A mutation may affect both the translation initiation of viral proteins and the synthesis of virus-specific RNAs.

Here we describe the results of our studies to understand the effect(s) of the 5'-terminal, TC-83-specific mutation on virus replication. Our data indicate that the G3A mutation does not have a major effect on the translation of viral nsPs. However, it significantly increased viral genome synthesis during replication, affecting the ratio of genomic and subgenomic RNA synthesis and together, these effects enhanced virus replication *in vitro.* Based on our results, the current knowledge of viral RNA synthesis and on previous studies implicating the role of type 1 IFN in virus attenuation, we propose a model for the 5'UTR mutation mediated attenuation of VEEV.

MATERIALS AND METHODS

Cell cultures

BHK-21 cells were kindly provided by Dr. Sondra Schlesinger (Washington University, St Louis, MO). NIH 3T3 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA). BHK-21 and NIH 3T3 cells were propagated in Alpha MEM supplemented with 10% fetal bovine serum (FBS) and vitamins.

Plasmid constructs

The parental plasmid with VEEV TC-83 genome and the VEE/SINV chimera encoding VEEV TRD-specific 5'UTR were described elsewhere (Petrakova et al., 2005). A VEE/SINV chimeric virus, denoted as pG3/VEE/SINV, encoded for the 5'UTR of the VEEV TRD strain, 3' UTR, subgenomic promoter and ns polyprotein-coding sequence from VEEV TC-83 and structural polyprotein-coding sequence from SINV Toto1101 (Rice et al., 1987). The second chimeric virus, denoted as pA3/VEE/SINV differed from pG3/VEE/SINV only by one nucleotide (A3), specific for the VEEV TC-83 5'UTR. In both

genomes, the poly(A) sequence was followed by a MluI restriction site. pA3/VEErep/Pac and pG3/VEErep/Pac replicons had viral structural genes replaced by a puromycin acetyltransferase (Pac) sequence (Petrakova et al., 2005).

pG3/Luc and pA3/Luc encoded the promoter for the SP6 DNA-dependent RNA polymerase, followed by nt 1-191 of the viral genome, fused with the entire ubiquitin gene and firefly luciferase-coding sequence. This protein-coding sequence was followed by VEEV TC-83-specific 3'UTR, poly(A) and MluI restriction site, required for linearization of the plasmid before the in vitro transcription reaction.

pG3/DI/Luc and pA3/DI/Luc plasmids encoded defective viral genomes under control of the SP6 promoter. These genomes contained the 5' terminal 519 nucleotides derived from VEEV TRD or VEEV TC-83 genomes, respectively, followed by nt 7291-7564 (encoding VEEV subgenomic promoter), a firefly luciferase gene, 3' terminal nt 11202-11446 of VEEV genome, poly(A) tail and MluI restriction site.

All plasmids encoding modified VEEV genomes and luciferase were constructed by standard PCR-based mutagenesis and cloning methods. After cloning into the plasmids, all of the PCR fragments were sequenced to exclude the possibility of spontaneous mutations.

RNA transcription

Plasmids were purified by centrifugation in CsCl gradients. Prior to transcription, they were linearized by MluI, and RNAs were synthesized in vitro by SP6 RNA polymerase in the presence of cap analog (Rice et al., 1987) under the conditions recommended by the manufacturer (Invitrogen). The yield and integrity of the transcripts were monitored by gel electrophoresis under non-denaturing conditions, followed by analysis of the RNA concentration on a

FluorChem imager (Alpha Innotech) and by UV/VIS spectroscopy. For virus rescue and analysis of DI RNA replication, the appropriate volumes of reaction mixtures were directly used for electroporation. For comparative studies of translation efficiencies of the Luc-coding RNAs, the transcripts were additionally purified using RNeasy columns (Qiagen), and RNA concentration was measured as described above.

RNA transfection

BHK-21 cells were electroporated with 2 µg of in vitro-synthesized, viral genome RNA using previously described conditions (Liljeström et al., 1991), and cells were seeded into 100-mm dishes. The released viruses were harvested 24 h post transfection. Titers were determined by a plaque assay on BHK-21 cells. For analysis of DI RNA replication, replicons and DI RNAs were co-electroporated as described in the figure legends. Equal amounts of cells were seeded into 35-mm dishes, and luciferase activity was measured at different times post transfection by using a luciferase assay kit according to the manufacturer's instructions (Promega). To assess the translation efficiency of the templates, equal amounts of A3/Luc and G3/Luc RNAs were electroporated into BHK-21 cells, equal aliquots of the cells were seeded into 35-mm dishes, and luciferase activity was measured at different times post transfection using Dual-Luciferase kit (Promega). To determine the stability of the RNAs, similar amounts of ^{32}P-labeled, in vitro-synthesized A3/Luc and G3/Luc RNAs were electroporated into BHK-21 cells, equal aliquots of the cells were seeded into 35-mm dishes. At the indicated times, RNAs were isolated by using TRizol reagent as recommended by the manufacturer (Invitrogen), followed by electrophoresis in agarose gel and autoradiography. Dried gels were further analyzed on a Storm phosphorimager (Molecular Dynamics). The radioactivity detected in the RNA bands was normalized to the 0-h samples isolated immediately after RNA

transfection. Replicons were packaged into infectious viral particles by co-electroporation of the in vitro-synthesized replicon and two helper RNAs (Volkova, Gorchakov, and Frolov, 2006) in BHK-21 cells. Media were harvested 24 h post electroporation, and titers of the packaged replicons were determined by infecting BHK-21 cells with different dilutions of the samples and measuring the numbers of infected, GFP-expressing cells after 8 h of incubation at 37 ^{o}C in a CO_2 incubator.

In vitro translation assay

Different amounts of in vitro-synthesized A3/Luc and G3/Luc RNAs were translated in 10 ml of the rabbit reticulocyte lysate (RRL) system according to the manufacturer's instructions (Promega). Aliquots of the reaction mixtures were taken after 1 h incubation at 30 ^{o}C, and luciferase activity was measured using luciferase assay kit according to the manufacture's protocol (Promega).

Analysis of virus replication

5 x 10^5 BHK-21 or NIH 3T3 cells were seeded into 35-mm dishes. After 4 h of incubation at 37 ^{o}C in 5% CO_2, monolayers were infected at a multiplicity of infection (MOI) of 10 PFU/cell for 1 h, washed three times with phosphate-buffered saline (PBS), and overlaid with 1 ml of complete medium. At the indicated times post infection, media was replaced by fresh media, and virus titers in the harvested samples were determined by plaque assay on BHK-21 cells as previously described (Lemm et al., 1990).

Analysis of viral RNA synthesis

BHK-21 cells were infected with chimeric viruses at an MOI of 10 PFU/cell. At the indicated times post infection, the intracellular RNAs were labeled with [^{3}H]uridine (20 mCi/ml), in the presence of 1 mg/ml of D-actinomycin (ActD)/ml for 4 h at 37^{o}C in 5% CO_2. RNAs were isolated from the cells using

TRIzol reagent according to the manufacturer's instructions (Invitrogen). The RNAs were denatured with glyoxal in dimethyl sulfoxide and analyzed by agarose gel electrophoresis as previously described (Bredenbeek et al., 1993). For quantitative analysis, the RNA bands were excised from the 2,5-diphenyloxazole (PPO)-impregnated dried gels, and the radioactivity was measured by liquid scintillation counting.

RESULTS

G3A mutation moderately enhances RNA translation efficiency

The NMR studies and enzymatic probing data (Chapter 2) demonstrated that the G3A mutation destabilized the 5'-terminal stem-loop in the VEEV genome. This destabilization of the 5'terminal sten-loop could have a strong effect on the translation of the encoded ns proteins. In addition, it is also likely that the destabilization of the stem-loop in the 5'terminus may also affect the stability of the viral RNA in cells. To assess the effect of the mutation on RNA stability and on protein translation, we designed reporter, luciferase-expressing constructs (Fig. 3.1 A) containing either TRD or TC-83-specific 5'UTR, and compared the efficiency of translation of the encoded proteins both in vitro and in vivo.

The reporter constructs contained the 5'-terminal, 1-191-nt of the VEEV genome, encoding either TC-83 or TRD 5'UTRs and the first 49 aa of nsP1, cloned under control of the SP6 promoter. The RNA sequence coding for the nsp1 was included to preserve the secondary structure architecture of the 5' termini. A firefly luciferase gene was fused in frame with the nsP1-coding sequence using the ubiquitine-coding sequence. Ubiqutin was used inframe with the upstream nsp1 and downstream luciferase to express luciferase in a free

form. These cassettes also contained VEEV-specific 3'UTR, and the 25-nt-long poly(A) tail to mimic the viral RNAs.

Initially, to determine if the nt3 mutation affected RNA stability in cells, we transfected BHK-21 cells with ^{32}P labeled RNA cassettes. Total cellular RNA was isolated at 0, 1, 2 and 4 h post transfection and analyzed by gel electrophoresis in denaturing conditions. Radioactive decay of the RNA was assessed using a phosphorimager (Fig. 3.1 B). Both TRD and TC-83 5'UTR encoding RNA cassettes exhibited the same rates of degradation, suggesting that the mutation did not affect the stability of the viral mRNAs in cells.

Rabbit reticulocyte lysate (RRL) translation system was used for in vitro translation of in vitro-synthesized, capped RNA cassettes. Different concentrations of the template capped RNAs were used in the experiments to avoid saturation. RNA cassettes having the TC-83-specific 5'UTR demonstrated a higher luciferase expression suggesting that the G3A mutation had a positive effect on RNA translation (Fig 3.2 A). The RNA cassettes were then transfected into BHK-21 cells and luciferase expression was then evaluated at different times post-electroporation (Fig 3.3 B). The translation efficiency of the cassettes observed in cells is biologically more relevant as it closely resembles translation of the viral RNA in cells. RNA coding for Renilla luciferase was co-transfected in certain experiments for normalization of the translation data. In multiple repeat experiments, RNA templates encoding TC-83-specific 5'UTR were slightly more efficient in translation of the downstream nucleotides than RNA cassettes encoding TRD-specific 5'UTR. These results clearly demonstrated that the nt3 mutation did not affect the stability of the viral RNAs in cells and moderately enhanced the translation of the encoded proteins as evidenced by increased luciferase expression.

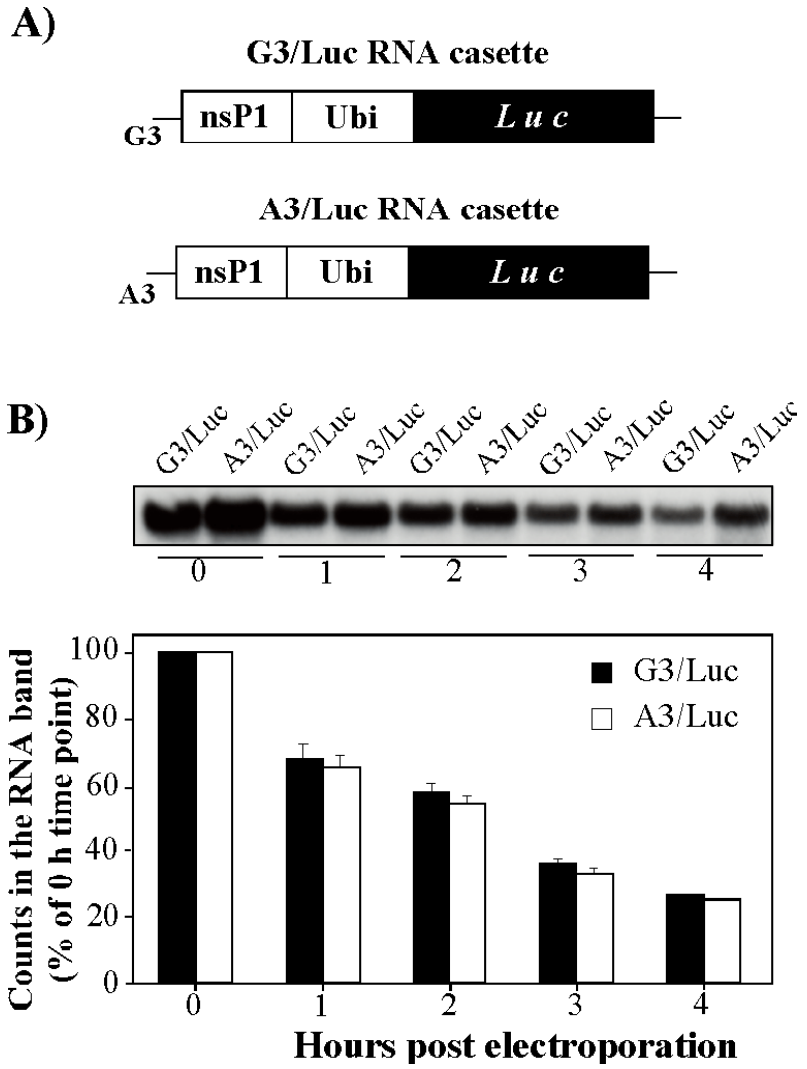


Figure 3.1. Effect of G3A mutation on RNA stability in cells. (A) The schematic representation of the firefly luciferase constructs used to evaluate the effect of G3A mutation on the template translation. (B) Analysis of RNA stability using labeled RNA transcripts.

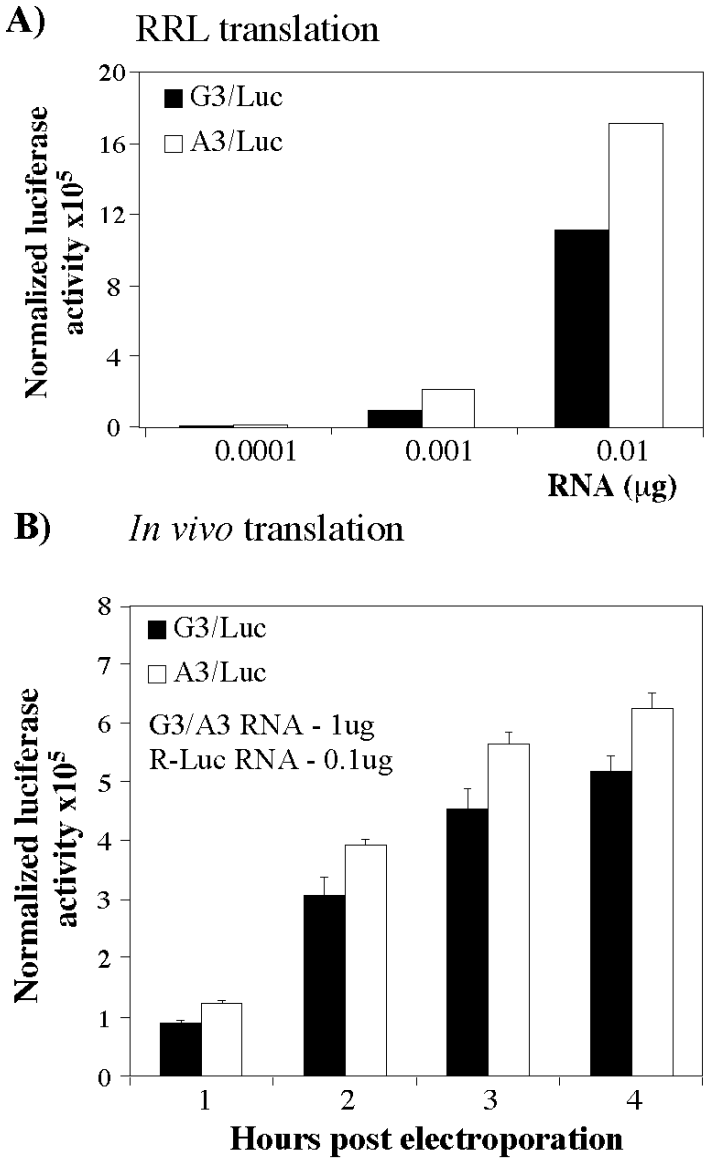


Figure 3.2. Effect of G3A mutation on RNA translation efficiency. (A) Luciferase activities of G3/Luc and A3/Luc capped template RNAs using Rabbit Reticulocyte Lysate reaction mixture (D) Firefly luciferase expression in BHK-21 cells transfected with the in vitro-synthesized, capped G3/Luc or A3/Luc RNA.

Effect of G3A mutation on virus-specific RNA synthesis

The 5’ terminus of the alphavirus genome and its complement in the negative strand RNA of the replicative intermediate function as part of the promoters for negative and positive strand RNA synthesis, respectively (Strauss and Strauss, 1994). 5’UTR mutations in other alphaviruses have been shown to affect virus replication and genomic RNA synthesis (Frolov, Hardy, and Rice, 2001; Gorchakov et al., 2004; Niesters and Strauss, 1990). Hence, it is likely that the G3A mutation could have a significant effect on viral RNA synthesis and virus replication. To identify the effect of the mutation on virus replication and RNA synthesis, we designed infectious cDNA constructs for recombinant alphavirus genomes encoding TRD and TC-83 specific 5’UTRs. The recombinant chimeric virus genomes (G3/VEE/SINV and A3/VEE/SINV) contained VEEV genome specific nonstructural protein genes and cis-acting RNA elements, while structural proteins encoded in the subgenomic RNA were derived from Sindbis virus (Fig 3.3 A). These chimeric viruses lacking VEEV-specific structural proteins were highly attenuated in mice and exhibited low cytopathogenicity in cells (Garmashova et al., 2007a; Petrakova et al., 2005). Although, *in vitro* synthesized RNAs encoding chimeric virus genomes demonstrated low cytopathogenicity upon transfection into BHK-21 cells, the chimeras (the resultant viruses) were still capable of replicating to high titers without forming pseudorevertants, and hence made for an attractive system to work with as they did not require high bio-containment.

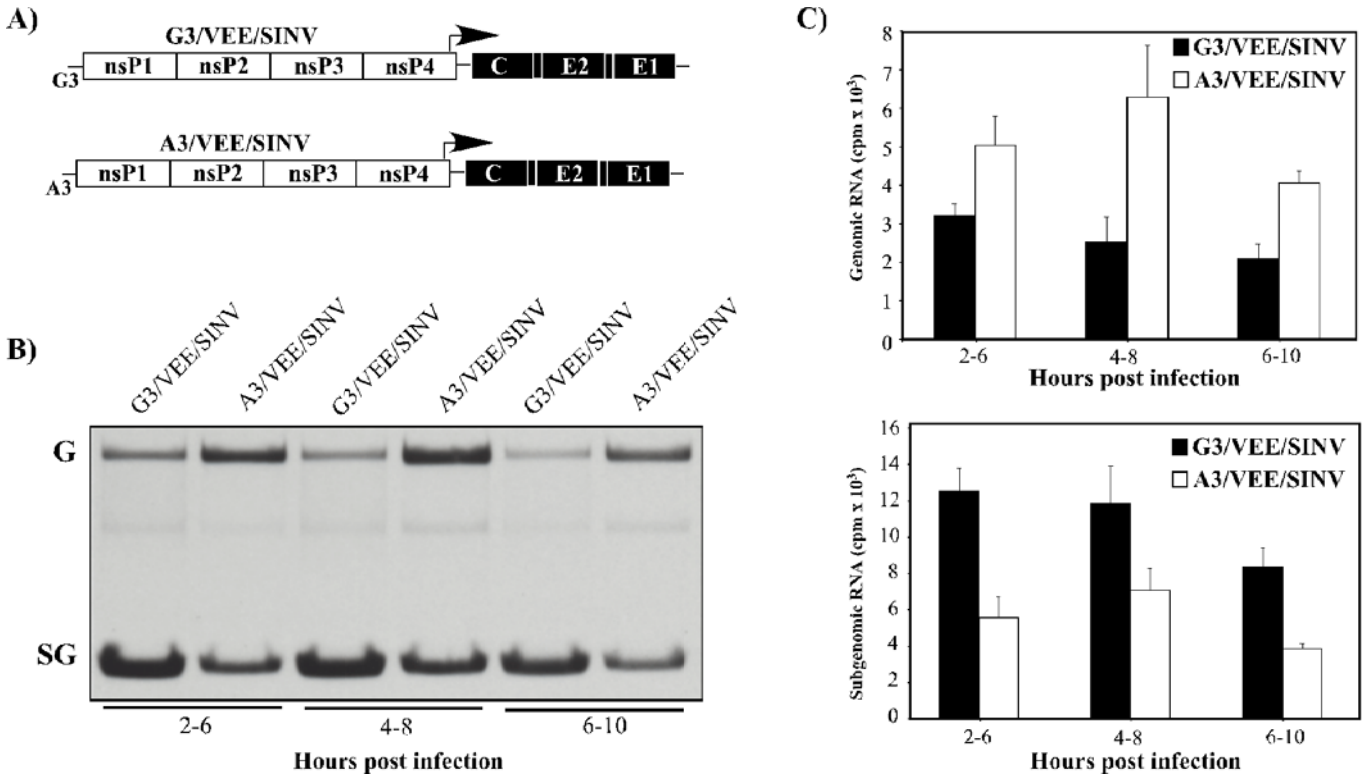


Figure 3.3. Effect of G3A mutation on virus-specific RNA synthesis. (A) Schematic representation of G3/VEE/SINV and A3/VEE/SINV chimeric virus genomes encoding TRD and TC-83-specific 5'UTRS respectively. Open boxes indicate VEEV-specific sequences and filled boxes indicate SINV-specific structural genes. (B) Analysis of virus-specific RNA synthesis in BHK-21 cells were infected with chimeric viruses. G and SG indicate positions of the viral genomic and subgenomic RNAs, respectively. (C) Comparison of genomic and sub-genomic RNA synthesis, calculated from the radioactivity in RNA bands excised from the gel.

First, we evaluated the infectivity of chimeric viral RNAs. The in vitro-synthesized RNAs exhibited the same infectivity as the VEEV TC-83 RNA in the infectious center assay (5-10x10^5 PFU/mg of transfected RNA) and universal plaque sizes, which indicated that no adaptive mutations were required for the viability of the chimeras. Virus stocks having titers above 10^9 PFU/ml were harvested at 24 h post transfection. Next, we evaluated the synthesis of viral genomic and subgenomic RNAs at different times post infection. BHK-21 cells were infected with chimeric viruses at an MOI of 10 and the virus specific RNAs were metabolically labeled with [^{3}H] uridine in the presence of ActD. The RNAs were isolated from cells, analyzed by agarose gel electrophoresis (Fig. 3.3 B), and the efficiency of virus-specific RNA synthesis was assessed by measuring radioactivity incorporated in the RNA. Our results demonstrated that the G3A mutation had a positive effect on the replication of viral genome but affected subgenomic RNA synthesis (Fig. 3.3 C). This increase in viral genome RNA synthesis resulted in a 6-fold decrease in the molar ratio of the subgenomic to genomic (SG:G) RNA synthesis.

A higher level of genomic RNA synthesis by viruses harboring the G3A mutation could be due to the enhanced translation efficiency of RNAs encoding the mutation resulting in increased synthesis of viral replicase that function in RNA synthesis and due to the change in promoter efficiency. Therefore, to further understand the effect of the mutation on virus replication, we designed i) VEEV replicons A3/VEErep/Pac and G3/VEErep/Pac and ii) defective viral genomes A3/DI/Luc and G3/DI/Luc. The VEEV replicons encoded the viral nonstructural proteins and the Pac gene, under the subgenomic promoter; while the DI genomes encoded the 5' 519 nucleotides and firefly luciferase under control of the subgenomic promoter (Fig. 3.4 A). The DI RNAs lacking viral nonstructural genes were incapable of self-replication, and thus, required trans-

complementation with nsPs. When co-transfected, the DI RNAs were expected to compete with the replicons for the viral nsPs, translated from the replicon genomes, for replication. The promoter sequences in the 5' terminus determined the efficiency of DI RNAs to compete for the viral nsPs and defined the level of luciferase expression. Different combinations of the in vitro-synthesized replicon and DI genomes were co-transfected into BHK-21 cells, and the luciferase activity was determined at different times post electroporation. A3/DI/Luc RNA, encoding TC-83-specific 5'UTR, was more efficient in utilizing the VEEV replicative enzymes supplied in trans, and synthesized more luciferase than G3/DI/Luc (Fig 3.4 B).

We then assessed the effect of the mutation on subgenomic RNA synthesis. We designed VEEV replicons A3/VEErep/Luc/GFP and G3/VEErep/Luc/GFP, encoding firefly luciferase and GFP under the control of the subgenomic promoters (Fig 3.5 A). Both replicons were packaged into infectious virus particles by using helper RNAs encoding viral capsid and glycoproteins. Packaged viruses were harvested 24 hours post transfection of cells with replicon and helper RNAs. Virus titers were assessed and cells were infected at an MOI of 10. Luciferase activity was determined at different times post infection and luciferase expression was higher in cells infected with replicons encoding TRD-specific 5'UTR (Fig. 3.5 B). A significant, 3 – 4 fold difference was observed by 10 and 12 h post transfection. Similar results were obtained by measuring GFP fluorescence levels by flow cytometry (data not shown), in which a 3-fold difference was detected in the mean fluorescence of GFP by 8 h post infection. This clearly suggested that the G3A mutation lowered subgenomic RNA synthesis.

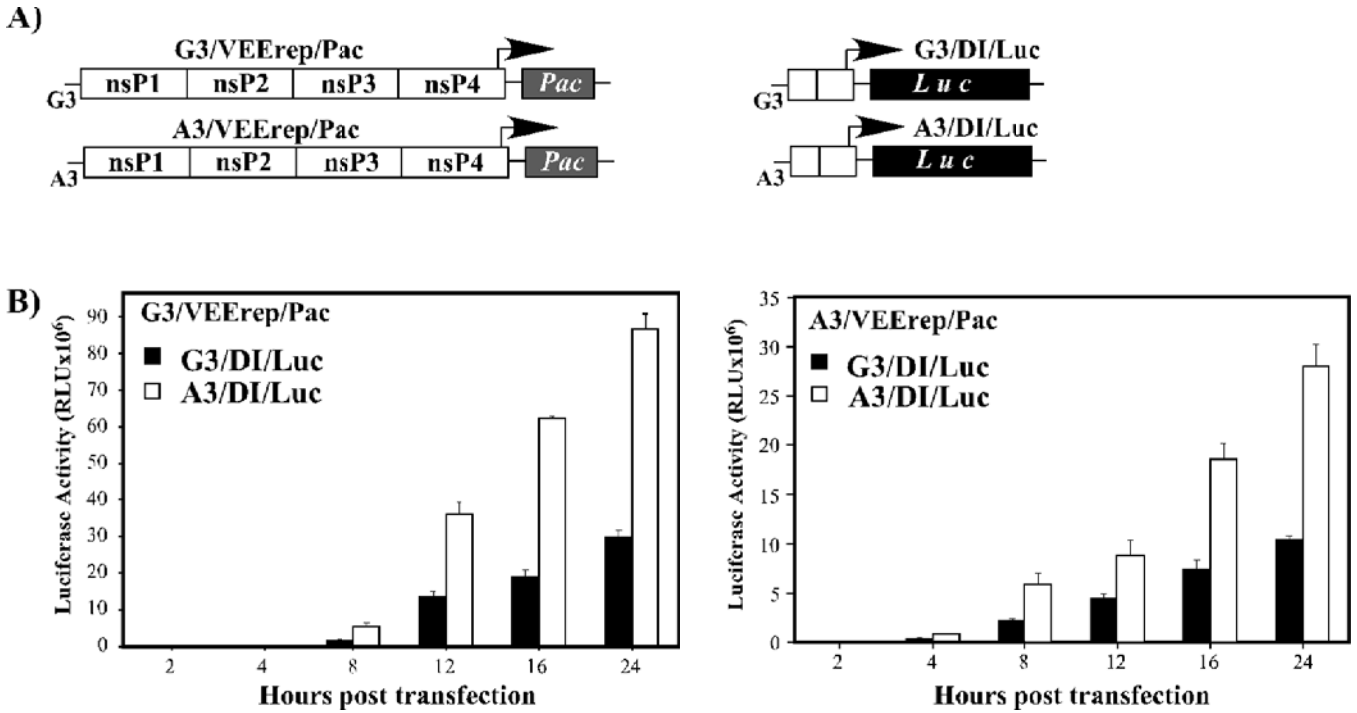


Figure 3.4. Replication of the DI RNAs encoding VEEV TRD- and VEEV TC-83-specific 5'UTRs, in the presence of VEEV replicons. (A) The schematic representation of the DI RNA and VEEV replicon genomes used in the study. (B and C) Efficiency of RNA synthesis by A3- and G3-DI RNAs measured from the luciferase activity.

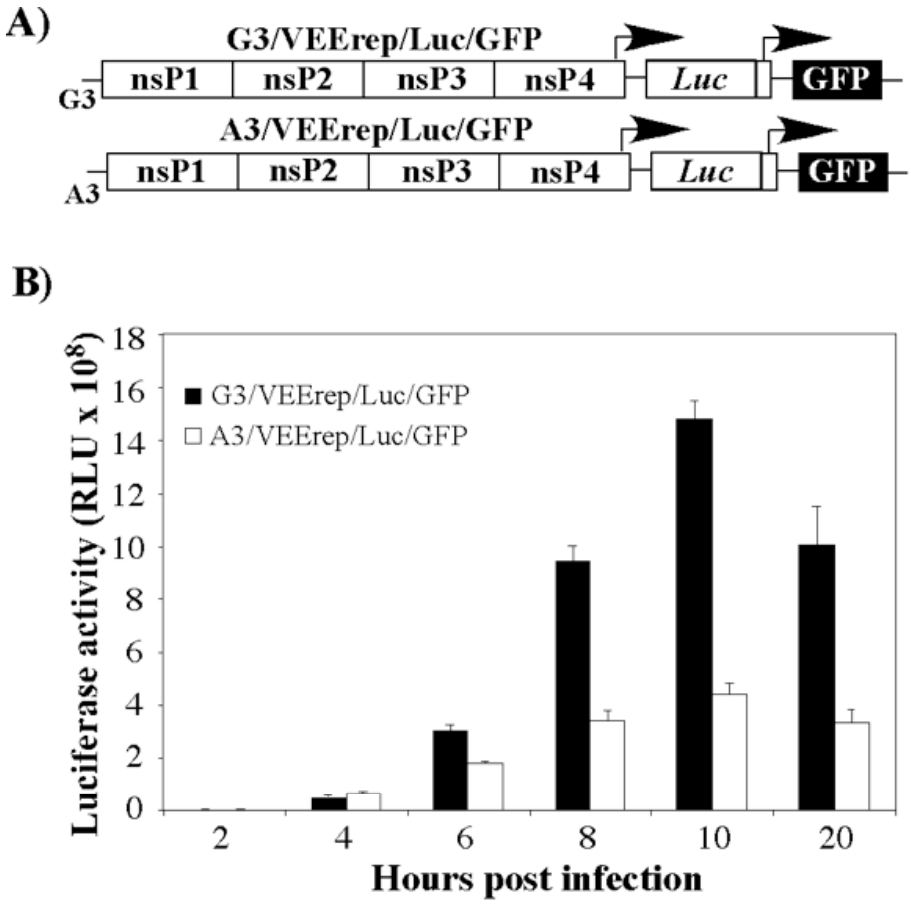


Figure 3.5. Expression of genes encoded by subgenomic RNA of VEEV replicons harboring either VEEV TRD- or VEEV TC-83-specific 5'UTRs. (A) Schematic representation of the replicon genomes. (B) Efficiency of subgenomic RNA translation measured by luciferase activity.

Together, these results suggest that the increased genome RNA synthesis observed in chimeric virus harboring the G3A mutation is a result of enhanced 5'UTR RNA promoter efficiency and not due to the increase in translation of viral polyprotein complex. Further, the observed decrease in subgenomic RNA synthesis suggests that the mutation in the 5'UTR improved the affinity of the promoter for viral replicase resulting in increased promoter activity in genomic RNA replication and thus, reduced the molar ratio of SG:G RNA synthesis.

Effect of G3A mutation on virus replication

Next, we assessed the replication rates of both A3/VEE/SINV and G3/VEE/SINV variants in BHK-21, NIH 3T3 and mosquito $C_7$10 cells to evaluate the effect of the G3A mutation on virus replication. Cells were infected with viruses at an MOI of 10 PFU/cell and media was replaced at specific times post-infection to evaluate virus replication rates. Both chimeric viruses demonstrated efficient replication in all cell types, but at a lower rate than the VEEV TC-83 control (Fig. 3.6). However, A3/VEE/SINV chimeric virus, encoding TC-83 specific 5'UTR, continued to grow at a better rate and to higher titers than G3/VEE/SINV, encoding the TRD- specific 5'UTR. These results correlate with the enhanced genomic RNA synthesis and suggest that the rate of genomic RNA synthesis is probably vital for increased virus replication.

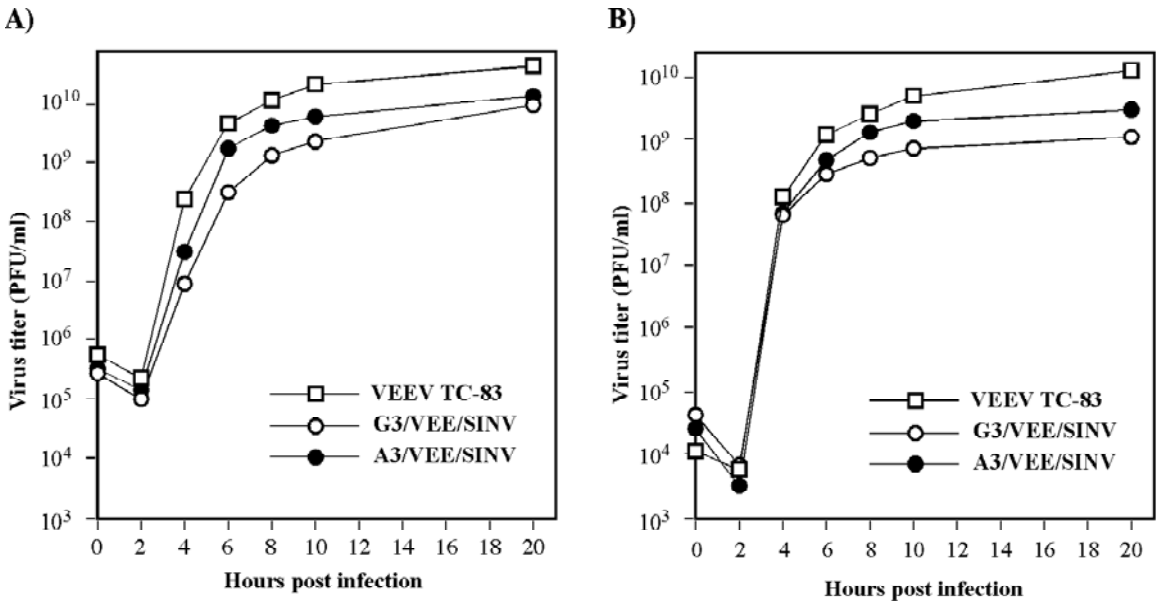


Figure 3.6 Analysis of chimeric virus replication in BHK-21 (A) and NIH-3T3 (B) cells

DISCUSSION

Alphaviruses are predominantly mosquito borne viruses that infect a wide range of hosts. In order to maintain the virus infectious cycle, these viruses are capable of replicating efficiently in many hosts to sustain a persistent infection. Virus replication in the hosts is accomplished by hijacking the host translational apparatus for translation of the viral proteins that form the virus replicase and the viral envelope. The genome encoded virus replicase then replicates the virus genome that is assembled to produce progeny viruses. In addition to their roles in virus replication and assembly, the viral proteins are involved in other aspects of infection and function in the development of cytopathogenicity, and down regulate anti-virus cellular responses (Frolov, 2004; Frolova et al., 2002; Garmashova et al., 2006; Strauss and Strauss, 1994). The differences among TRD and TC-83 strains regarding efficiency of VEEV infection, the virulence of the virus, is determined by an nt3 mutation in the 5'UTR and a T120R mutation in the envelope glycoprotein E2 (Kinney et al., 1993). Other data also demonstrate that the G3A mutation in the VEEV 5'UTR strongly affects the pathogenicity of the virus (Kinney et al., 1993; White et al., 2001) and the ability of the VEEV-specific replicons to cause cytopathogenic effects (Petrakova et al., 2005). White and co-workers showed that the G3A mutation results in increased sensitivity to INF-α/β and plays a major role in attenuation of the virus, however the molecular mechanism for increased sensitivity to interferon and subsequent attenuation is not clear. Our previous experiments (Chapter 2) demonstrate that the G3A mutation reduced the stability of the 5'-terminal stem-loop and, most likely, caused a similar change in the 3'end of the negative strand intermediate. The very 5' end of the VEEV genome has been shown to contain two functional elements (Ou, Strauss, and Strauss, 1983), one of which is located in the 5'UTR. The 5'UTR (more precisely, its complement in the negative strand of the viral genome) was defined as a core promoter, and some of the mutations in this

sequence or its replacement by the 5'UTR derived from other alphaviruses, have deleterious effects on RNA replication (Frolov, Hardy, and Rice, 2001; Gorchakov et al., 2004; Niesters and Strauss, 1990). Accordingly, it is highly likely that the G3A mutation could affect multiple processes of virus replication. Our experiments were directed towards elucidating the possible molecular mechanism for virus attenuation by studying the effects of the mutation on various processes involved in virus replication. The findings presented in this chapter provide a plausible explanation for attenuation of the TC-83 strain and for the variances in the cytopathogenicity of the replicons differing only in the nt3 of the 5'UTR. Further, the results suggest that the secondary structure of the 5' terminal RNA fragment plays crucial role(s) in virus replication.

Our first experiments, which focused on identifying the effect of the nt3 mutation on translation of the downstream-encoded proteins, suggested that the G3A mutation moderately enhanced the replication of viral proteins. Thermal melting experiments monitored by NMR and UV-spectroscopy revealed that the TRD-specific 5'terminal stem-loop was more stable. Stem-loops at the very 5'terminus of the genomes generally are known to affect the translation efficiency (Babendure et al., 2006; Kozak, 2005). Compared to mRNAs encoding stable stem-loops, stem-loops of lower stability have higher translation efficiency (Babendure et al., 2006; Vega Laso et al., 1993). Additionally, the position of the stem-loop from the cap has also been shown to affect translation efficiency (Babendure et al., 2006); with stem-loops starting just one nucleotide from the cap having the least translation efficiency. Given that the TRD-specific stem-loop has higher stability and is one nucleotide from the cap one would expect mRNAs encoding it to have low translation efficiency compared to those encoding TC-83-specific 5'UTR. However, we observed that viral RNA cassettes encoding TC-83-specific 5'UTR were only modestly more efficient in translating downstream nucleotides than the ones encoding TRD 5'stem-loop (Fig. 3.2 A, B). Though this

was surprising, our results were similar to those observed in Sindbis virus encoding similar mutations affecting the pathogenicity and the stability of the 5' stem. A G5A mutation observed in SINV Toto1101 and the culture adapted strain of SINV was predicted to destabilize the 5'stem-loop (McKnight et al., 1996), but it did not enhance translation efficiency (Nickens and Hardy, 2008). Another nt8 mutation in SINV was shown to determine the pathogenicity of the virus and stabilized the 5'-terminal stem (Dubuisson et al., 1997), but it did not have any effect on translation efficiency. Together these results suggest that mutations in the alphavirus 5'UTR that affect the pathogenicity of the virus may not essentially affect translation of the viral non-structural polyprotein.

Evaluation of RNA replication levels of chimeric viruses encoding the nt3 mutation revealed that the G3A mutation in the VEEV genome enhanced genomic RNA replication but down regulated transcription of the subgenomic RNA (Fig. 3.3 C). Further, our analysis of viral RNA synthesis using DI RNAs and viral replicons demonstrated that the promoters for genomic RNA synthesis and subgenomic RNA synthesis appear to compete for the viral replicase. In the DI RNA replication studies, A3/DI/Luc demonstrated higher luciferase expression compared to G3/DI/Luc (Fig. 3.4 B). However, studies using packaged replicons, A3VEErep/Luc/GFP and G3VEErep/Luc/GFP, demonstrated that sgRNA synthesis and subsequent luciferase expression was higher for G3VEErep/Luc/GFP (Fig. 3.5 B). The higher luciferase expression observed for A3/DI/Luc suggests that the RNA synthesis is likely much higher than what was observed due to the lower levels of sgRNA synthesis in the replicon encoding the nt3 mutation. Earlier studies to characterize the sequence requirements of the sgRNA promoter hypothesized that the sequence at the 3'terminus of the minus strand could affect the sgRNA synthesis due to preferential association of the viral enzyme complex (Raju et al., 1999). To date, it is not clear how the viral replicase recognizes the sgRNA promoter to initiate translation, however this

preference for the promoter in the 3'end of the negative strand could sufficiently explain the decrease in the synthesis of the sgRNA as the synthesis of both the plus-strand genomic RNA and the sgRNA require the same fully processed viral replicative enzymes (nsPs). Further, in addition to the decrease in sgRNA synthesis, it is likely that the G3A mutation might affect the synthesis of the negative strand RNA (Gorchakov et al., 2004; Nickens and Hardy, 2008), and this aspect of the RNA synthesis needs to be evaluated.

The decrease in sgRNA synthesis resulted in decreased synthesis of the downstream proteins as observed in the studies using packaged replicons (Fig. 3.4B). Logically one is likely to assume that a decrease in structural protein synthesis might affect virus replication by limiting the structural scaffold needed to form virions. However, chimeric viruses encoding the G3A mutation exhibited modestly higher replication rates and hence increased virus titers as a result of enhanced genomic RNA synthesis (Fig. 3.6). The increase in virus replication with increase in RNA synthesis suggests that the virus replication is probably determined by the amount of genomic RNA synthesized and not by the amount of sgRNA synthesized or the levels of structural proteins translated for packaging the virus.

Although chimeric virus encoding nt3 mutation demonstrated a modest increase in virus replication in cell culture, the mutation significantly affected sgRNA synthesis and viral structural proteins, and VEEV TC-83 encoding the nt3 mutation was attenuated in mice having a fully competent type I IFN system (Kinney et al., 1993; White et al., 2001). Recent studies of nsp3 associated viral replication complexes (Gorchakov et al., 2008) revealed the presence of dsRNAs in these complexes and the amount of dsRNA was shown to increase with increase in virus replication in a time dependent manner. The G3A mutation increases replication of both viral genome and virus itself in tissue culture. The increase in viral RNA synthesis would likely increase the amount dsRNA

associated complexes in the cells resulting in inducing cellular innate immune response. VEEV capsid on the other hand was found to interfere with nuclear-cytoplasmic trafficking and inhibited translation of cellular messenger and ribosomal RNAs (Garmashova et al., 2007b). Modifications of the defined 35-aa-long capsid-specific peptide or its replacement, with one derived from the SINV capsid, made the virus dramatically less cytopathic and strongly attenuated (Garmashova et al., 2007a). Viruses encoding TRD-specific 5'UTR demonstrated 3 – 4 fold enhanced synthesis of structural proteins, suggesting that increased synthesis of VEEV capsid is likely a method to combat virus-induced immune response by blocking nuclear transport.

Thus, an increased RNA synthesis and decreased synthesis of VEEV capsid in VEEV encoding the nt3 mutation, sufficiently explain the G3A induced higher sensitivity to IFN-α/β (White et al., 2001). Although this likely outlines a molecular mechanism for the nt3 mutation mediated attenuation of VEEV, a thorough and extensive investigation of the hypotheses is warranted.

CHAPTER 4

STRUCTURAL REQUIREMENTS WITHIN THE VEEV 5'UTR ENCODED PROMOTER FOR EFFICIENT VIRUS REPLICATION

INTRODUCTION

RNA viruses encoding a positive strand genome adopt similar strategies for replication. The genomic RNA serves as a template for synthesis of a negative sense intermediate which then serves as a template for synthesis of plus-strand genomic RNA that is packaged in the progeny viruses. Although most positive strand RNA viruses depend on host machinery for translation of their mRNA sense genome, they usually code for genes that are involved in genome replication. The encoded viral RNA dependent RNA polymerase complex (RdRp-complex) replicates the RNA genome associating specifically with the viral genome and its negative strand intermediate. It is believed that the viral RdRp-complex recognize the viral genomic RNA and/or its negative strand intermediate by direct or indirect interaction with specific elements that are present in the template RNA. These elements are often short sequence with no apparent secondary structure, and in some cases possess simple RNA structures like hairpins, pseudoknots or cloverleaf-like structures. Although much is known about the structure of viral RdRps and their initiation complexes (Ahlquist, 2002; Ferrer-Orta et al., 2006; Wu and White, 2007), very little is known about the process by which these RdRps recognize the template RNA sequences and their structural requirements of such elements for efficient RNA replication.

Venezuelan Equine Encephalitis a pathogenic member of the *Alphavirus* genus in the *Togaviridae* family causes widespread epidemics of viral

encephalitis in both humans and animals that can result in death or neurological disorders (Strauss and Strauss, 1994). VEEV has a non-segmented, positive-sense RNA genome that is 11.4 kb in length and contains a 5'-methylguanylate cap and a 3'-polyadenylate tail (Strauss and Strauss, 1994). The 5’ two-thirds of the genome codes for the nonstructural proteins (nsp1-4), and the 3’ one-third codes for the structural proteins (capsid, E1 and E2) which are translated from a subgenomic mRNA. Viral RNA replication is highly regulated and proceeds through initial translation of the encoded nsPs by the host translation machinery to produce nsP1-4 that form the viral replication complex. This replication complex then recognizes the positive sense genomic RNA and utilizes it as template to synthesize the negative strand intermediate in an iterative process. The newly synthesized minus-strand then serves as template for synthesis of the plus-strand genome and transcription of the sub-genomic RNA. While differential processing of the viral nsPs regulates this iterative process of plus- and minus-strand synthesis (Strauss and Strauss, 1994), little is known about the process of RNA template recognition and the role of various promoter elements.

Alphavirus promoter elements include, i) a 19-nt long conserved sequence element (CSE) at the 3’end of the genome adjacent to the poly (A) tail (Ou, Strauss, and Strauss, 1981; Ou, Trent, and Strauss, 1982); ii) a subgenomic promoter in the negative strand intermediate (Ou et al., 1982)and iii) a genomic promoter for synthesis of the positive- and negative-strand viral genome, located at the 3’end of the negative strand and 5’end of the positive strand respectively (Strauss and Strauss, 1994). The genomic promoter contains two elements – the 5’terminal sequence encoded in the 5’UTR and a 51nt CSE located ~150nt downstream of the 5’UTR in the nsP1-coding region. Previous studies have shown that the 51-nt CSE functions more as a replication enhancer in a virus- and cell-dependent manner (Fayzulin and Frolov, 2004; Michel et al., 2007). However, there have been no studies on the second element encoded by the

5’UTR to address its requirement for virus replication. The G3→A mutation identified in the vaccine strain (TC-83) clearly highlights the importance of the 5’UTR element in virus pathogenicity and virulence (Kinney et al., 1993; White et al., 2001). Our previous studies to understand the effect of this mutation on RNA structure and virus replication suggest that the nt3 mutation affects the stem-loop RNA structure in the 5’UTR. This change in the RNA secondary structure in turn enhances the rate of genomic RNA synthesis and virus replication in VEE/SINV chimeric viruses suggesting that the mutation favorably enhances the interaction of the 5’UTR with the viral replication complex increasing genomic RNA replication levels.

Based on these findings, we hypothesized that the secondary structure and stability of the stem-loop in the 5’UTR play a crucial role in virus replication. Here we describe experiments to evaluate the role of RNA sequence, secondary structure and stability of the stem-loop in virus replication using viral RNA replicons and VEE/SINV chimeric viruses. Our results suggest that the nucleotide sequence in the 5’UTR along with the structure and stability of the stem are critical for efficient virus replication. Modifications affecting either of these resulted in virus that were unable to replicate efficiently and evolved into pseudorevertant viruses that contained compensatory mutations to the 5’UTR or non-structural proteins.

MATERIALS AND METHODS

Cell culture

BHK-21 cells were kindly provided by Dr. Sondra Schlesinger (Washington University, St Louis, MO). NIH 3T3 cells were obtained from the American Type Tissue Culture Collection (Manassas, VA). BHK-21 and NIH 3T3

cells were propagated in Alpha MEM supplemented with 10% fetal bovine serum (FBS) and vitamins.

Plasmid constructs

p(G3)/VEE/SINV and p(A3)/VEE/SINV, encoding the genome of (G3)VEE/SINV and (A3)VEE/SINV chimeric virus genomes were described in Chapter 2. VEE/SINV chimeric virus, pG3/VEE/SINV, encoded 5'UTR of the VEEV TRD strain, 3' UTR, subgenomic promoter and ns polyprotein-coding sequence from VEEV TC-83 and structural polyprotein-coding sequence from SINV Toto1101 (Rice et al., 1987). pA3/VEE/SINV differed from pG3/VEE/SINV only by one nucleotide (A3), specific for the VEEV TC-83 5'UTR. In both genomes, the poly(A) sequence was followed by a MluI restriction site. Other 5'UTR mutants had essentially the same design, but differed in the 5'-terminal sequences of the genome.

pG3Ubi/Luc encoded the promoter for the SP6 DNA-dependent RNA polymerase, followed by nt 1-191 of the viral genome, fused with the entire ubiquitin gene and firefly luciferase-coding sequence. This protein-coding sequence was followed by VEEV TC-83-specific 3'UTR, poly(A) and MluI restriction site, required for linearization of the plasmid before in vitro transcription reaction.

pG3/DI/Luc and pA3/DI/Luc plasmids encoded defective viral genomes under control of the SP6 promoter. These genomes contained the 5' terminal 519 nucleotides derived from VEEV TRD or VEEV TC-83 genomes, respectively, followed by nt 7291-7564 (encoding VEEV subgenomic promoter), a firefly luciferase gene, 3' terminal nt 11202-11446 of VEEV genome, poly(A) tail and MluI restriction site. pA3/VEErep/Pac and pG3/VEErep/Pac replicons had viral structural genes replaced by a puromycin acetyltransferase (Pac) sequence (Petrakova et al., 2005).

All plasmids encoding modified VEEV genomes and luciferase were constructed by standard PCR-based mutagenesis and cloning methods. After cloning into the plasmids, all of the PCR fragments were sequenced to exclude the possibility of spontaneous mutations.

RNA transcription

Plasmids were purified by centrifugation in CsCl gradients. Plasmids were lineraized by MluI, and RNAs were synthesized in vitro by SP6 RNA polymerase in the presence of cap analog (Rice et al., 1987) under the conditions recommended by the manufacturer (Invitrogen). The yield and integrity of the transcripts were monitored by gel electrophoresis under non-denaturing conditions, followed by analysis of the RNA concentration on a FluorChem imager (Alpha Innotech) and by spectroscopy. For virus rescue and analysis of DI RNA replication, the appropriate volumes of reaction mixtures were directly used for electroporation. For comparative studies of translation efficiencies of the Luc-coding RNAs, the transcripts were additionally purified using RNeasy columns (Qiagen), and RNA concentration was measured as described above.

RNA transfection

BHK-21 cells were electroporated with 2 μg of in vitro-synthesized RNA transcripts using previously described conditions (Liljestrom et al., 1991). For analysis of DI RNA replication, 2 μg of replicon and DI RNAs were co-electroporated using the same conditions. Equal amounts of cells were seeded into 35-mm dishes, and luciferase activity was measured at different times post transfection by using a Luciferase assay kit according to the manufacturer's instructions (Promega). To assess the translation efficiency of the templates, equal amounts of Ubi/Luc RNAs were electroporated into BHK-21 cells, and equal aliquots of the cells were seeded into 35-mm dishes. At the indicated time

points, cells were lysed, and luciferase activity was measured by using a Dual-Luciferase assay kit according to the manufacturer's instructions (Promega).

Infectious center assay

In standard experiments, 2 µg of in vitro-synthesized viral genome RNA was transfected into BHK-21 cells using previously described conditions (Liljestrom et al., 1991). Ten-fold dilutions of electroporated cells were seeded into 6-well Costar plates containing naive BHK-21 cells. After a 1 h incubation at 37°C, the cells were overlaid with 2 ml of 0.5% agarose (Invitrogen) containing MEM supplemented with 3% FBS. Plaques were stained with crystal violet after 2 days of incubation at 37° C. Before staining, some of the plaques were randomly isolated for analysis of the pseudorevertants. Remaining electroporated cells were seeded into 100-mm tissue culture dishes for generating viral stocks or into 35-mm dishes to evaluate the rates of virus replication. At the time points indicated in the corresponding figures, media were replaced, and virus titers in the corresponding samples were assessed by a plaque assay on BHK-21 cells (Lemm et al., 1990).

Analysis of virus replication

BHK-21, and $C_7$10 cells were seeded at a concentration of 5 x10^5 or 10^6 cells per 35-mm dish, respectively. Cells were infected at a multiplicity of infection of 10 PFU/cell for 1 h, washed with phosphate-buffered saline, and overlaid with 1 ml of complete medium. At the indicated times, media were replaced by fresh media, and virus titers in the harvested samples were determined by a plaque assay on BHK-21 cells as described elsewhere (Lemm et al., 1990).

Selection of pseudorevertants and sequencing of viral genomes

In order to identify adaptive mutations accumulating in viral genomes in response to mutations in the 5'UTR, plaques were isolated from agarose overlay directly in the infectious center assay. Viruses were then eluted into 1 ml of alpha-MEM supplemented with 10% FBS, and 0.5 ml of this media was used to infect naïve BHK-21 cells in 35-mm dish dishes. After 16-to-24 h incubation at 37^{o}C in 5% CO_2, media was harvested and RNA was isolated from the cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The isolated RNAs were used to generate cDNA fragments for the 5' terminus using commercially available FirstChoice RLM-Race kit (Ambion Inc). The amplified DNA fragments were purified by agarose gel electrophoresis, and cloned into the pRS2 plasmid. Plasmids isolated from multiple colonies were used for sequencing. For some of the viruses, PCR fragments covering the entire ns polyprotein-coding region were synthesized, purified by agarose gel electrophoresis and sequenced.

Analysis of viral RNA synthesis

BHK-21 cells were infected with chimeric viruses at an MOI of 10 PFU/cell. At the times indicated in the figure legends, viral RNAs were labeled with [^{3}H] uridine (20 mCi/ml) in the presence of 1 µg of D-actinomycin (ActD)/ml for 4 h at 37^{o} C in 5% CO_2. RNAs were isolated from the cells using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The RNAs were denatured with glyoxal in dimethyl sulfoxide and analyzed by agarose gel electrophoresis using the previously described conditions (Bredenbeek et al., 1993). For quantitative analysis, the RNA bands were excised from the 2,5-diphenyloxazole (PPO)-impregnated gels, and the radioactivity was measured by liquid scintillation counting.

RESULTS

A critical role for the 5'-terminal nucleotide sequence in VEEV replication

Our previous results demonstrated that the nt3 mutation in TC-83 altered the structure of the stem-loop in the 5'UTR and enhanced both genomic RNA synthesis and rate of viral replication. This suggested that the change in the stem-loop structure increased the preferential association of the viral replication complex with the complement of the 5'UTR in the 3'end of the minus strand resulting in increased genomic RNA synthesis, thus enhancing virus replication. However, it was not clear as to how the mutation enhanced RNA replication: was it the change in the sequence; or the change in the structure and stability of the stem-loop in the 5'UTR that enhanced RNA synthesis. It was thus essential to identify the essential features of the promoter in the 5'UTR that was critical for efficient genomic RNA synthesis and virus replication. We performed extensive mutational analysis to identify the key features and the importance of the nucleotide sequence, secondary structure and stability of the stem-loop that are essential for virus replication.

The 5'UTR sequence among various VEEV isolates is highly conserved and begin with an AUG except for TC-83 where nt 3 is an A. On the other hand, the 5'UTR sequence among several isolated alphaviruses demonstrates a low level of identity (Fig. A1) (Ou, Strauss, and Strauss, 1983); however, the sequence of the first few nucleotides are very similar. All of the genomes start with an AU di-nucleotide, with G being the third nucleotide in the sequence followed by the -GCGG-sequence. This conservation of the sequence in the first few nucleotides suggests that they may be critical for RNA recognition by the viral replication and hence play a role in RNA synthesis and virus replication. In our initial experiments, we assessed the importance of the 5' terminal sequence in RNA and virus replication.

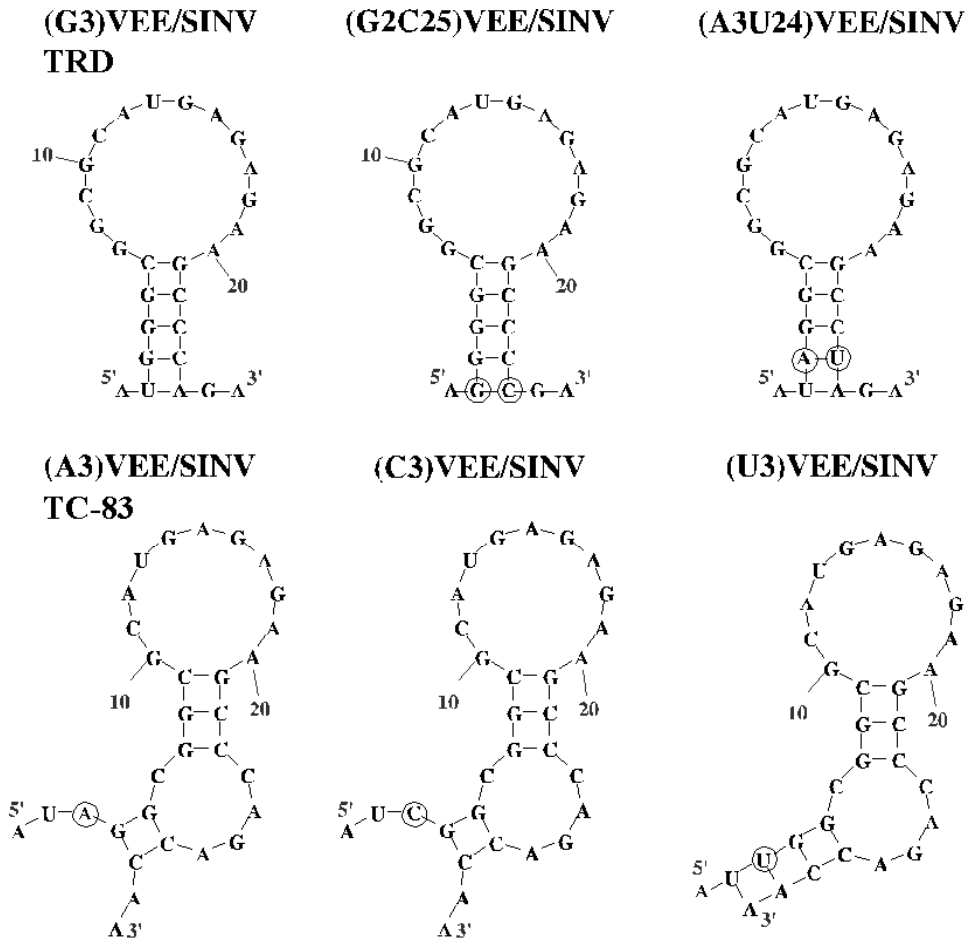


Figure 4.1: 5’ terminal nucleotide mutations. Mfold predicted RNA secondary structures for each 5’terminal nucleotide modification.

First, we evaluated the importance of nt2 by introducing a U→G mutation. To preserve the 5’-terminal RNA secondary structure and mimic the VEEV TRD-specific folding of the 5’UTR, the designed (G2C25)VEE/SINV variant contained the U2→G and a compensatory A25→C mutation (Fig. 4.1). Although, the infectivity of this in vitro-synthesized RNA was almost 3 orders of magnitude lower than that of the (G3)VEE/SINV RNA, the virus titers recovered at 48 h post electroporation were ~10^7 suggesting that the recovered media contained true or

pseudorevertant viruses, and the plaques detected in the infectious center assay were formed by pseudorevertants. Four plaques were randomly selected in the infectious center assay and the 5'UTRs of the isolated variants were sequenced to identify any adaptive mutations. Direct sequencing of the PCR product suggested the presence of multiple variants in each plaque. Therefore, the 5'UTR-containing fragments were cloned into the plasmid and multiple clones were sequenced. The sequencing results demonstrated that (G2C25)VEE/SINV genome acquired a spectrum of new sequences that contained multiple AUG repeats at the 5' terminus (Table 4.1).

Plaque #	5' sequence	Number of clones
Orig. (G2C25)VEE/SINV	AgGGGCGGCGCAUGAGAGAAGCCCcGA	
Pl. 1	**AUG**AgGGGCGGCGCAUGAGAGAAGCCCcGA	2/4
	AUGAUGAgGGGCGGCGCAUGAGAGAAGCCCcGA	1/4
	AUAAAGAAGUGGUCAUGAgGGGCGGCGCAUGAGAGAAGCCCcGA	1/4
Pl. 2	**AUGAUG**AgGGGCGGCGCAUGAGAGAAGCCCcGA	1/6
	AUGAUGAUGAgGGGCGGCGCAUGAGAGAAGCCCcGA	3/6
	AUGAUGAUGAUGAgGGGCGGCGCAUGAGAGAAGCCCcGA	2/6
Pl. 3	**AUG**AgGGGCGGCGCAUGAGAGAAGCCCcGA	2/4
	AUGAUGAUGAgGGGCGGCGCAUGAGAGAAGCCCcGA	2/4
Pl. 4	**AUGAUGAUG**AgGGGCGGCGCAUGAGAGAAGCCCcGA	3/3

Table 4.1: Sequences of 5' ends in the genomes of (G2C25)VEE/SINV pseudorevertants isolated from the plaques. 5' terminal sequence additions are boxed and presented in bold

Next, we evaluated the nucleotide preference at nt3 for efficient virus replication by replacing TRD-specific G3 by U, and C. In both the cases, the predicted stem-loops had a low free energy of folding (ΔG); -5.1 kcal/mol for (U3)VEE/SINV and -3.8 kcal/mol for (C3)VEE/SINV); and folded into secondary structure similar to that of TC-83 specific 5'stem-loop (Fig. 4.1). Both

(U3)VEE/SINV and (C3)VEE/SINV were viable, and their RNA demonstrated the same efficiency of plaque formation in the infectious center assay as (G3)VEE/SINV and (A3)VEE/SINV (Fig. 4.2 A). Sequencing the 5'ends of the genomes isolated from variants in randomly selected plaques detected no reversions or other mutations suggesting that the mutations did not have any deleterious effect on virus replication. However, in contrast to G3A, both G3U and G3C were not beneficial for both virus and RNA replication (Fig. 4.2 B, A2). In addition to the U and C mutations, we designed another G3A mutation that had an additional compensatory mutation (C24U) to retain the overall secondary structure of the 5' end of VEEV TRD genome while encoding a 5' terminal sequence similar to VEEV TC-83. The (A3U24)VEE/SINV demonstrated RNA infectivity virus replication and RNA synthesis similar to that of (A3)VEE/SINV (Fig. A3) suggesting that an A at the third nucleotide in the RNA sequence irrespective of the stem-loop structure enhances RNA synthesis and virus replication.

We then evaluated the significance of the nucleotide sequence in the stem, by switching the G-C base pairs with C-G base-pairs (Fig. 4.3 A). The replacement of even one G-C base pair with a C-G base pair in (Rev1BP)VEE/SINV altered the rates of virus replication (Fig. 4.5B). Switching two ((Rev2BP)VEE/SINV) or four base pairs ((Rev4BP)VEE/SINV) significantly reduced the virus replication. The in vitro-synthesized RNAs demonstrated dramatically lower infectivities in the infectious center assay, and viruses were replicating to very low titers (Fig. 4.3 B) and formed pinpoint-sized plaques. The tiny plaques detected in the infectious center assay and virus replication analysis suggested that the virus recovered from the media may require several passages to restore virus replication. Hence, we did not detect formation of the true or pseudorevertants. These results suggested that the sequence at the 5' end of the genome is crucial for RNA recognition by viral replicase.

A)

Chimera	RNA infectivity (PFU/μg)	Virus titers (PFU/ml) 48 h PEP	Pseudorevertants
(G3)VEE/SINV	$5x10^5$	$4.5x10^9$	—
(C3)VEE/SINV	$2.5x10^6$	$3x10^8$	—
(U3)VEE/SINV	$2.5x10^6$	$2.5x10^9$	—

B)

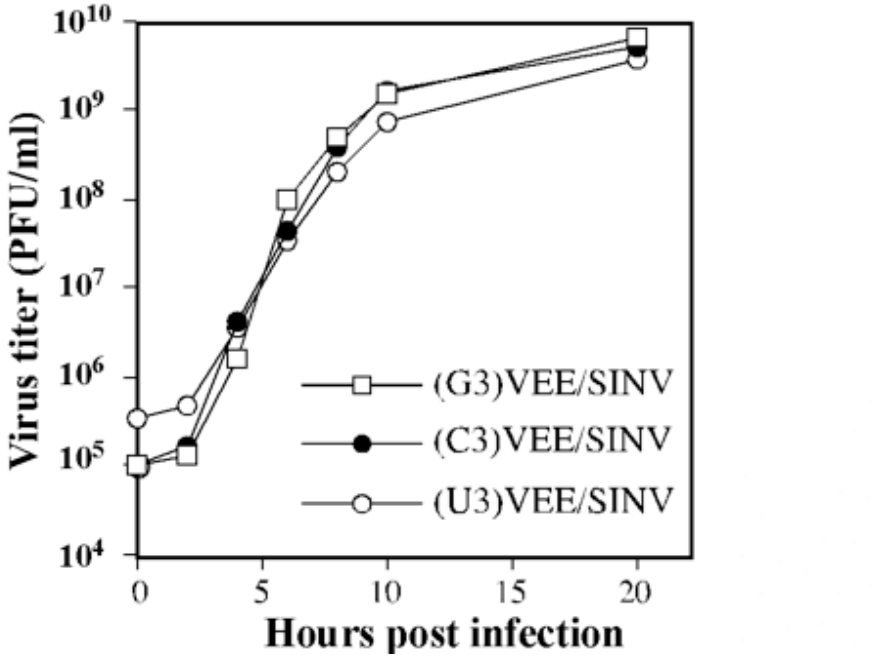


Figure 4.2: Analysis of the effects of nt3-specific mutations on VEE/SINV viability and replication. (A) RNA infectivity in the infectious center assay. (B) Replication analysis of chimeric viruses encoding nt3 specific mutations in BHK-21 cells.

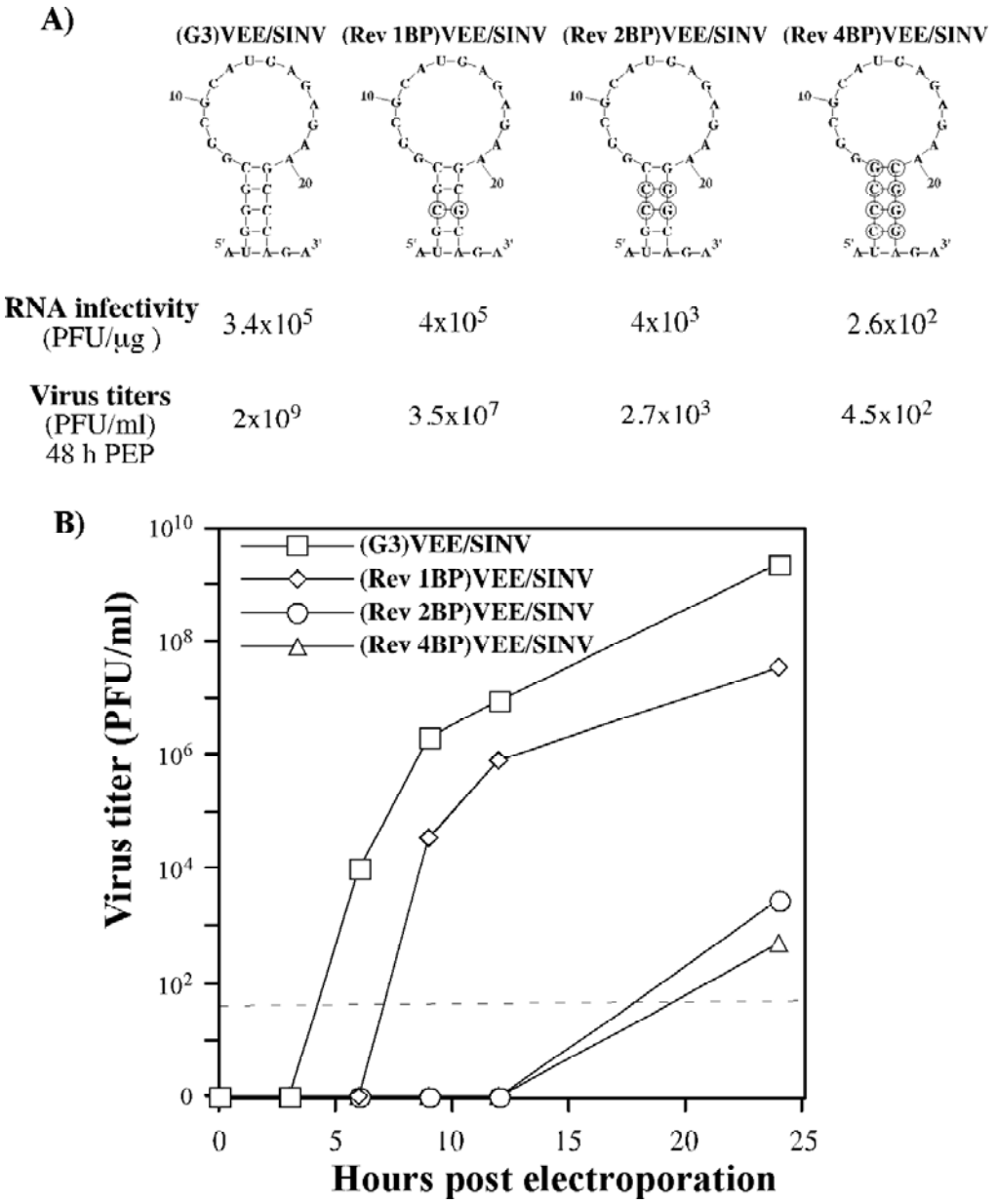


Figure 4.3: Sequence requirements within the stem for efficient VEE/SINV replication. (A) Predicted 5'-terminal secondary structures of the designed mutant genomes and RNA infectivity of the in vitro-synthesized RNAs. (B) Analysis of mutant chimeric virus replication in BHK-21 cells

Modifications to the loop sequence do not affect virus replication

The results of the previous experiments suggested that the sequence at the 5’ end of the genome leading to the loop in the hairpin loop was crucial for virus replication. Next we designed a loop mutant to detect the significance of the loop in virus replication. To identify if the sequence in loop was important for virus replication, we modified the loop in (A3U24)VEE/SINV. Compared to (G3)VEE/SINV, (A3U24)VEE/SINV variant demonstrated higher genomic RNA synthesis and lower SG:G RNA synthesis ratio and hence was expected to be a better model for detecting decrease in the 5’UTR-specific RNA promoter function. In the resulting (Loop)VEE/SINV, we reversed the sequence of nucleotides in the loop by replacing the loop nucleotides with the same sequence cloned in the opposite orientation (Fig. 4.4 A). This modification of the loop did not affect the overall, predicted secondary structure of both 5’UTR and its complement at the 3’ end of the negative-strand intermediate. The in vitro-synthesized (Loop)VEE/SINV RNA demonstrated similar RNA infectivity and virus growth rates as the (A3U24)VEE/SINV RNA (Fig. 4.4 B). Further, it did not affect RNA replication (Fig. 4.4 C) and SG:G ratio of RNA synthesis was similar to that of (A3U24)VEE/SINV. These results indicated that changes to the loop sequence did not affect virus replication or genomic RNA synthesis, suggesting that the loop nucleotides were not involved in RNA recognition by the viral replicase.

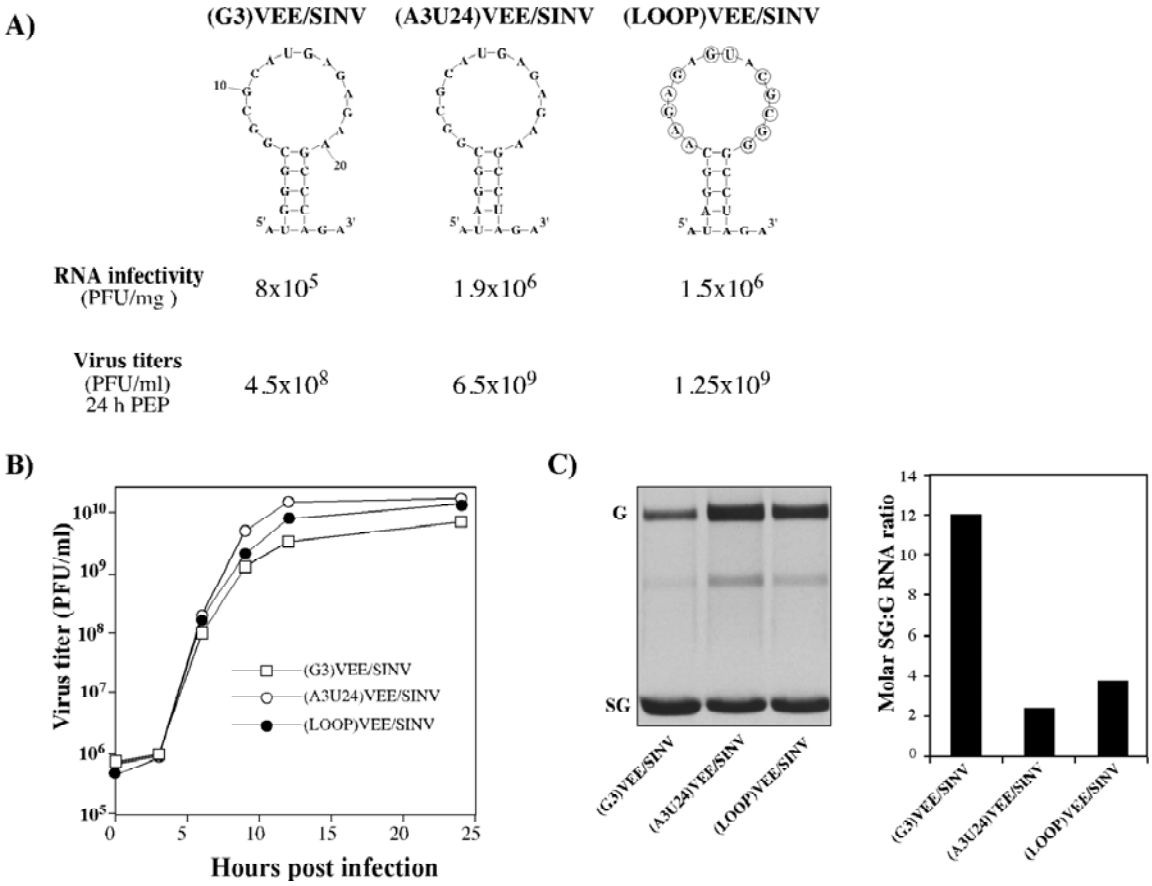


Figure 4.4: Modifications to the loop do not affect RNA synthesis and chimeric virus replication. (A) Predicted 5'-terminal secondary structures of the designed mutant genomes and RNA infectivity of the in vitro-synthesized RNAs. (B) Chimeric virus replication was analyzed by infecting BHK-21 cells at an MOI of 10 Pfu/ml. Media was replaced at indicated times post-infection and virus replication was assessed. (C) Analysis of virus specific RNA synthesis. RNAs were metabolically labeled between 4 and 8 h post infection as described in Materials and methods. RNAs were then isolated and analyzed by agarose gel electrophoresis. G and SG indicate positions of the viral genomic and subgenomic RNAs, respectively. The molar ratio of SG:G RNA is presented

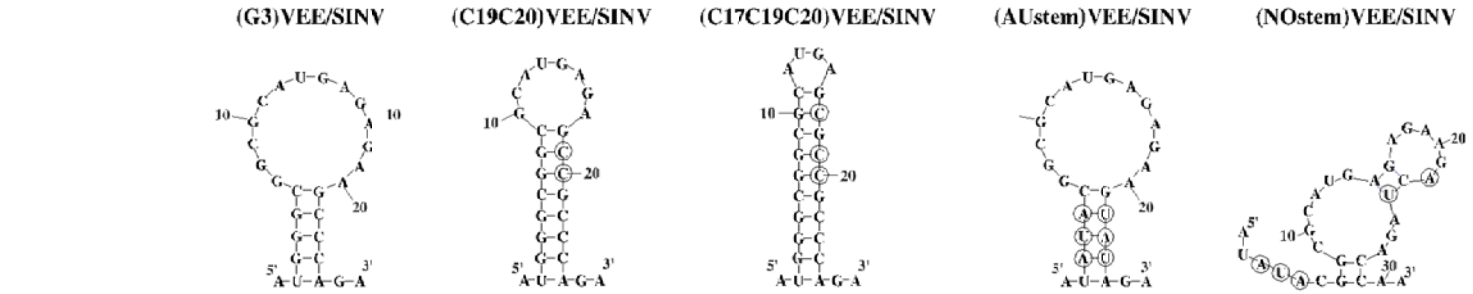
Stability of the 5’ stem-loop affects virus replication

The 5’stem-loop in VEEV TRD genome is G-C-rich and has 4 consecutive G-C base-pairs in the RNA stem leading to the loop. Previous studies in various prokaryotic and eukaryotic systems have shown that, translation is affected when there are stable stem-loop structures close to the 5’ methyl G cap (Babendure et al., 2006; Kozak, 1986; Vega Laso et al., 1993). However, in our previous studies (described in Chapter 3), we observed that the nt3 mutation that lowers the stability of the 5’stem-loop in VEEV TC-83, did not significantly increase the translation efficiency, but enhanced both virus and RNA replication. Substitution of G3 with U or C resulted in 5’UTRs encoding stem-loops similar to that in TC-83, however they did not enhance genomic RNA synthesis or virus replication. However, lowering the stability of TRD-like stem-loop in the (A3U24)VEE/SINV mutant enhanced both RNA synthesis and virus replication. These results suggest that the stability and the structure of the stem-loop are critical for efficient virus replication. Therefore, we designed another set of mutants that had both higher and lower free energy of folding for the stem-loop in the 5’UTR to assess the effect of stem stability on RNA and virus replication.

Stem-loops with higher stability were designed by replacing two or three As; A17, A19 and A20; in the TRD-specific 5’stem-loop with Cs to promote the base-pairing, thus extending the stem (Fig. 4.5). The ΔG for the 5’ stem-loops in (C19C20)VEE/SINV (-18.4 kcal/mol) and (C17C19C20)VEE/SINV (-23.5 kcal/mol) genomes were much lower than that of the TRD-like stem-loop in (G3)VEE/SINV (-8 kcal/mol). We also designed two other mutants that encoded stems with lower stability. The first one, (AUstem)VEE/SINV, encoded a stem consisting mainly of A-U pairs and in the second, (NOstem)VEE/SINV, the original stem was completely destroyed by multiple point mutations. The

minimum free energies of the 5' ends for these two mutants were -1.8 and -0.4 kcal/mol respectively, higher than that of (G3)VEE/SINV.

Modifications increasing the stability of the 5'stem-loop proved to be harmful and dramatically lowered RNA infectivity in the infectious center assay (Fig. 4.5). The magnitude of reduction in RNA infectivity correlated with the decrease in ΔG. The virus titers in media recovered 48 h post transfection was over 10^5 for both the variants, suggesting that it contained pseudorevertant viruses that acquired adaptive mutations to replicate efficiently in tissue culture. On the other hand, both mutants with lower stem-stabilities were viable, and demonstrated infectivities comparable to that of the original (G3)VEE/SINV (Fig. 4.5). However, viruses generated were incapable of causing profound CPE, and were unable to develop clear plaques under agarose cover in the infectious center assay suggesting that the phenotype was highly unstable. The media recovered 48h post transfection contained variants capable of forming clear plaques and the virus titers were over 10^9 and 10^7 for (AUstem)VEE/SINV and (NOstem)VEE/SINV, suggesting the development of pseudorevertants.



	(G3)VEE/SINV	(C19C20)VEE/SINV	(C17C19C20)VEE/SINV	(AUstem)VEE/SINV	(NOstem)VEE/SINV
RNA infectivity (PFU/mg)	$8x10^5$	$2x10^2$	30	$9x10^5$	$3x10^5$
Virus titers (PFU/ml) 48 h PEP	$4x10^9$	$2.5x10^7$	$6x10^5$	$1.5x10^9$	$3x10^7$
Pseudorevertants	-	+	+	+	+

Figure 4.5: Effect of mutations affecting the stability of the 5'-terminal stem on VEE/SINV viability. Mutations affecting the stability of 5'stem-loop and their respective Mfold predicted secondary structures. Nucleotide modifications are indicated by open circles. RNA infectivities in the infectious center assay and titers of the viruses at 48 h post transfection are presented.

A)

Plaque #	5' sequence	Number of clones
Orig. (C19C20)VEE/SINV	AUgGGCGGCGCAUGAGAGccGCCCAGA	
Pl. 1	**AUGAUGAUG**AUgGGCGGCGCAUGAGAGccGCCCAGA	1/4
	----------AUGAGAGccGCCCAGA	3/4
Pl. 2	**AUGAUGAUG**AUgGGCGGCGCAUGAGAGccGCCCAGA	3/4
	AUGAUGAUGAUGAUgGGCGGCGCAUGAGAGccGCCCAGA	1/4
Pl. 3	**AUGAUGAUG**AUgGGCGGCGCAUGAGAGccGCCCAGA	2/3
	AUGAUAAUGAUGAUgGGCGGCGCAUGAGAGccGCCCAGA	1/3

B)

Plaque #	5' sequence	Number of clones
Orig. (C17C19C20)VEE/SINV	AUgGGCGGCGCAUGAGcGccGCCCAGA	
Pl. 1	**AUGAUGAUGAUG**AUgGGCGGCGCAUGAGcGccGCCCAGA	3/3
	AUGAUGAUGAUGAUgGGCGGCGCAUGAGcGccGCCCAGA	2/3
Pl. 2	**AUGAUGAUGAUGAUGAUG**AUgGGCGGCGCAUGAGcGccGCCCAGA	1/3

Table 4.2: 5' sequence insertions due to increased stability of the stem. Sequences of 5' ends in the genomes of (C19C20)VEE/SINV (A) and (C17C19C20)VEE/SINV (B) pseudorevertants isolated from randomly selected plaques. 5' terminal insertions are highlighted by bold typecase and underlined

Random plaques were selected from infectious center assay, and the 5' genomic ends were sequenced for variants thus isolated. Sequencing results suggested the presence of multiple quasispecies in plaque isolates, an indication that the viruses were still evolving. Most of the variants thus isolated contained multiple AU or AUG insertions at the 5' ends (Table 4.2, 4.3), with a few having different 5' ends. One of the plaque-purified variants in (C19C20)VEE/SINV (Table 4.2, Pl. 1) had short 5'UTR, where the 5'-terminal sequence was deleted but its genome still started with an AUG. Another variant isolated from

(AUstem)VEE/SINV (Table 4.3, Pl. 1) contained an additional AUAG in the 5'end, while most of the other pseudorevertants contained multiple AU repeats. In the case of viruses recovered from 2 plaques of the (NOstem)VEE/SINV (Table 4.3, Pl. 1 & Pl. 2), the 5' termini contained a heterologous sequence that could potentially form stem-loops, similar to that in the (G3)VEE/SINV genome.

Taken together, the results suggested that the presence of the RNA stem with particular stability in the 5'terminus of VEEV genome plays an important role in genomic RNA replication. Modification affecting the structure and stability affect virus replication and results in pseudorevertants that contain 5' UTRs with adaptive mutations. Isolation of variants with multiple AUG/AU repeats in response to both different stem-loop modifications suggests that there might be a universal way of VEEV evolution in response to the 5'UTR modification. However, we could not rule out the possibility that other mutations in the protein coding regions may be required for their viability.

A)

Plaque #	5' sequence	Number of clones
Orig. (AUstem)VEE/SINV	AUauaCGGCGCAUGAGAGAAGuauAGACC	
Pl. 1	**AUAU**AUauaCGGCGCAUGAGAGA**C**GuauAGACC	2/5
	AUAUAUauaCGGCGCAUGAGAGAAGuauAGACC	1/5
	AUAGAUauaCGGCGCAUGAGAGAAGuauAGACC	2/5
Pl. 2	**AUAUAU**AUauaCGGCGCAUGAGAGA**C**GuauAGACC	1/1
Pl. 3	**AUAUAU**AUauaCGGCGCAUGAGAGAAGuauAGACC	1/2
	AUAUAUAUauaCGGCGCAUGAGAG**C**AGuauAGAC**U**	1/2

B)

Plaque #	5' sequence	Number of clones
Orig. (NOstem)VEE/SINV	AUauaCGGCGCAUGAGAGAAGaCuAGACCA	
Pl. 1	**AUAUAUAUAUAUAUAU**AUauaCGGCGCAUGAGAGAAGaCuAGACCA	1/2
	AU**CGG**CGGCGCAUGAGAGAAG**C**C**C**AGACCA	1/2
Pl. 2	**AUGGUCAU**AUauaCGGCGCAUGAGAGAAGaCuAGACCA	3/4
	AUAUauaCGGCGCAUGAGAGAAGaCuAGACCA	1/4
Pl. 3	**AU**AUauaCGGCGCAUGAGAGAAGaCuAGACCA	2/2
Pl. 4	**AU**AUauaCGGCGCAUGAGAGAAGaCuAGACCA	1/2
	AUAUAUauaCGGCGCAUGAGAGAAGaCuAGACCA	1/2

Table 4.3: 5' sequence insertions due to decreased stability of the stem. Sequences of 5' ends in the genomes of (AU-stem)VEE/SINV (A) and (NO-stem)VEE/SINV (B) pseudorevertants isolated from randomly selected plaques. 5' terminal insertions are highlighted by bold typecase and underlined

5’ adaptations observed in the pseudorevertants enhance RNA synthesis and virus replication

The 5’ sequence insertions in the isolated pseudorevertants ranged from multiple AU or AUG insertions to insertion of heterologous sequence and deletion of 5’sequence. In some cases, variants isolated from the same plaque contained multiple repeating AU/AUGs suggesting continuous evolution and it was likely that higher replication rates were achieved by with several repeats. We designed (C19C20)VEE/SINV genome with 1, 2 or 3 AUG insertions to identify the significance of these additions on virus replication. As a control, we also designed (G3)VEE/SINV variants with one or more AU repeats to see if such additions enhance virus replication in (G3)VEE/SINV.

Infectious center assay of the in vitro-synthesized RNAs demonstrated infectivity was similar to that of (G3)VEE/SINV (Fig. 4.6 A). Infectivities of variants with AUG insertions were more than three orders of magnitude higher than that of the original (C19C20)VEE/SINV construct suggesting that addition of one more AUG to the 5’ terminus of the (C19C20)VEE/SINV genome was sufficient to rescue the virus, and no additional mutations in the nonstructural genes and/or CSEs was required. Virus replication was evaluated by electroporation, and an increase in the number of AUG repeats correlated with the increase in virus replication rates (Fig. 4.6 B), with the variant containing 3x(AUG) replicating almost as efficiently as the (G3)VEE/SINV chimera encoding TRD-specific stem-loop. We detected variants having 4 and 6 AUG repeats in the plaque isolates of (C19C20)VEE/SINV and (C17C19C20)VEE/SINV, suggesting that they could achieved even higher replication rates. However, on the other hand addition of one or more AUs did not have a significant effect on genomic RNA synthesis and virus replication rates of (AU/G3)VEE/SINV.

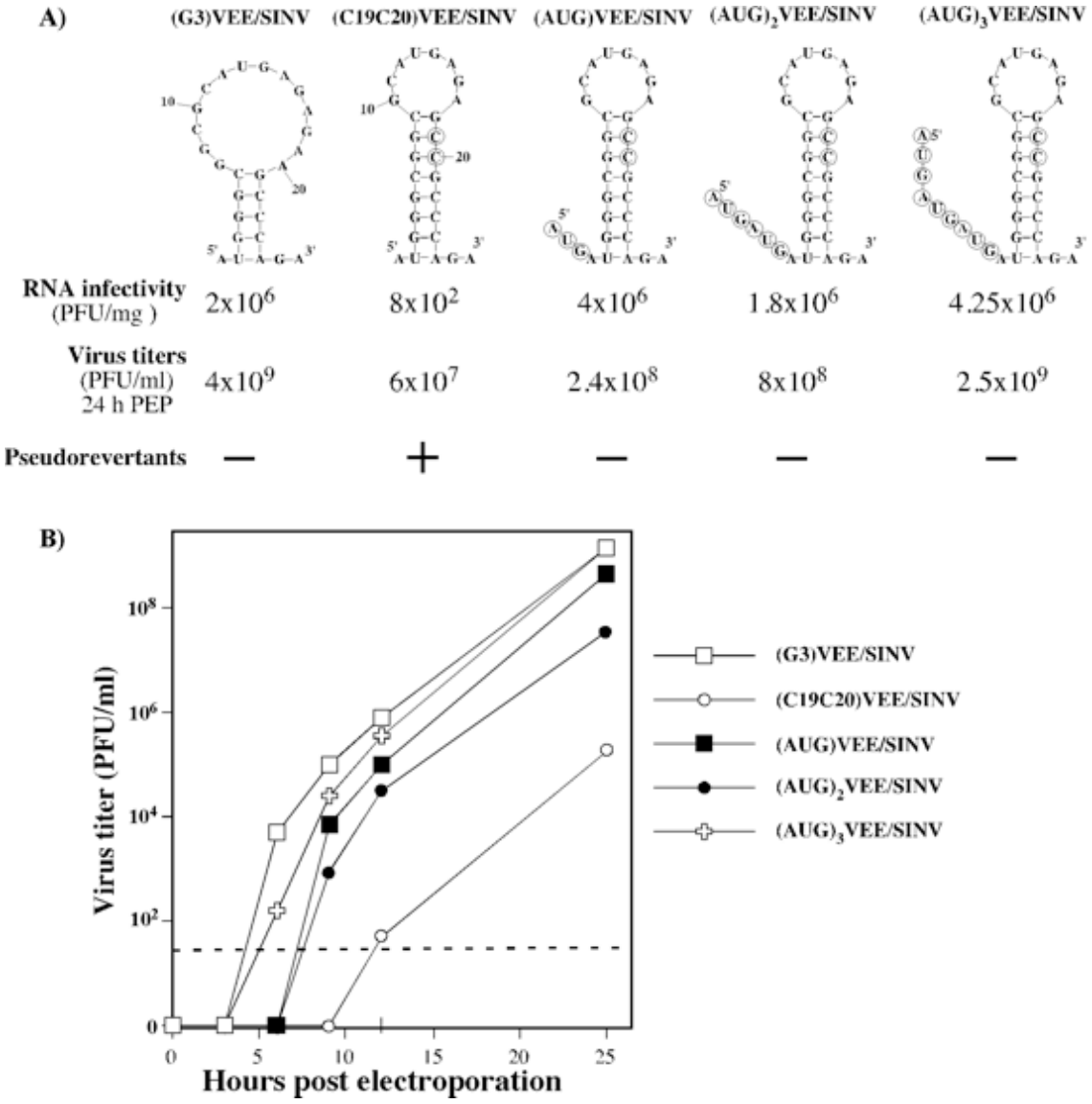


Figure 4.6: Replication of (C19C20)VEE/SINV variants having additional AUG repeats at the 5' terminus of the genome. A) Mfold predicted 5'UTR secondary structures of (C19C20)VEE/SINV variants having additional AUG repeats, RNA infectivities in the infectious center assay and virus titers at 24 h post RNA transfection. B) Chimeric virus replication evaluated in BHK-21 cells by electroporation of in vitro-synthesized RNAs. At the indicated times, media were replaced and titers of virus in the harvested samples were measured by a plaque assay in BHK-21 cells. Dashed line indicates the limit of detection

In addition to variants with multiple AU/AUG insertions, we also engineered the variant in which the 5' sequence was partially deleted. The (Del)VEE/SINV mutant was viable, and the in vitro synthesized RNAs demonstrated infectivity of $2x10^5$ PFU/mg that was similar to that of (G3)VEE/SINV. The (Del)VEE/SINV variant developed tiny pinpoint plaques, did not replicate to higher titers and sequencing of the 5'ends revealed no sequence changes suggesting that it was an inefficient variant that developed in response to the changes in stem-loop stability. However, after just one cycle of passaging it evolved into a better replicating virus, and was capable of forming larger plaques. Sequencing of the 5' ends of the genomes of the recovered variants once again revealed no changes in the 5'UTR, but the adaptive mutations were found nsp1, nsp2 or nsp4 (Fig. 4.7 A). These mutations were mapped to the carboxy terminus of the nsP1, and amino terminus of nsP2 adjacent to the cleavage site between nsP1 and nsP2. The mutation in the nsP4 was mapped close to the peptide sequence that was critical for initiation and synthesis of sgRNA (Li and Stollar, 2004; Li and Stollar, 2007). This suggested that strong modifications in the promoter sequence affecting virus replication could also be compensated mutations in different virus-specific nsPs that form the viral replicase. We then evaluated the effects of non-structural protein specific mutations on (Del)VEE/SINV replication. (Del)VEE/SINV variants encoding nsP1 and nsP4 mutations demonstrated growth rates higher than that of the original (Del)VEE/SINV (Fig. 4.7 B). In all, our results suggest that 5' stem-loop modifications that severely affect virus replication can also be overcome by compensatory mutations in the nsPs, in addition to 5' sequence insertions that enhance virus replication.

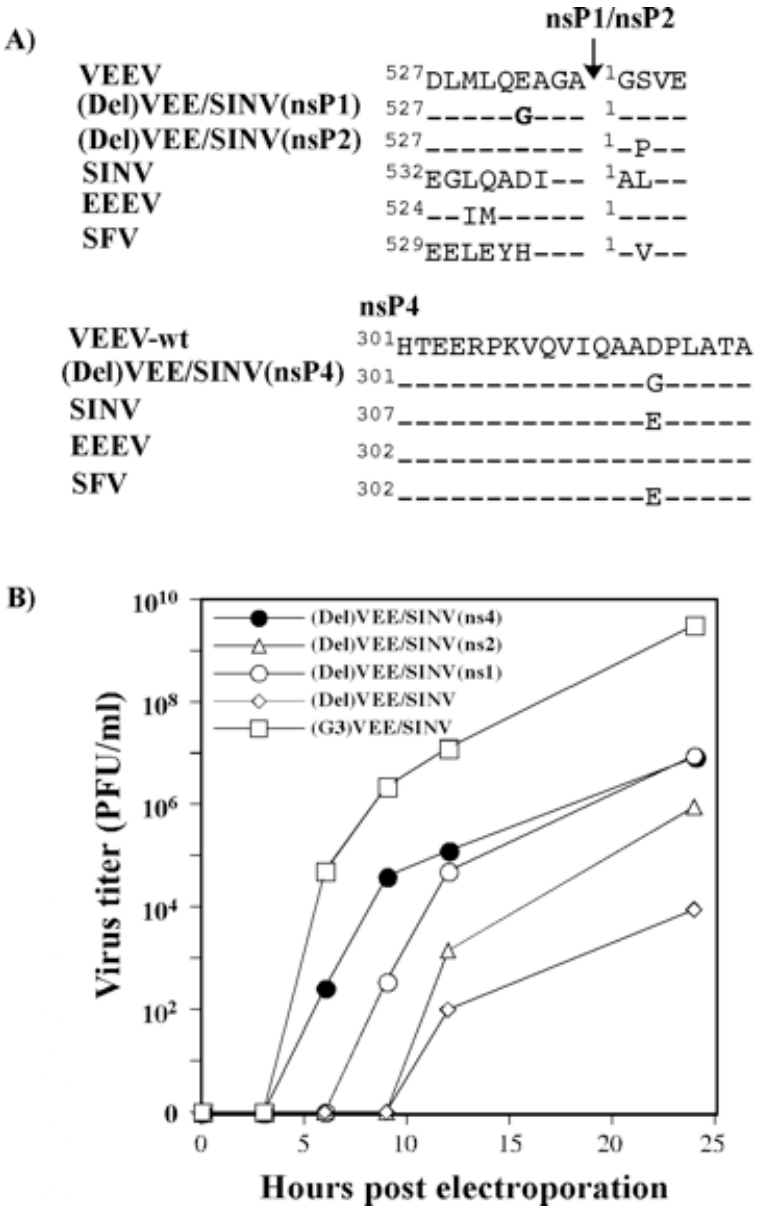


Figure 4.7: Analysis of (Del)VEE/SINV variant A) Mutations found in the nsP1/nsP2 junction and fragment of nsP4 of (Del)VEE/SINV plaque isolates adapted for growth in BHK-21 cells is shown in alignment with that of several alphaviruses. VEEV, Venezuelan equine encephalitis virus; EEEV, eastern equine encephalitis virus; SFV, Semliki Forest virus; SINV, Sindbis virus. Residues identical to those in the VEEV sequence are denoted by dashes. B) Chimeric virus replication evaluated in BHK-21 cells by electroporation of in vitro-synthesized RNAs.

5’ modifications affect RNA replication but do not affect translation of the encoded polyprotein

5’ modifications affecting the sequence and stability of the stem had a negative effect on virus replication. This negative effect could also be due to lower activity of the RNA promoter elements encoded in the 5’UTR. However, a detailed characterization of the variants encoding such 5’modifications and multiple AUG repeats was complicated by the likelihood of their further evolution leading to appearance of virus quasispecies. To clearly understand the effect of the mutations on RNA replication, we designed defective viral genomes encoding different 5’ UTRs, partial nucleotide sequence coding for the nsP1 gene (475 nt), and a firefly luciferase gene under control of the subgenomic promoter (Fig. 4.8). The DI RNAs did not encode full-length nonstructural proteins, and were capable of replication only in the presence of nsPs supplied in trans by helper VEEV replicon (VEErep/Pac). Upon delivery into the same cell, replicon genomes were translated to produce VEEV nsPs which could then be used for the replication of both the replicon and DI RNA and transcription of the subgenomic RNA, expressing luciferase. The efficiency of DI RNA replication and luc expression is independent of the 5’UTR function in translation initiation, but is determined by the promoter sequences at the DIs’ 5’ ends, the ability of the latter promoter to utilize the replicative enzymes.

The VEEV replicon and the DI genomes were co-transfected into BHK-21 cells, and luciferase activity was evaluated at different times post electroporation (Fig 4.8). The DI RNAs having stable 5’-terminal stems [(C19C20)DI/Luc and (C17C19C20)DI/Luc] demonstrated low levels of luciferase expression, but addition of one or more AUGs to (C19C20) variant enhanced luciferase expression. This suggested that addition of AUGs in (AUG)DI/Luc, $(AUG)_2$DI/Luc and $(AUG)_3$DI/Luc that increased luciferase expression is due to enhanced RNA replication levels of these DIs. In the case of other stem mutants, (G2C25)DI/Luc

RNA did not demonstrate any detectable replication, but the (NOstem)/DI/Luc RNA was surprisingly efficient in luciferase expression and its ability to express luciferase correlated with high RNA infectivity of the corresponding viral genome in the infectious center assay.

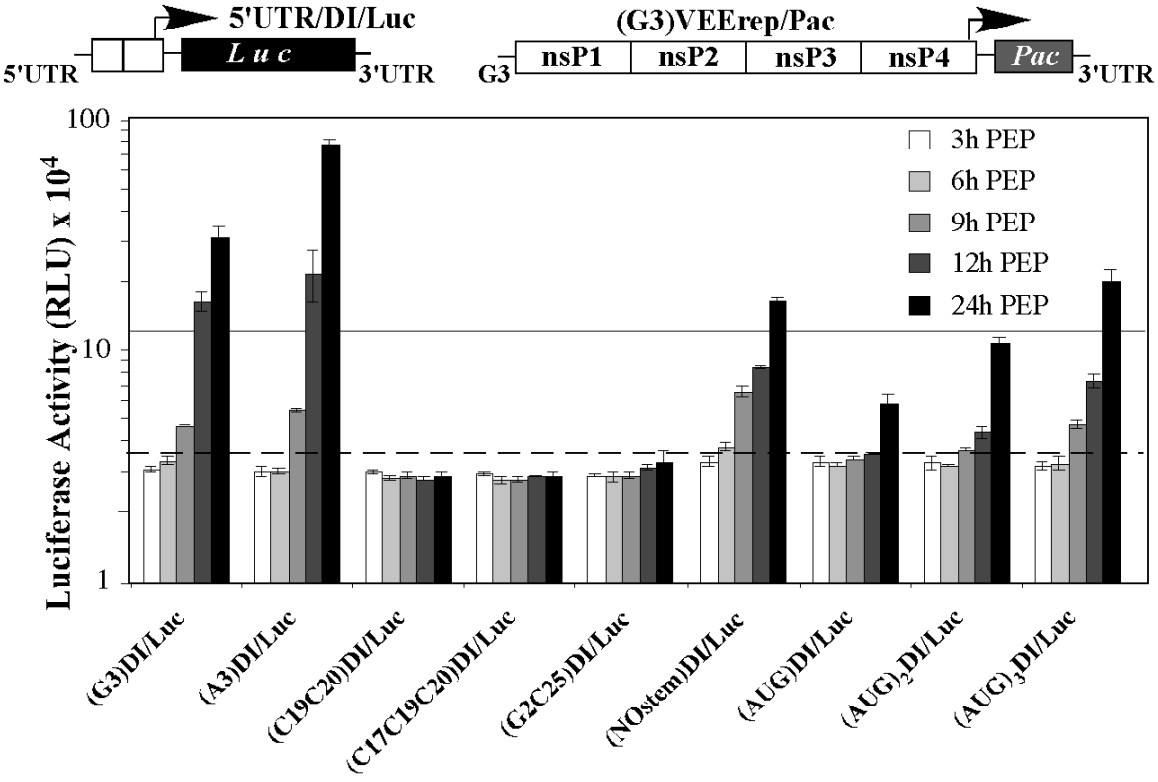


Figure 4.8: Effects of 5'UTR mutations on RNA replication. Schematic representation of DI RNAs firefly luciferase reported and replicon genomes encoding VEEV non-structural proteins used to evaluate the effect of the 5'UTR-specific mutations on RNA replication. Average firefly luciferase activities detected at indicated times post electroporation are presented in the graph. Dashed line indicates the average background.

The negative effects of the 5'-terminal RNA modifications on virus replication and RNA synthesis indicate that the mutations directly lower the activity of the RNA promoter elements encoded in the 5'UTR. However,

downregulation of replication can also be due to decreased translation of the ns polyprotein, especially in the case of mutations that increase the stability of the stem-loop structure in the 5'UTR. To distinguish between the effects of the mutation on RNA replication and/or translation, we initially designed a number of cassettes, which were incapable of self-replication and contained different 5'UTRs. The cassettes encoded a firefly luciferase gene, fused in frame with an Ubi sequence and the 147-nt-long fragment of VEEV nsP1 gene under the control of the 5'UTRs (Fig. 4.9). The latter sequence encoding the nsP1 coding region was required to preserve the natural folding of the RNA in the 5' terminus, and the Ubi gene was required for promoting production of luciferase in a more active, free form. Equal amounts of in vitro-synthesized capped RNAs were co-transfected into BHK-21 cells by electroporation along with Renilla luciferase-encoding RNA. The activity of both luciferases was evaluated at different times post transfection (Fig. 4.9). The results demonstrated that all of the 5' UTR modifications, except for that in (C17C19C20)VEE/SINV variant having the most stable stem, downregulated the translation less than two-fold. The (C17C19C20)VEE/SINV variant downreguated translation by at least 3 folds, and this can be attributed to the presence of a stable stem-loop at the 5'end. The luciferase expression for (AUG)VEE/SINV-derived 5'UTR was higher than that determined for similar constructs having two or three AUGs. Thus, the results of these experiments demonstrated that the modifications of the very 5' terminus of the genome, affected viral RNA replication rather than the translation of the VEEV nsPs. Additional 5'-terminal AUG repeats, acquired by mutants with more stable 5'-terminal RNA secondary structures, did not enhance the translation of the encoded proteins, but increased the efficiency of the RNA replication that resulted in the generation of viable viruses.

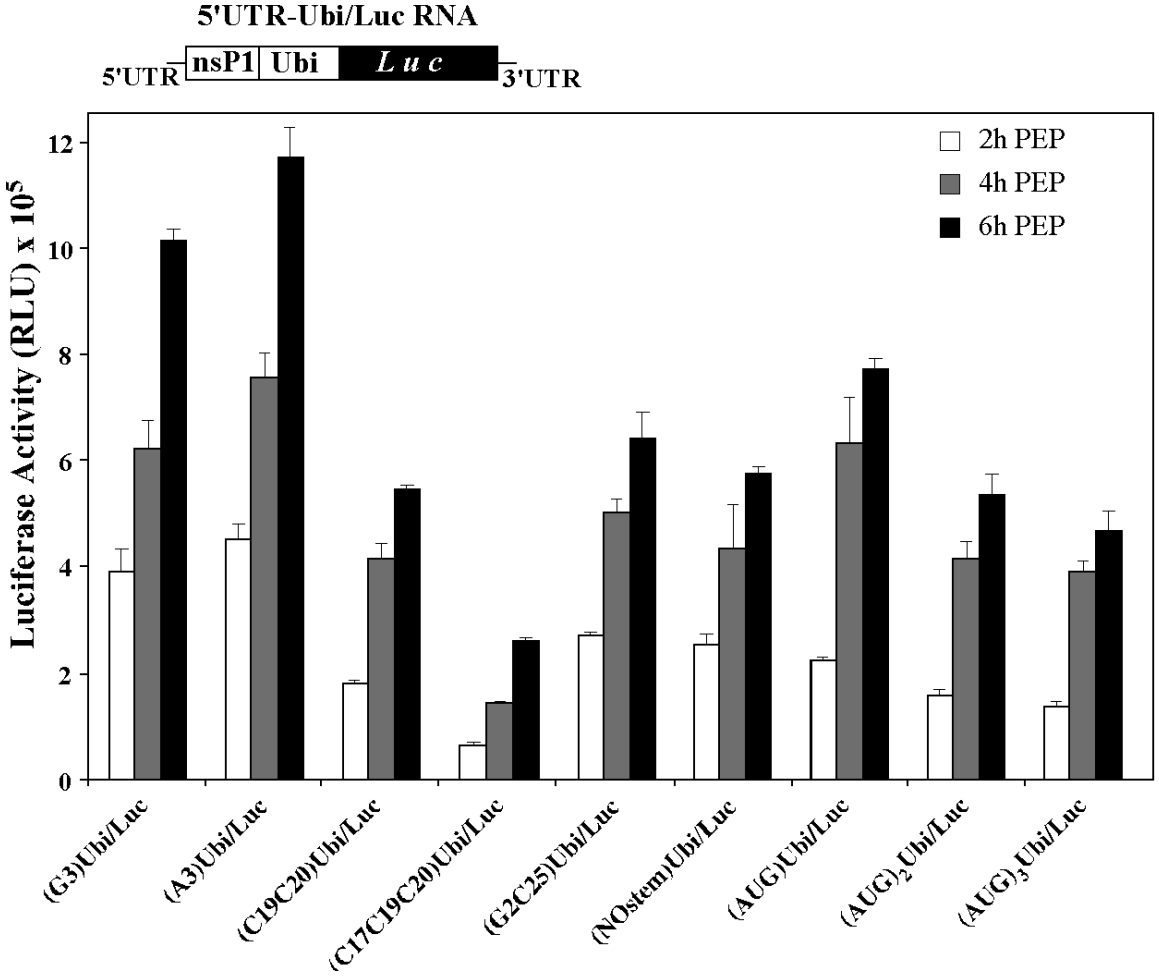


Figure 4.9: Effects of 5'UTR mutations on translation. Schematic representation of the firefly luciferase reporter construct used to evaluate the effect of the 5'UTR-specific mutations on translation of downstream sequence. Normalized luciferase activity determined using a Dual-Luciferase system (Promega) at indicated times post electroporation presented.

DISCUSSION

Genomic RNA replication in alphaviruses depends directly on the promoter element encoded in the 5'UTR and its complement located at the 3' termini of the negative-strand intermediate. In addition to its promoter activity, the 5'UTR in alphavirus plays a key role in several processes of virus replication. The

5’UTR directs translation of viral nsPs, and thus, its structure ultimately determines accumulation of the replication- and other virus-specific protein complexes. The SINV-specific 5’UTR also plays a key role in the negative-strand synthesis (Frolov, Hardy, and Rice, 2001; Gorchakov et al., 2004; Nickens and Hardy, 2008) and can be considered to be part of the promoter for synthesis of the replicative intermediate. The same function might be attributed to the 5’UTRs of other alphaviruses. Sequence comparison of several alphavirus 5’UTRs suggested that the 5’UTR folds into a stem-loop that is critical for virus replication (Ou, Strauss, and Strauss, 1983), however, the structure and functions of the promoter are not well understood.

VEEV TRD genome-specific 5’UTR was predicted to fold into a stem-loop structure, and its structure was confirmed by NMR and enzymatic analysis in our previous studies (Chapter 3). However, the structural features of the stem-loop in TC-83 that enhanced virus and RNA replication were not clear. Hence, we performed extensive mutational analysis to identify the sequence and structural requirements of the 5’stem-loop for efficient virus and RNA replication. Our results suggest that the sequence, structure, and stability of the stem in the hairpin-loop at the 5’end are critical for efficient virus replication. Mutations changing the sequence of the loop did not have any significant effect on virus and RNA replication (Fig. 4.4) implying that loop sequence is probably not critical for virus replication.

The AU sequence at the 5’end of the genome are conserved across several alphaviruses and are required for efficient virus replication. The U2G mutation proved deleterious to virus replication and resulted in pseudorevertants that acquired adaptive mutations at the 5’ends. Sequencing of the 5’ ends in the pseudorevertants revealed that most of them evolved to acquire multiple AUG insertions and the genomes now began with a 5’AU (Table. 4.1). Modification of G3 to U or C or A did not affect virus replication. The virus replication rates and

genomic RNA levels of the (A3U24) variant was almost similar to that of (A3)VEE/SINV that encoded TC-83 specific 5'UTR (Fig. A3), suggesting that the sequence along with the stability of the stem-loop are crucial for efficient virus replication. Severe modifications of the sequence in the stem, proved detrimental as the virus demonstrated low replication rates and were incapable of forming definite plaques (Fig. 4.3).

The stability of the stem-loop proved to be crucial for virus replication. Mutations that increased or decreased the stability of the stem-loop proved to be harmful. In infectious center assay, the in vitro synthesized RNAs demonstrated low RNA infectivity (Fig. 4.5) and had low virus titers 48 h post transfection as the resultant viruses had low replication rates compared to chimeric virus genomes encoding TRD-specific 5'UTRs, (G3)VEE/SINV. Most of these viruses acquired adaptive mutations and resultant viruses were able to replicate better with the addition of one or more AU/AUG repeats to the 5'ends. Mutant viruses' encoding stable stem-loops acquired one or more AUG repeats (Table. 4.2), while mutants with destabilized stem acquired multiple (AU) repeats (Table. 4.3). Analysis of these pseudoreverants suggested that addition of even one AUG was sufficient to rescue the virus (Fig. 4.6). Increase in the number of repeats correlated with increase in virus and RNA replication rates but did not have a positive effect on translation of the downstream polypeptide (Fig. 4.9) suggesting that the positive effect on virus replication resulted from enhanced RNA promoter efficiency that increased the level of RNA replication. 5' terminal additions enhanced virus replication rates only for viruses that were incapable of efficient replication, however, AU additions to the 5' end of (G3)VEE/SINV did not significantly enhance virus replication or genomic RNA synthesis compared to the parental (G3)VEE/SINV (Fig. A4).

Previous studies of 5' and 3' UTR elements in Sindbis virus, have shown that the genomic ends can acquire AU-rich sequence in response to mutations in

these elements (Hardy and Rice, 2005; Raju et al., 1999) or in response to replacement of the natural ends with those from a related viruses (Gorchakov et al., 2004). Such AU additions were suggested as a possible method for genome repair as it was shown to rescue and enhance virus replication (Raju et al., 1999). In our studies, different stem-modifications gave rise to similar repeat sequences that rescued viruses suggesting that VEEV replicase, like other alphavirus replicase, is conservative and is capable of using only a limited number of nucleotide sequences for genome repair. However, addition of sequences to the 5'end as in this case is not possible directly. Based on this and on previous observations (Gorchakov et al., 2004; Nickens and Hardy, 2008), we propose that even after strong modifications of the 5'-terminal fragments, alphaviruses sustain some ability for negative-strand RNA synthesis and acquire heterologous sequences at the 3' end of the negative-strand RNA intermediate. The particular sequences capable of functioning as promoter for positive-strand RNA synthesis are then selected during the subsequent rounds of RNA replication. Recent studies have demonstrated that the core catalytic domain of Sindbis nsP4 possesses terminal adenylyltransferase activity (Tomar et al., 2006). Experiments using the full-length nsP4 further affirmed this finding (Rubach et al., 2009). It is likely that full-length nsP4 by itself or in complex with other nsPs could possess terminal nucleotydyltransferase activity by which it could add combinations of nucleotides to the 3'end of the template negative strands in case of templates that encode poor promoters for plus-strand synthesis.

The high frequency of perfect repeats suggests another possible mechanism for 5 terminal insertions. RNA replication in alphaviruses follows de novo, primer-independent, initiation for RNA synthesis from the template strand. Modifications of the stem might make initiation of the positive strand synthesis inefficient. The replication complex likely dissociates from the template after

synthesizing the first two or three nucleotides and then re-initiates RNA synthesis with the previously synthesized oligonucleotide still in place, resulting in a non-templated sequence being added to the 5' terminus of the positive strand RNA. This putative mechanism could explain the appearance of many, but not all of the heterologous sequences, because some in some of them the repeats are not perfect. Thus, the template re-initiation mechanism and the terminal nucleotydyltransferase mechanism might function synergistically in the 5'UTR modification.

In addition to sequence insertions to the 5' ends of the genomes to rescue viruses, some of the variants acquired compensatory mutations in the viral non-structural protein regions. These mutations were identified in nps4 or the carboxy terminus of nsP1, or amino terminus of nsP2 (Fig. 4.7A). The mutations in nsP1 and nsP2 were close to the nsP1/nsP2 cleavage site that is critical for synthesis of free nsP1 that functions in plus strand RNA synthesis (Strauss and Strauss, 1994). The nsP4 mutation on the other hand was found close to the –LVRRLNA-peptide (Li and Stollar, 2004; Li and Stollar, 2007) that was found to be the polypeptide sequence that was critical for recognition of the sgRNA promoter and for initiation sgRNA synthesis. Further, it was suggested that both the nsP4 might have distinct sites that are involved recognition of promoters for plus strand synthesis and sgRNA synthesis as mutations to this region did not affect sgRNA synthesis (Li and Stollar, 2007). Reconstructed (Del)VEE/SINV variants encoding nsP4 mutation replicated better, suggesting that the mutation occurred in the region of nsP4 that is essential for recognition of the promoter for plus strand RNA synthesis (Fig. 4.7B).

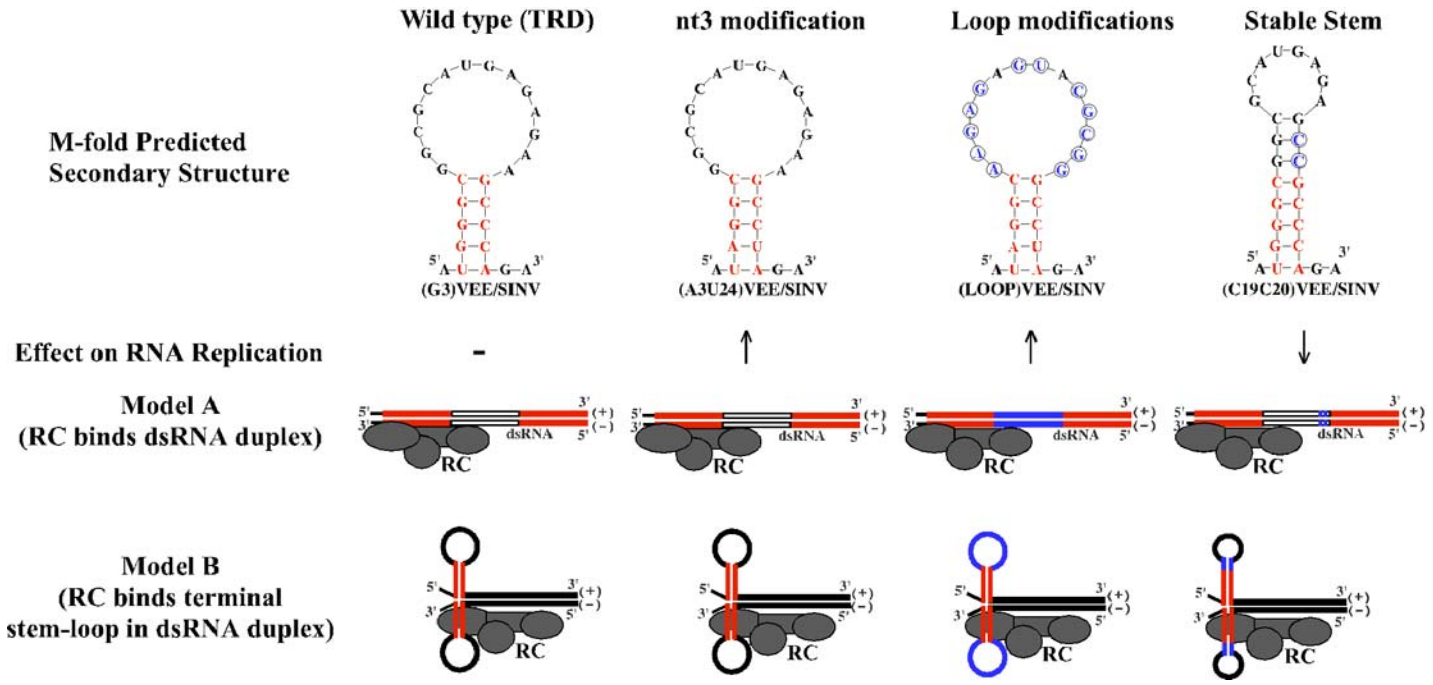


Figure 4.10: Model for interaction of the genomic ends in the dsRNA intermediate with VEEV replication complex (RC) during genomic RNA replication. Model A is based on the assumption that, in the replicative intermediate, the 5' end of the positive strand and the 3' end of the negative strand exist as a complete dsRNA duplex. According to this model, VEEV RC recognizes the very terminus of the dsRNA duplex and the following sequence. Model B depicts the dsRNA terminus as two ssRNA fragments, which fold into stem-loop structures. According to this model, VEEV RC binds to the 3' terminal unpaired fragment and the stem (but not the loop) of the negative-strand RNA for initiation of positive-strand RNA synthesis. Model A cannot explain the contrasting effects on RNA replication between loop replacement in (LOOP)VEE/SINV, and point mutations in (C19C20)VEE/SINV and (C17C19C20)VEE/SINV; while Model B supports the results observed for these modifications and hence is the right model for interaction of the dsRNA termini with VEEV RC.

The current model for RNA replication in alphavirus suggests that the plus-strand genomic RNA serves as the template for synthesis of the minus-strand. The single-stranded negative-strand intermediate is then used as the template for synthesis of progeny viral genomes and for the transcription of the sgRNA. Our data suggests that during RNA replication, the 5' terminal sequence of encoding the stem region of the hairpin loop functions as a critical element of the promoter. The compliment of this region in the 3'end of the minus strand is likely the site where the replication complex binds to initiate plus strand RNA synthesis. Negative effect of stem mutations is overcome by the addition of AU or AUG repeats to the 5'UTR of VEEV genome signifying that the structure and stability of this region is as critical as its sequence. Recent studies of alphavirus replication complexes suggest that the negative strand intermediate is in fact double-stranded (Gorchakov et al., 2008). Our results on the other hand, strongly support the possibility that the 5' terminus of positive strand and the 3' terminus of the negative strand in the VEEV do not form the dsRNA duplex, but are present as single-stranded RNA folded into stem-loops. Based on this two hypothetical models for RNA replication: model A and B as in Fig. 4.10 can be defined. Model A represents the case where the promoters for RNA synthesis in terminal ends of the plus-strand RNA and the minus strand, are double stranded. Model B represents the scenario where the 5' and the 3' ends of the positive and negative strands in this dsRNA intermediate are likely to fold into individual stem-loop structures rather than base-pair with each other. Results from our studies strongly support Model B, as model A does not explain the effect of the mutations that increase or decrease the stability of the 5' stem, on viral RNA replication. The results of other research groups also support the hypothesis that the promoter region in the 5'UTR is not present as a completely double-stranded RNA as in model A. Previously, it has been shown that the single-stranded negative strand of the genome, is recognized by the nsPs and utilized as a

template for the positive-strand RNA synthesis in vitro and the 3' end of the negative strand RNA contains binding sites for a number of cellular proteins (Pardigon, Lenches, and Strauss, 1993; Pardigon and Strauss, 1992). Further, data from SINV-related studies also suggest that the entire 5' terminus in Sindbis virus genome can be replaced by heterologous tRNA- or subgenomic RNA-derived sequences (Monroe and Schlesinger, 1983; Strauss and Strauss, 1994). It is highly unlikely that these completely different sequences can function as promoters in a double-stranded duplex form, however, only their ability to fold into individual stem-loops corroborates their activity as promoters.

In summary, our results demonstrate that the sequence, RNA structure, and stabiliy of the stem-loop in the 5'UTR play a critical role in VEEV replication. The 5' terminal AU overhang and the sequence of the stem form the core promoter for viral RNA replication. Mutations increasing or decreasing the stability of the stem have a deleterious effect on virus and RNA replication. VEEV overcomes such deleterious effects by introducing compensatory AU or AUG repeat sequences or by the addition of heterologous sequence that promotes the formation of a stem-loop at the 5' ends. In some cases, compensatory mutations are introduced in the viral nsPs to restore virus replication. Further, our data clearly demonstrates that the 5' genomic ends of the plus-strand and the corresponding 3' end in the negative strand are not part of the dsRNA duplex but fold into individual stem-loops that are critical for RNA and virus replication.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

The G3→A mutation in the 5'UTR of VEEV had previously been shown to be the determinant of virus attenuation observed in the vaccine strain TC-83. The studies described in this dissertation form an attempt to elucidate the molecular basis for VEEV attenuation mediated by the nt3 mutation, by studying the effect of the mutation on RNA secondary structure and its role in virus replication.

Using a combination of biophysical and biochemical approaches, I first investigated the effect of the mutation on viral 5'UTR RNA secondary structure. Enzymatic probing experiments revealed that the nt3 mutation altered the structure and lowered the stability of the 5'stem-loop in the VEEV 5'UTR. Thermal melting experiments using UV spectroscopy further confirmed this finding as RNA fragments corresponding to TC-83-specific stem-loop had lower melting temperatures compared to those of TRD (virulent strain). By analyzing the 1D imino region of NMR spectra collected at different temperatures, I was able to identify specific regions of base-pairing in the TC-83 and TRD 5'UTR RNAs, that were disrupted with increase in temperature. Furthermore, comparison of the TRD and TC-83 31nt 2D NOESY spectra clearly revealed the differences in RNA tertiary structure.

Next, I performed experiments to identify the effects of the nt3 mutation on virus replication. Using luciferase cassettes encoding viral 5'UTRs I showed that the G3A mutation moderately enhanced viral RNA translation. Then, using chimeric viral genomes and defective viral-RNA genomes, I demonstrated that the nt3 mutation increased viral genomic RNA synthesis and enhanced virus

replication in host cells. However, subgenomic RNA synthesis and subgenomic RNA translation were significantly lower for chimeric viruses and DI genomes encoding TC-83-specific 5'UTR. Based on the current knowledge of alphavirus replication complexes, I propose a model for attenuation of the vaccine strain: The increase in genomic RNA synthesis observed in TC-83 contributes to an increase in dsRNA levels in host cells, and most likely activates cellular innate immunity response. On the other hand, lower levels of subgenomic RNA translation contributes to a decline in the levels of capsid protein in cells, thus rendering the virus unable to shut off host cell transcription and block nuclear import. These two factors together contribute to a failure in shutting down the activation of cellular immune responses in the case of VEEV TC-83 infections (Fig. 5.1).

The G3A mutation in TC-83 changed the structure and lowered stability of the 5'stem-loop, but resulted in increased RNA synthesis and RNA translation levels, suggesting that the structure of the RNA at the 5'end determines viral RNA replication levels. These findings prompted me to investigate the significance of the RNA sequence and structure of the 5' stem-loop for efficient virus replication. By introducing single or multiple base substitutions, I showed that the sequence, secondary structure, and stability of the stem in the 5' hairpin loop determine viral RNA replication levels and virus replication titers. I also demonstrated that while the sequence and stability of the stem was crucial the sequence in the loop was not important as mutations to the loop did not affect either RNA or virus replication. Further, characterization of pseudorevertants isolated from RNA infectivity assays revealed that the virus overcomes deleterious effects of 5'modifications by evolving to acquire additional adaptive mutations that restore RNA and virus replication thus rescuing the virus. I discovered that the virus restores RNA and virus replication by introducing compensatory mutations in the nsPs that form the viral replicase. This particular

finding suggested that VEEV nsP1, nsP2 and nsP4 are involved in RNA promoter recognition to initiate RNA replication. Interestingly, I also observed that multiple AU/AUG repeats were added to the very 5' terminus of the genome in the pseudorevertants, in response to stem-modifications. The results from my experiments together suggested that the 5'terminal AU overhang along with the sequence in the stem formed the core promoter for viral RNA replication. It is likely that the viral replicase may bind to the complement of this region in the negative-strand to initiate plus-strand synthesis. The sequence, structure, and the stability of the stem are thus crucial for efficient RNA and virus replication.

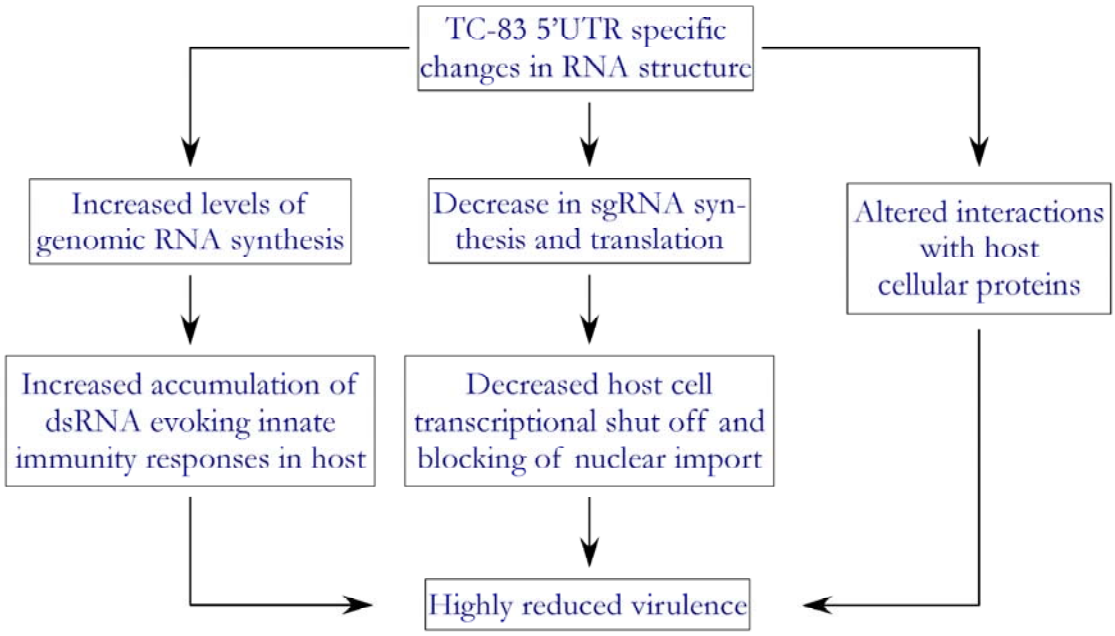


Figure 5.1: A model for attenuation of VEEV TC-83.

MODEL FOR INTERACTION OF THE VEEV 5'UTR WITH THE VIRAL REPLICASE AND ITS ROLE IN VIRAL RNA SYNTHESIS

The current model for viral RNA replication assumes that the genomic RNA and the negative strand intermediate are both single stranded. However, recent studies have confirmed that the negative strand intermediate is in fact a dsRNA intermediate. My results specifically provide evidence that the genomic ends of VEEV are not part of the double-stranded RNA duplex, but are folded into individual stem-loops. This is further supported by the findings of other research groups as discussed in Chapter 4. Based on these, I propose a model for interaction of the 5'UTR with the viral replicase in which the replicase uses the double stranded negative strand intermediate as template for plus strand synthesis, but initiates replication at the single stranded stem-loop structure in the UTR (Fig. 5.2).

FUTURE DIRECTIONS

Results from my studies have been summarized in two independent manuscripts. The first manuscript describing the effect of the nt3 mutation on RNA secondary structure and virus replication has been published in Virology. The second manuscript is currently under revision.

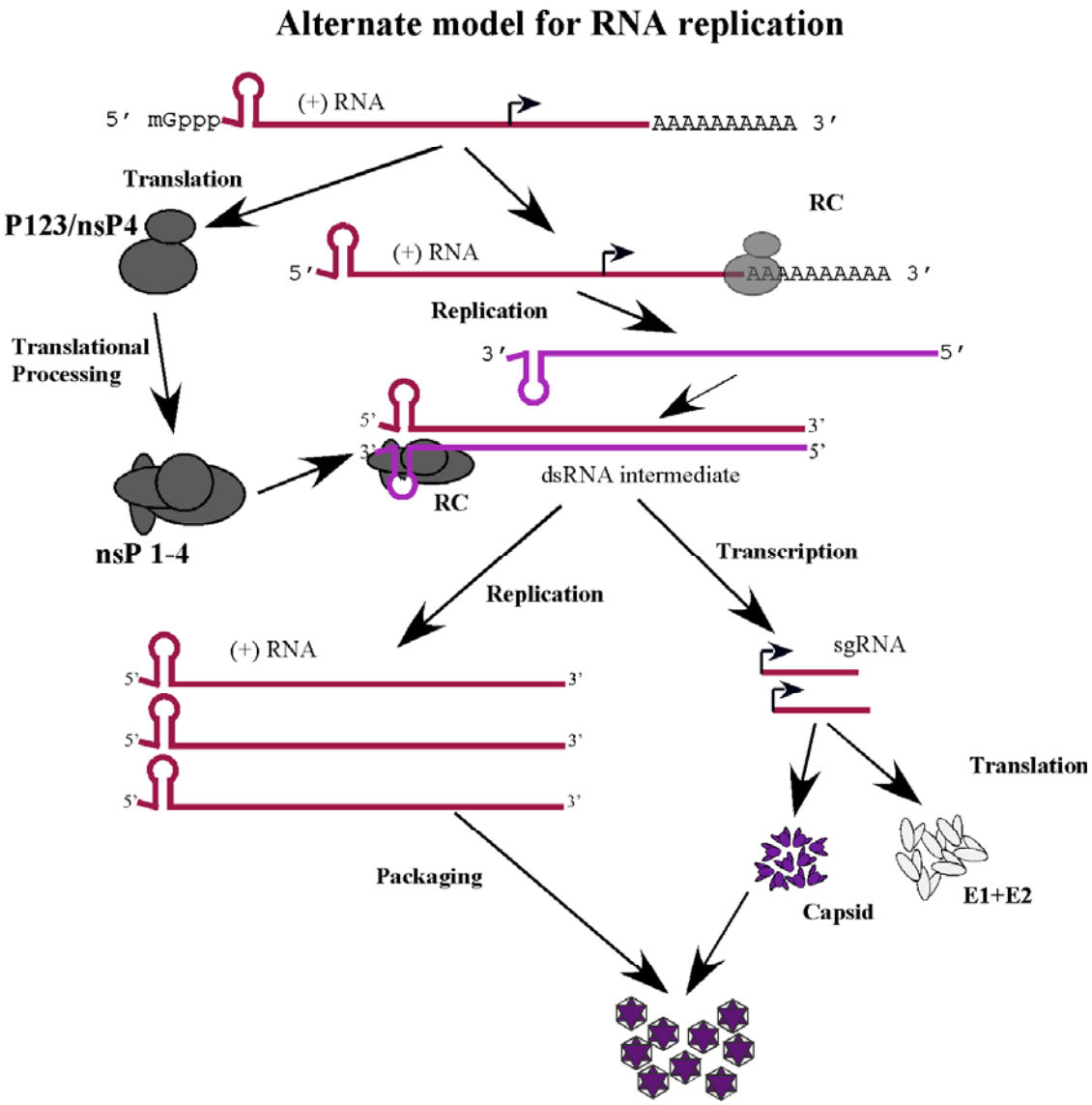


Figure 5.1: A model for interaction of the 5'UTR with the viral replicase and its role in viral RNA synthesis. The negative strand intermediate is represented by a dsRNA duplex, which is used as the template for synthesis of progeny plus strand and for transcription of the sgRNA. The viral replicase initiates plus strand synthesis by binding to the complement of the 5'UTR stem-loop structure on the negative strand.

The major goal of this project was to address the gap in the knowledge of how the 5'UTR mutation in VEEV TRD leads to molecular changes resulting in the shift from virulence to attenuation in the vaccine strain, TC-83. My results provide a glimpse of how the G3→A mutation in the 5'UTR affects RNA structure, that subsequently affects viral RNA synthesis and replication ultimately mediating attenuation. However, only a thorough investigation into the molecular biology of VEEV infection will provide a detailed picture as to why the nt3 mutation is a critical determinant of VEEV virulence. A complete systematic analysis of viral infection using microarrays would likely reveal other processes that may be involved in viral attenuation.

My studies have demonstrated the significance of the sequence and secondary structure of VEEV 5'UTR RNA. The results also provide evidence for the direct interaction of the 5'UTR with the virus non-structural proteins that are part of the replicase. Although the structure of the viral RNA replicase has been well studied, it is still not clear how the protein components of the replicase recognize the terminal ends of the genome to initiate replication. Structural analysis of such RNA-protein complexes would prove vital for enhancing our understanding of how these moieties interact to initiate viral RNA replication.

APPENDIX

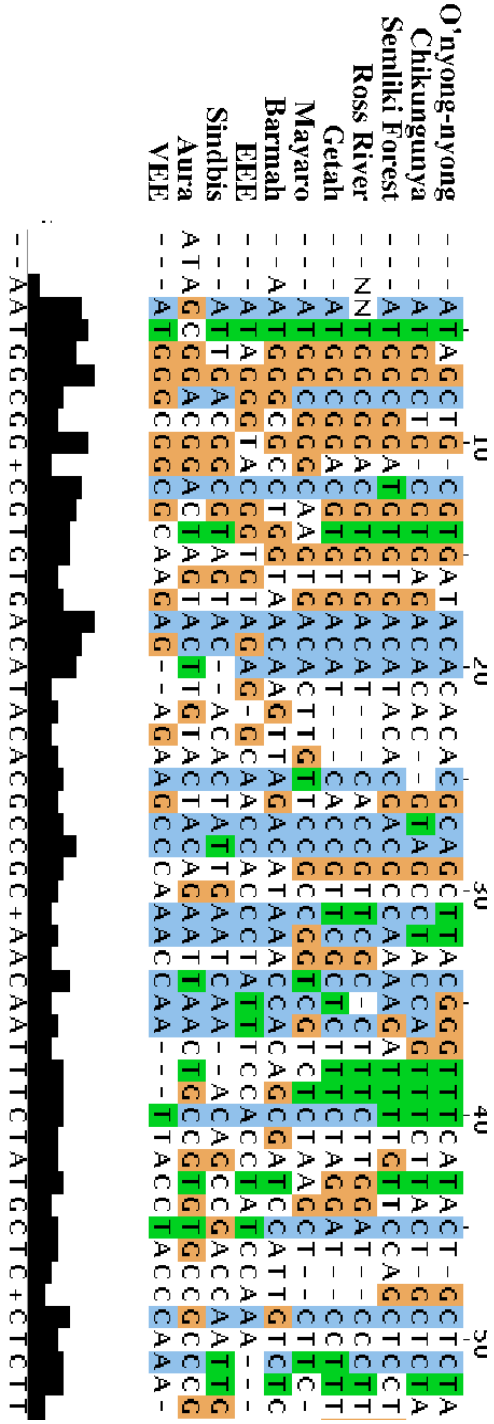


Figure A.1: Sequence alignment of alphavirus 5'UTR regions

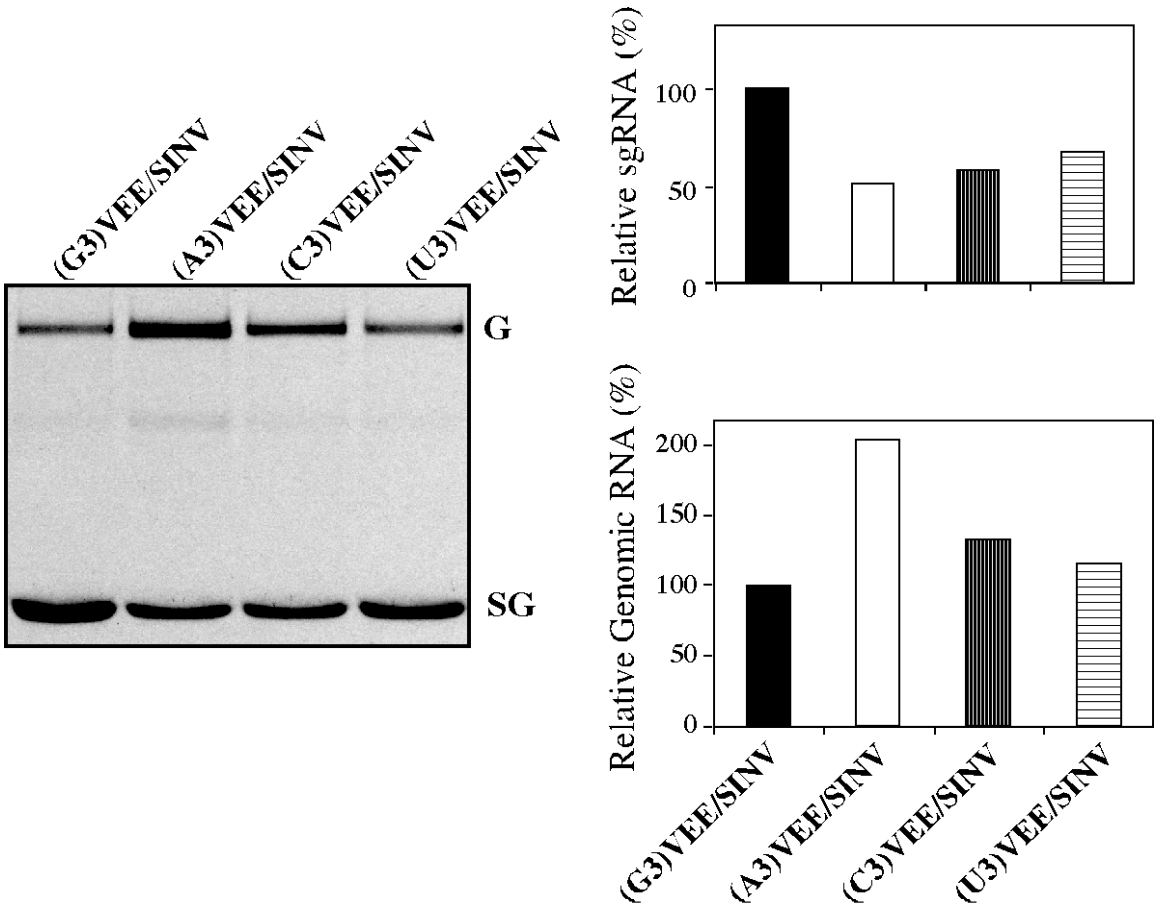


Figure A.2: Analysis of RNA synthesis in viruses encoding different nucleotides at nt3. BHK-21 cells were infected with chimeric viruses at an MOI of 10 Pfu/ml, and RNA was metabolically labeled between 4 and 8h post infection. RNAs were then isolated and analyzed by agarose gel electrophoresis. Relative amounts of genomic and subgenomic RNA synthesized is presented. G and SG indicate positions of the viral genomic and subgenomic RNAs, respectively.

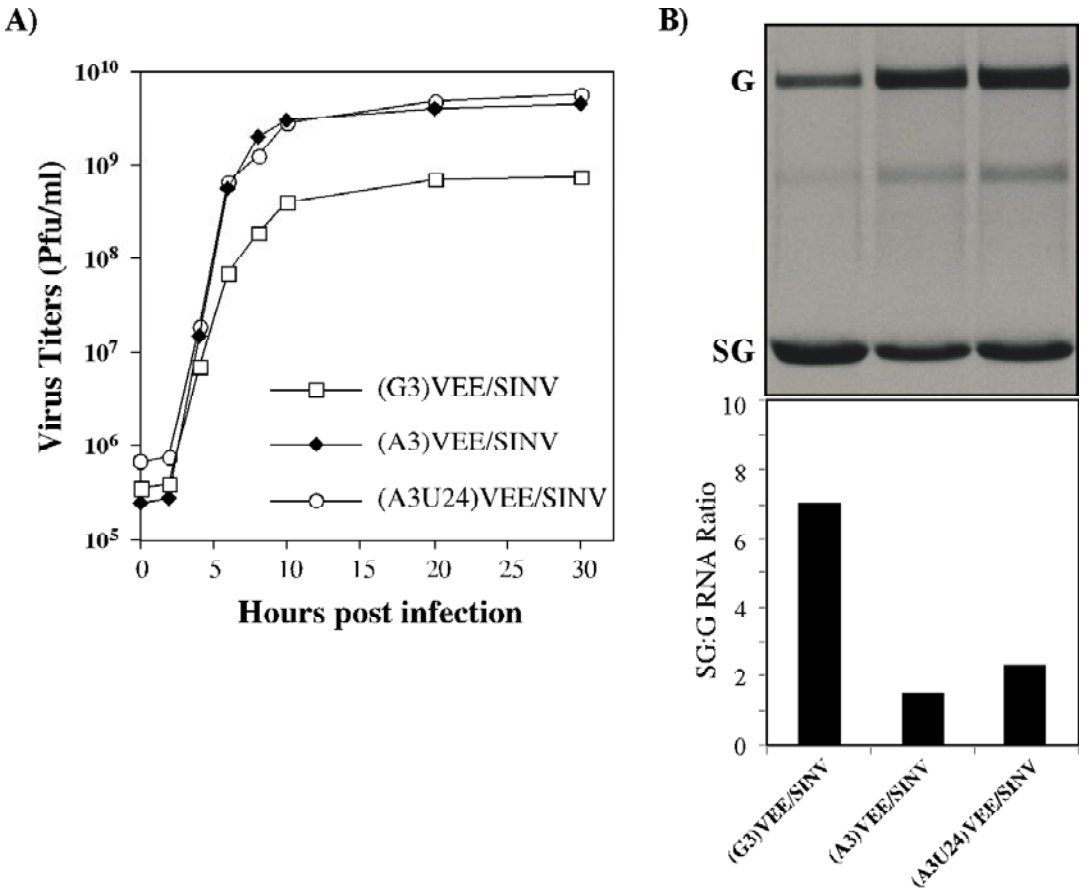


Figure A.3: A3 enhances virus replication and RNA synthesis. (A) Replication of chimeric viruses encoding 'A' at nt3 was analyzed by infecting BHK-21 cells at an MOI of 10 Pfu/ml. Media was replaced at indicated times post-infection and virus replication was assessed. (B) Virus specific RNA synthesis was analyzed by metabolically labeling viral RNAs between 4 and 8 h post infection as described in Materials and methods. RNAs were then isolated and analyzed by agarose gel electrophoresis. G and SG indicate positions of the viral genomic and subgenomic RNAs, respectively. The molar ratio of SG:G RNA is presented

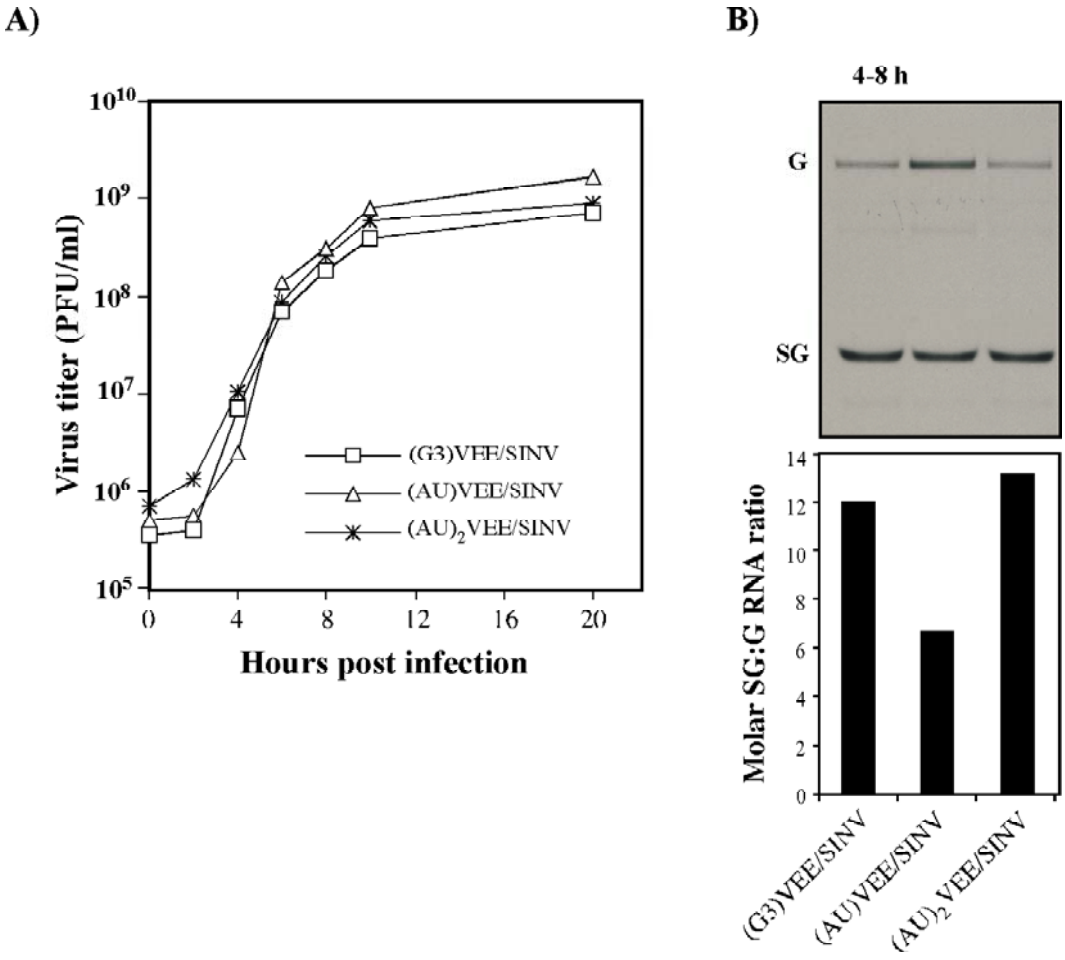


Figure A.4: Effect of AU additions to the 5'terminus of (G3)VEE/SINV. (A) Replication of (G3)VEE/SINVviruses encoding 5'AU additions. BHK-21 cells were infected at an MOI of 10 Pfu/ml, media was replaced at indicated times post-infection and virus replication was assessed. (B) Analysis of RNA synthesis by (G3)VEE/SINV viruses encoding AU additions to the 5'terminus. RNAs were metabolically labeled between 4 and 8 h post infection, isolated and analyzed by agarose gel electrophoresis. G and SG indicate positions of the viral genomic and subgenomic RNAs, respectively. The molar ratio of SG:G RNA is presented.

REFERENCES

Abell, B. A., and Brown, D. T. (1993). Sindbis virus membrane fusion is mediated by reduction of glycoprotein disulfide bridges at the cell surface. *J Virol* **67**(9)**,** 5496-501.

Ahlquist, P. (2002). RNA-dependent RNA polymerases, viruses, and RNA silencing. *Science* **296**(5571)**,** 1270-3.

Ahola, T., Kujala, P., Tuittila, M., Blom, T., Laakkonen, P., Hinkkanen, A., and Auvinen, P. (2000). Effects of palmitoylation of replicase protein nsP1 on alphavirus infection. *J Virol* **74**(15)**,** 6725-33.

Ahola, T., Laakkonen, P., Vihinen, H., and Kaariainen, L. (1997). Critical residues of Semliki Forest virus RNA capping enzyme involved in methyltransferase and guanylyltransferase-like activities. *J Virol* **71**(1)**,** 392-7.

Ahola, T., Lampio, A., Auvinen, P., and Kaariainen, L. (1999). Semliki Forest virus mRNA capping enzyme requires association with anionic membrane phospholipids for activity. *Embo J* **18**(11)**,** 3164-72.

Babendure, J. R., Babendure, J. L., Ding, J. H., and Tsien, R. Y. (2006). Control of mammalian translation by mRNA structure near caps. *RNA* **12**(5)**,** 851-61.

Baird, S. D., Turcotte, M., Korneluk, R. G., and Holcik, M. (2006). Searching for IRES. *RNA* **12**(10)**,** 1755-85.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* **136**(2)**,** 215-33.

Barth, B. U., Suomalainen, M., Liljestrom, P., and Garoff, H. (1992). Alphavirus assembly and entry: role of the cytoplasmic tail of the E1 spike subunit. *J Virol* **66**(12)**,** 7560-4.

Barton, D. J., Sawicki, D. L., and Sawicki, S. G. (1990). Association of alphavirus replication with the cytoskeletal framework and transcription in vitro in the absence of membranes. *In* "New aspects of positive-strand RNA viruses" (M. A.

Brinton, and F. X. Heinz, Eds.), pp. 75-79. American Society for Microbiology, Washington, DC.

Becher, P., Orlich, M., and Thiel, H. J. (2000). Mutations in the 5' nontranslated region of bovine viral diarrhea virus result in altered growth characteristics. *J Virol* **74**(17)**,** 7884-94.

Beck, C. E., and Wyckoff, R. W. (1938). Venezuelan Equine Encephalomyelitis. *Science* **88**(2292)**,** 530.

Berge, T. O., Banks, I. S., and Tigertt, W. D. (1961). Attenuation of Venezuelan equine encephalomyelitis virus by in vitro cultivation in guinea pig heart cells. *Am. J. Epidemiol.* **73**(2)**,** 209-218.

Bernard, K. A., Klimstra, W. B., and Johnston, R. E. (2000). Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology* **276**(1)**,** 93-103.

Bowen, G. S., and Calisher, C. H. (1976). Virological and serological studies of Venezuelan equine encephalomyelitis in humans. *J Clin Microbiol* **4**(1)**,** 22-7.

Brault, A. C., Powers, A. M., Holmes, E. C., Woelk, C. H., and Weaver, S. C. (2002). Positively charged amino acid substitutions in the e2 envelope glycoprotein are associated with the emergence of venezuelan equine encephalitis virus. *J Virol* **76**(4)**,** 1718-30.

Brault, A. C., Powers, A. M., Ortiz, D., Estrada-Franco, J. G., Navarro-Lopez, R., and Weaver, S. C. (2004). Venezuelan equine encephalitis emergence: enhanced vector infection from a single amino acid substitution in the envelope glycoprotein. *Proc Natl Acad Sci U S A* **101**(31)**,** 11344-9.

Bredenbeek, P. J., Frolov, I., Rice, C. M., and Schlesinger, S. (1993). Sindbis virus expression vectors: Packaging of RNA replicons by using defective helper RNAs. *J. Virol.* **67,** 6439-6446.

Brierley, I., Pennell, S., and Gilbert, R. J. (2007). Viral RNA pseudoknots: versatile motifs in gene expression and replication. *Nat Rev Microbiol* **5**(8)**,** 598-610.

Burke, D. S., Ramsburg, H. H., and Edelman, R. (1977). Persistence in humans of antibody to subtypes of Venezuelan equine encephalomyelitis (VEE) virus after immunization with attenuated (TC-83) VEE virus vaccine. *J Infect Dis* **136**(3)**,** 354-9.

Carleton, M., Lee, H., Mulvey, M., and Brown, D. T. (1997). Role of glycoprotein PE2 in formation and maturation of the Sindbis virus spike. *J Virol* **71**(2)**,** 1558-66.

Cole, F. E., Jr., May, S. W., and Eddy, G. A. (1974). Inactivated Venezuelan equine encephalomyelitis vaccine prepared from attenuated (TC-83 strain) virus. *Appl Microbiol* **27**(1)**,** 150-3.

Cutler, D. F., and Garoff, H. (1986). Mutants of the membrane-binding region of Semliki Forest virus E2 protein. I. Cell surface transport and fusogenic activity. *J Cell Biol* **102**(3)**,** 889-901.

Dal Canto, M. C., and Rabinowitz, S. G. (1981). Central nervous system demyelination in Venezuelan equine encephalomyelitis infection. *J Neurol Sci* **49**(3)**,** 397-418.

Davis, N. L., Powell, N., Greenwald, G. F., Willis, L. V., Johnson, B. J., Smith, J. F., and Johnston, R. E. (1991). Attenuating mutations in the E2 glycoprotein gene of Venezuelan equine encephalitis virus: construction of single and multiple mutants in a full-length cDNA clone. *Virology* **183**(1)**,** 20-31.

de Groot, R. J., Rumenapf, T., Kuhn, R. J., Strauss, E. G., and Strauss, J. H. (1991). Sindbis virus RNA polymerase is degraded by the N-end rule pathway. *Proc Natl Acad Sci U S A* **88**(20)**,** 8967-71.

DeTulleo, L., and Kirchhausen, T. (1998). The clathrin endocytic pathway in viral infection. *EMBO J* **17**(16)**,** 4585-93.

Ding, M. X., and Schlesinger, M. J. (1989). Evidence that Sindbis virus NSP2 is an autoprotease which processes the virus nonstructural polyprotein. *Virology* **171**(1)**,** 280-4.

Dubuisson, J., Lustig, S., Akov, Y., and Rice, C. M. (1997). Genetic determinants of Sindbis virus neuroinvasiveness. *J. Virol.* **71,** 2636-2646.

Dubuisson, J., and Rice, C. M. (1993). Sindbis virus attachment: isolation and characterization of mutants with impaired binding to vertebrate cells. *J Virol* **67**(6)**,** 3363-74.

Ehrenkranz, N. J., and Ventura, A. K. (1974). Venezuelan equine encephalitis virus infection in man. *Annu Rev Med* **25,** 9-14.

Engler, R. J., Mangiafico, J. A., Jahrling, P., Ksiazek, T. G., Pedrotti-Krueger, M., and Peters, C. J. (1992). Venezuelan equine encephalitis-specific immunoglobulin responses: live attenuated TC-83 versus inactivated C-84 vaccine. *J Med Virol* **38**(4)**,** 305-10.

Fata, C. L., Sawicki, S. G., and Sawicki, D. L. (2002). Modification of Asn374 of nsP1 suppresses a Sindbis virus nsP4 minus-strand polymerase mutant. *J Virol* **76**(17)**,** 8641-9.

Fayzulin, R., and Frolov, I. (2004). Changes of the secondary structure of the 5' end of the Sindbis virus genome inhibit virus growth in mosquito cells and lead to accumulation of adaptive mutations. *J Virol* **78**(10)**,** 4953-64.

Ferrer-Orta, C., Arias, A., Escarmis, C., and Verdaguer, N. (2006). A comparison of viral RNA-dependent RNA polymerases. *Curr Opin Struct Biol* **16**(1)**,** 27-34.

Friebe, P., Lohmann, V., Krieger, N., and Bartenschlager, R. (2001). Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. *J Virol* **75**(24)**,** 12047-57.

Frolov, I. (2004). Persistent infection and suppression of host response by alphaviruses. *Arch Virol Suppl*(18)**,** 139-47.

Frolov, I., Agapov, E., Hoffman, T. A., Jr., Pragai, B. M., Lippa, M., Schlesinger, S., and Rice, C. M. (1999). Selection of RNA replicons capable of persistent noncytopathic replication in mammalian cells. *J Virol* **73**(5)**,** 3854-65.

Frolov, I., Hardy, R., and Rice, C. M. (2001). Cis-acting RNA elements at the 5' end of Sindbis virus genome RNA regulate minus- and plus-strand RNA synthesis. *RNA* **7**(11)**,** 1638-51.

Frolov, I., and Schlesinger, S. (1996). Translation of Sindbis virus mRNA: analysis of sequences downstream of the initiating AUG codon that enhance translation. *J Virol* **70**(2)**,** 1182-90.

Frolova, E. I., Fayzulin, R. Z., Cook, S. H., Griffin, D. E., Rice, C. M., and Frolov, I. (2002). Roles of nonstructural protein nsP2 and Alpha/Beta interferons in determining the outcome of Sindbis virus infection. *J Virol* **76**(22)**,** 11254-64.

Froshauer, S., Kartenbeck, J., and Helenius, A. (1988). Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. *J Cell Biol* **107**(6 Pt 1)**,** 2075-86.

Garmashova, N., Atasheva, S., Kang, W., Weaver, S. C., Frolova, E., and Frolov, I. (2007a). Analysis of Venezuelan equine encephalitis virus capsid protein function in the inhibition of cellular transcription. *J Virol* **81**(24)**,** 13552-65.

Garmashova, N., Gorchakov, R., Frolova, E., and Frolov, I. (2006). Sindbis virus nonstructural protein nsP2 is cytotoxic and inhibits cellular transcription. *J Virol* **80**(12)**,** 5686-96.

Garmashova, N., Gorchakov, R., Volkova, E., Paessler, S., Frolova, E., and Frolov, I. (2007b). The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. *J Virol* **81**(5)**,** 2472-84.

Garneau, N. L., Sokoloski, K. J., Opyrchal, M., Neff, C. P., Wilusz, C. J., and Wilusz, J. (2008). The 3' untranslated region of sindbis virus represses deadenylation of viral transcripts in mosquito and Mammalian cells. *J Virol* **82**(2)**,** 880-92.

George, J., and Raju, R. (2000). Alphavirus RNA genome repair and evolution: molecular characterization of infectious sindbis virus isolates lacking a known conserved motif at the 3' end of the genome. *J Virol* **74**(20)**,** 9776-85.

Gorchakov, R., Garmashova, N., Frolova, E., and Frolov, I. (2008). Different types of nsP3-containing protein complexes in Sindbis virus-infected cells. *J Virol* **82**(20)**,** 10088-101.

Gorchakov, R., Hardy, R., Rice, C. M., and Frolov, I. (2004). Selection of functional 5' cis-acting elements promoting efficient sindbis virus genome replication. *J Virol* **78**(1)**,** 61-75.

Greene, I. P., Paessler, S., Austgen, L., Anishchenko, M., Brault, A. C., Bowen, R. A., and Weaver, S. C. (2005). Envelope glycoprotein mutations mediate equine amplification and virulence of epizootic venezuelan equine encephalitis virus. *J Virol* **79**(14)**,** 9128-33.

Griffin, D. E. (2007). Alphaviruses. 5th ed. *In* "Fields Virology" (D. M. Knipe, and P. M. Howley, Eds.), Vol. 1, pp. 1023 - 1067. 2 vols. Lippncott, Williams & Wilkins, New York, NY.

Grimson, A., Srivastava, M., Fahey, B., Woodcroft, B. J., Chiang, H. R., King, N., Degnan, B. M., Rokhsar, D. S., and Bartel, D. P. (2008). Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. *Nature* **455**(7217)**,** 1193-7.

Hardy, R. W. (2006). The role of the 3' terminus of the Sindbis virus genome in minus-strand initiation site selection. *Virology* **345**(2)**,** 520-31.

Hardy, R. W., and Rice, C. M. (2005). Requirements at the 3' end of the sindbis virus genome for efficient synthesis of minus-strand RNA. *J Virol* **79**(8)**,** 4630-9.

Hardy, W. R., and Strauss, J. H. (1989). Processing the nonstructural polyproteins of sindbis virus: nonstructural proteinase is in the C-terminal half of nsP2 and functions both in cis and in trans. *J Virol* **63**(11)**,** 4653-64.

Hofacker, I. L. (2003). Vienna RNA secondary structure server. *Nucleic Acids Res* **31**(13)**,** 3429-31.

Ivanova, L., Le, L., and Schlesinger, M. J. (1995). Characterization of revertants of a Sindbis virus 6K gene mutant that affects proteolytic processing and virus assembly. *Virus Res* **39**(2-3)**,** 165-79.

Jahrling, P. B., and Stephenson, E. H. (1984). Protective efficacies of live attenuated and formaldehyde-inactivated Venezuelan equine encephalitis virus vaccines against aerosol challenge in hamsters. *J Clin Microbiol* **19**(3)**,** 429-31.

Johnston, R. E., and Peters, C. J. (1996). Alphaviruses. *In* "Virology, 3rd Edition" (B. N. Fields, D. M. Knipe, and P. M. Howley, Eds.), pp. 843-898. Lippincott-Raven, New York.

Johnston, R. E., and Smith, J. F. (1988). Selection for accelerated penetration in cell culture coselects for attenuated mutants of Venezuelan equine encephalitis virus. *Virology* **162**(2)**,** 437-43.

Kieft, J. S. (2008). Viral IRES RNA structures and ribosome interactions. *Trends Biochem Sci* **33**(6)**,** 274-83.

Kinney, R. M., Chang, G.-J., Tsuchiya, K. R., Sneider, J. M., Roehrig, J. T., Woodward, T. M., and Trent, D. W. (1993). Attenuation of Venezuelan equine encephalitis virus strain TC-83 is encoded by the 5'-non coding region and the E2 envelope glycoprotein. *J. Virol.* **67,** 1269-1277.

Klimstra, W. B., Nangle, E. M., Smith, M. S., Yurochko, A. D., and Ryman, K. D. (2003). DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. *J Virol* **77**(22)**,** 12022-32.

Knapp, G. (1989). Enzymatic approaches to probing of RNA secondary and tertiary structure. *Methods Enzymol* **180,** 192-212.

Knipfer, M. E., and Brown, D. T. (1989). Intracellular transport and processing of Sindbis virus glycoproteins. *Virology* **170**(1)**,** 117-22.

Kobiler, D., Rice, C. M., Brodie, C., Shahar, A., Dubuisson, J., Halevy, M., and Lustig, S. (1999). A single nucleotide change in the 5' noncoding region of Sindbis virus confers neurovirulence in rats. *J Virol* **73**(12)**,** 10440-6.

Kolokoltsov, A. A., Fleming, E. H., and Davey, R. A. (2006). Venezuelan equine encephalitis virus entry mechanism requires late endosome formation and resists cell membrane cholesterol depletion. *Virology* **347**(2)**,** 333-42.

Kozak, M. (1986). Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc Natl Acad Sci U S A* **83**(9)**,** 2850-4.

Kozak, M. (2005). Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* **361,** 13-37.

Kuhn, R. J., Hong, Z., and Strauss, J. H. (1990). Mutagenesis of the 3' nontranslated region of Sindbis virus RNA. *J Virol* **64**(4)**,** 1465-76.

Kulasegaran-Shylini, R., Atasheva, S., Gorenstein, D. G., and Frolov, I. (2009a). Structural and functional elements of the promoter encoded by 5'UTR of Venezuelan equine encephalitis virus genome. *J Virol* **(In Press; Epub June 10 2009)**.

Kulasegaran-Shylini, R., Thiviyanathan, V., Gorenstein, D. G., and Frolov, I. (2009b). The 5'UTR-specific mutation in VEEV TC-83 genome has a strong effect on RNA replication and subgenomic RNA synthesis, but not on translation of the encoded proteins. *Virology* **387**(1)**,** 211-221.

Laakkonen, P., Ahola, T., and Kaariainen, L. (1996). The effects of palmitoylation on membrane association of Semliki forest virus RNA capping enzyme. *J Biol Chem* **271**(45)**,** 28567-71.

Lastarza, M. W., Grakoui, A., and Rice, C. M. (1994). Deletion and duplication mutations in the C-terminal nonconserved region of Sindbis virus nsP3: effects on phosphorylation and on virus replication in vertebrate and invertebrate cells. *Virology* **202**(1)**,** 224-32.

LaStarza, M. W., Lemm, J. A., and Rice, C. M. (1994). Genetic analysis of the nsP3 region of Sindbis virus: evidence for roles in minus-strand and subgenomic RNA synthesis. *J Virol* **68**(9)**,** 5781-91.

Lemm, J. A., Durbin, R. K., Stollar, V., and Rice, C. M. (1990). Mutations which alter the level or structure of nsP4 can affect the efficiency of Sindbis virus replication in a host-dependent manner. *J Virol* **64**(6)**,** 3001-11.

Lemm, J. A., and Rice, C. M. (1993a). Assembly of functional Sindbis virus RNA replication complexes: Requirement for coexpression of P123 and P34. *J. Virol.* **67,** 1905-1915.

Lemm, J. A., and Rice, C. M. (1993b). Roles of nonstructural polyproteins and cleavage products in regulating Sindbis virus RNA replication and transcription. *J Virol* **67**(4)**,** 1916-26.

Lemm, J. A., Rumenapf, T., Strauss, E. G., Strauss, J. H., and Rice, C. M. (1994). Polypeptide requirements for assembly of functional Sindbis virus replication complexes: a model for the temporal regulation of minus- and plus-strand RNA synthesis. *Embo J* **13**(12)**,** 2925-34.

Leon, C. A. (1975). Sequelae of Venezuelan equine encephalitis in humans: a four year follow-up. *Int J Epidemiol* **4**(2)**,** 131-40.

Levine, B., Jiang, H. H., Kleeman, L., and Yang, G. (1996). Effect of E2 envelope glycoprotein cytoplasmic domain mutations on Sindbis virus pathogenesis. *J Virol* **70**(2)**,** 1255-60.

Li, G. P., La Starza, M. W., Hardy, W. R., Strauss, J. H., and Rice, C. M. (1990). Phosphorylation of Sindbis virus nsP3 in vivo and in vitro. *Virology* **179**(1)**,** 416-27.

Li, G. P., and Rice, C. M. (1989). Mutagenesis of the in-frame opal termination codon preceding nsP4 of Sindbis virus: studies of translational readthrough and its effect on virus replication. *J Virol* **63**(3)**,** 1326-37.

Li, M. L., and Stollar, V. (2004). Identification of the amino acid sequence in Sindbis virus nsP4 that binds to the promoter for the synthesis of the subgenomic RNA. *Proc Natl Acad Sci U S A* **101**(25)**,** 9429-34.

Li, M. L., and Stollar, V. (2007). Distinct sites on the Sindbis virus RNA-dependent RNA polymerase for binding to the promoters for the synthesis of genomic and subgenomic RNA. *J Virol* **81**(8)**,** 4371-3.

Liljestrom, P., Lusa, S., Huylebroeck, D., and Garoff, H. (1991). In vitro mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-molecular-weight membrane protein modulates virus release. *J Virol* **65**(8)**,** 4107-13.

Liljeström, P., Lusa, S., Huylebroeck, D., and Garoff, H. (1991). *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the small 6,000-

molecular-weight membrane protein modulates virus release. *J. Virol.* **65,** 4107-4113.

Linger, B. R., Kunovska, L., Kuhn, R. J., and Golden, B. L. (2004). Sindbis virus nucleocapsid assembly: RNA folding promotes capsid protein dimerization. *RNA* **10**(1)**,** 128-38.

Liu, L. N., Lee, H., Hernandez, R., and Brown, D. T. (1996). Mutations in the endo domain of Sindbis virus glycoprotein E2 block phosphorylation, reorientation of the endo domain, and nucleocapsid binding. *Virology* **222**(1)**,** 236-46.

Loewy, A., Smyth, J., von Bonsdorff, C. H., Liljestrom, P., and Schlesinger, M. J. (1995). The 6-kilodalton membrane protein of Semliki Forest virus is involved in the budding process. *J Virol* **69**(1)**,** 469-75.

Lopez, S., Yao, J. S., Kuhn, R. J., Strauss, E. G., and Strauss, J. H. (1994). Nucleocapsid-glycoprotein interactions required for assembly of alphaviruses. *J Virol* **68**(3)**,** 1316-23.

Marsh, M., Kielian, M. C., and Helenius, A. (1984). Semliki forest virus entry and the endocytic pathway. *Biochem Soc Trans* **12**(6)**,** 981-3.

Mayuri, Geders, T. W., Smith, J. L., and Kuhn, R. J. (2008). Role for conserved residues of Sindbis virus nsP2 methyltransferase-like domain in regulation of minus-strand synthesis and development of cytopathic infection. *J Virol* **82**(15)**,** 7284-97.

McKinney, R. W., Berge, T. O., Sawyer, W. D., Tigertt, W. D., and Crozier, D. (1963). Use of an Attenuated Strain of Venezuelan Equine Encephalomyelitis Virus for Immunization in Man. *Am J Trop Med Hyg* **12,** 597-603.

McKnight, K. L., Simpson, D. A., Lin, S. C., Knott, T. A., Polo, J. M., Pence, D. F., Johannsen, D. B., Heidner, H. W., Davis, N. L., and Johnston, R. E. (1996). Deduced consensus sequence of Sindbis virus strain AR339: mutations contained in laboratory strains which affect cell culture and in vivo phenotypes. *J Virol* **70**(3)**,** 1981-9.

Melton, J. V., Ewart, G. D., Weir, R. C., Board, P. G., Lee, E., and Gage, P. W. (2002). Alphavirus 6K proteins form ion channels. *J Biol Chem* **277**(49)**,** 46923-31.

Merits, A., Vasiljeva, L., Ahola, T., Kaariainen, L., and Auvinen, P. (2001). Proteolytic processing of Semliki Forest virus-specific non-structural polyprotein by nsP2 protease. *J Gen Virol* **82**(Pt 4)**,** 765-73.

Mi, S., Durbin, R., Huang, H. V., Rice, C. M., and Stollar, V. (1989). Association of the Sindbis virus RNA methyltransferase activity with the nonstructural protein nsP1. *Virology* **170**(2)**,** 385-91.

Michel, G., Petrakova, O., Atasheva, S., and Frolov, I. (2007). Adaptation of Venezuelan equine encephalitis virus lacking 51-nt conserved sequence element to replication in mammalian and mosquito cells. *Virology* **362**(2)**,** 475-87.

Migliaccio, G., Pascale, M. C., Leone, A., and Bonatti, S. (1989). Biosynthesis, membrane translocation, and surface expression of Sindbis virus E1 glycoprotein. *Exp Cell Res* **185**(1)**,** 203-16.

Milligan, J. F., Groebe, D. R., Witherell, G. W., and Uhlenbeck, O. C. (1987). Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res* **15**(21)**,** 8783-98.

Monroe, S. S., and Schlesinger, S. (1983). RNAs from two independently isolated defective interfering particles of Sindbis virus contain a cellular tRNA sequence at their 5' ends. *Proc Natl Acad Sci U S A* **80**(11)**,** 3279-83.

Mukhopadhyay, S., Zhang, W., Gabler, S., Chipman, P. R., Strauss, E. G., Strauss, J. H., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2006). Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. *Structure* **14**(1)**,** 63-73.

Mulvey, M., and Brown, D. T. (1996). Assembly of the Sindbis virus spike protein complex. *Virology* **219**(1)**,** 125-32.

Navaratnarajah, C. K., and Kuhn, R. J. (2007). Functional characterization of the Sindbis virus E2 glycoprotein by transposon linker-insertion mutagenesis. *Virology* **363**(1)**,** 134-47.

Nickens, D. G., and Hardy, R. W. (2008). Structural and functional analyses of stem-loop 1 of the Sindbis virus genome. *Virology* **370**(1)**,** 158-72.

Nicola, A. V., Chen, W., and Helenius, A. (1999). Co-translational folding of an alphavirus capsid protein in the cytosol of living cells. *Nat Cell Biol* **1**(6)**,** 341-5.

Niesters, H. G., and Strauss, J. H. (1990). Defined mutations in the 5' nontranslated sequence of Sindbis virus RNA. *J. Virol.* **64,** 4162-4168.

Ou, J. H., Rice, C. M., Dalgarno, L., Strauss, E. G., and Strauss, J. H. (1982). Sequence studies of several alphavirus genomic RNAs in the region containing the start of the subgenomic RNA. *Proc Natl Acad Sci U S A* **79**(17)**,** 5235-9.

Ou, J. H., Strauss, E. G., and Strauss, J. H. (1981). Comparative studies of the 3'-terminal sequences of several alpha virus RNAs. *Virology* **109**(2)**,** 281-9.

Ou, J. H., Strauss, E. G., and Strauss, J. H. (1983). The 5'-terminal sequences of the genomic RNAs of several alphaviruses. *J Mol Biol* **168**(1)**,** 1-15.

Ou, J. H., Trent, D. W., and Strauss, J. H. (1982). The 3'-non-coding regions of alphavirus RNAs contain repeating sequences. *J Mol Biol* **156**(4)**,** 719-30.

Pardigon, N., Lenches, E., and Strauss, J. H. (1993). Multiple binding sites for cellular proteins in the 3' end of Sindbis alphavirus minus-sense RNA. *J Virol* **67**(8)**,** 5003-11.

Pardigon, N., and Strauss, J. H. (1992). Cellular proteins bind to the 3' end of Sindbis virus minus-strand RNA. *J Virol* **66**(2)**,** 1007-15.

Paredes, A., Alwell-Warda, K., Weaver, S. C., Chiu, W., and Watowich, S. J. (2001). Venezuelan equine encephalomyelitis virus structure and its divergence from old world alphaviruses. *J Virol* **75**(19)**,** 9532-7.

Paredes, A., Alwell-Warda, K., Weaver, S. C., Chiu, W., and Watowich, S. J. (2003). Structure of isolated nucleocapsids from venezuelan equine encephalitis virus and implications for assembly and disassembly of enveloped virus. *J Virol* **77**(1)**,** 659-64.

Peranen, J., and Kaariainen, L. (1991). Biogenesis of type I cytopathic vacuoles in Semliki Forest virus-infected BHK cells. *J Virol* **65**(3)**,** 1623-7.

Peranen, J., Laakkonen, P., Hyvonen, M., and Kaariainen, L. (1995). The alphavirus replicase protein nsP1 is membrane-associated and has affinity to endocytic organelles. *Virology* **208**(2)**,** 610-20.

Perera, R., Owen, K. E., Tellinghuisen, T. L., Gorbalenya, A. E., and Kuhn, R. J. (2001). Alphavirus nucleocapsid protein contains a putative coiled coil alpha-helix important for core assembly. *J Virol* **75**(1)**,** 1-10.

Petrakova, O., Volkova, E., Gorchakov, R., Paessler, S., Kinney, R. M., and Frolov, I. (2005). Noncytopathic replication of Venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in Mammalian cells. *J Virol* **79**(12)**,** 7597-608.

Pfeffer, M., Kinney, R. M., and Kaaden, O. R. (1998). The alphavirus 3'-nontranslated region: size heterogeneity and arrangement of repeated sequence elements. *Virology* **240**(1)**,** 100-8.

Powers, A. M., Brault, A. C., Shirako, Y., Strauss, E. G., Kang, W., Strauss, J. H., and Weaver, S. C. (2001). Evolutionary relationships and systematics of the alphaviruses. *J Virol* **75**(21)**,** 10118-31.

Raju, R., Hajjou, M., Hill, K. R., Botta, V., and Botta, S. (1999). In vivo addition of poly(A) tail and AU-rich sequences to the 3' terminus of the Sindbis virus RNA genome: a novel 3'-end repair pathway. *J Virol* **73**(3)**,** 2410-9.

Raju, R., and Huang, H. V. (1991). Analysis of Sindbis virus promoter recognition in vivo, using novel vectors with two subgenomic mRNA promoters. *J Virol* **65**(5)**,** 2501-10.

Randall, R., Maurer, F. D., and Smadel, J. E. (1949). Immunization of laboratory workers with purified Venezuelan equine encephalomyelitis vaccine. *J Immunol* **63**(3)**,** 313-8.

Rice, C. M., Levis, R., Strauss, J. H., and Huang, H. V. (1987). Production of infectious RNA transcripts from Sindbis virus cDNA clones: Mapping of lethal

mutations, rescue of a temperature-sensitive marker, and *in vitro* mutagenesis to generate defined mutants. *J. Virol.* **61**(12)**,** 3809-3819.

Rikkonen, M., Peranen, J., and Kaariainen, L. (1994). ATPase and GTPase activities associated with Semliki Forest virus nonstructural protein nsP2. *J Virol* **68**(9)**,** 5804-10.

Rivas, F., Diaz, L. A., Cardenas, V. M., Daza, E., Bruzon, L., Alcala, A., De la Hoz, O., Caceres, F. M., Aristizabal, G., Martinez, J. W., Revelo, D., De la Hoz, F., Boshell, J., Camacho, T., Calderon, L., Olano, V. A., Villarreal, L. I., Roselli, D., Alvarez, G., Ludwig, G., and Tsai, T. (1997). Epidemic Venezuelan equine encephalitis in La Guajira, Colombia, 1995. *J Infect Dis* **175**(4)**,** 828-32.

Rubach, J. K., Wasik, B. R., Rupp, J. C., Kuhn, R. J., Hardy, R. W., and Smith, J. L. (2009). Characterization of purified Sindbis virus nsP4 RNA-dependent RNA polymerase activity in vitro. *Virology* **384**(1)**,** 201-8.

Russo, A. T., White, M. A., and Watowich, S. J. (2006). The crystal structure of the Venezuelan equine encephalitis alphavirus nsP2 protease. *Structure* **14**(9)**,** 1449-58.

Ryman, K. D., Klimstra, W. B., and Johnston, R. E. (2004). Attenuation of Sindbis virus variants incorporating uncleaved PE2 glycoprotein is correlated with attachment to cell-surface heparan sulfate. *Virology* **322**(1)**,** 1-12.

Salonen, A., Ahola, T., and Kaariainen, L. (2005). Viral RNA replication in association with cellular membranes. *Curr Top Microbiol Immunol* **285,** 139-73.

Sanz, M. A., and Carrasco, L. (2001). Sindbis virus variant with a deletion in the 6K gene shows defects in glycoprotein processing and trafficking: lack of complementation by a wild-type 6K gene in trans. *J Virol* **75**(16)**,** 7778-84.

Schlesinger, M. J., London, S. D., and Ryan, C. (1993). An in-frame insertion into the Sindbis virus 6K gene leads to defective proteolytic processing of the virus glycoproteins, a trans-dominant negative inhibition of normal virus formation, and interference in virus shut off of host-cell protein synthesis. *Virology* **193**(1)**,** 424-32.

Shirako, Y., and Strauss, J. H. (1994). Regulation of Sindbis virus RNA replication: uncleaved P123 and nsP4 function in minus-strand RNA synthesis, whereas cleaved products from P123 are required for efficient plus-strand RNA synthesis. *J Virol* **68**(3)**,** 1874-85.

Shirako, Y., and Strauss, J. H. (1998). Requirement for an aromatic amino acid or histidine at the N terminus of Sindbis virus RNA polymerase. *J Virol* **72**(3)**,** 2310-5.

Singh, I., and Helenius, A. (1992). Role of ribosomes in Semliki Forest virus nucleocapsid uncoating. *J Virol* **66**(12)**,** 7049-58.

Sjoberg, M., and Garoff, H. (2003). Interactions between the transmembrane segments of the alphavirus E1 and E2 proteins play a role in virus budding and fusion. *J Virol* **77**(6)**,** 3441-50.

Smith, A. L., and Tignor, G. H. (1980). Host cell receptors for two strains of Sindbis virus. *Arch Virol* **66**(1)**,** 11-26.

Soderlund, H., and Ulmanen, I. (1977). Transient association of Semliki Forest virus capsid protein with ribosomes. *J Virol* **24**(3)**,** 907-9.

Strauss, J. H., and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiol Rev* **58**(3)**,** 491-562.

Sudia, W. D., and Newhouse, V. F. (1975). Epidemic Venezuelan equine encephalitis in North America: a summary of virus-vector-host relationships. *Am J Epidemiol* **101**(1)**,** 1-13.

Tellinghuisen, T. L., and Kuhn, R. J. (2000). Nucleic acid-dependent cross-linking of the nucleocapsid protein of Sindbis virus. *J Virol* **74**(9)**,** 4302-9.

Thal, M. A., Wasik, B. R., Posto, J., and Hardy, R. W. (2007). Template requirements for recognition and copying by Sindbis virus RNA-dependent RNA polymerase. *Virology* **358**(1)**,** 221-32.

Tomar, S., Hardy, R. W., Smith, J. L., and Kuhn, R. J. (2006). Catalytic core of alphavirus nonstructural protein nsP4 possesses terminal adenylyltransferase activity. *J Virol* **80**(20)**,** 9962-9.

Ulmanen, I., Soderlund, H., and Kaariainen, L. (1976). Semliki Forest virus capsid protein associates with the 60S ribosomal subunit in infected cells. *J Virol* **20**(1)**,** 203-10.

Vasiljeva, L., Merits, A., Auvinen, P., and Kaariainen, L. (2000). Identification of a novel function of the alphavirus capping apparatus. RNA 5'-triphosphatase activity of Nsp2. *J Biol Chem* **275**(23)**,** 17281-7.

Vega Laso, M. R., Zhu, D., Sagliocco, F., Brown, A. J., Tuite, M. F., and McCarthy, J. E. (1993). Inhibition of translational initiation in the yeast Saccharomyces cerevisiae as a function of the stability and position of hairpin structures in the mRNA leader. *J Biol Chem* **268**(9)**,** 6453-62.

Vihinen, H., Ahola, T., Tuittila, M., Merits, A., and Kaariainen, L. (2001). Elimination of phosphorylation sites of Semliki Forest virus replicase protein nsP3. *J Biol Chem* **276**(8)**,** 5745-52.

Vihinen, H., and Saarinen, J. (2000). Phosphorylation site analysis of Semliki forest virus nonstructural protein 3. *J Biol Chem* **275**(36)**,** 27775-83.

Volkova, E., Gorchakov, R., and Frolov, I. (2006). The efficient packaging of Venezuelan equine encephalitis virus-specific RNAs into viral particles is determined by nsP1-3 synthesis. *Virology* **344**(2)**,** 315-27.

Vonderheit, A., and Helenius, A. (2005). Rab7 associates with early endosomes to mediate sorting and transport of Semliki forest virus to late endosomes. *PLoS Biol* **3**(7)**,** e233.

Vournakis, J. N., Celantano, J., Finn, M., Lockard, R. E., Mitra, T., Pavlakis, G., Troutt, A., van den Berg, M., and Wurst, R. M. (1981). Sequence and structure analysis of end-labeled RNA with nucleases. *Gene Amplif Anal* **2,** 267-98.

Wahlberg, J. M., and Garoff, H. (1992). Membrane fusion process of Semliki Forest virus. I: Low pH-induced rearrangement in spike protein quaternary structure precedes virus penetration into cells. *J Cell Biol* **116**(2)**,** 339-48.

Wang, Y. F., Sawicki, S. G., and Sawicki, D. L. (1994). Alphavirus nsP3 functions to form replication complexes transcribing negative-strand RNA. *J Virol* **68**(10)**,** 6466-75.

Warrier, R., Linger, B. R., Golden, B. L., and Kuhn, R. J. (2008). Role of sindbis virus capsid protein region II in nucleocapsid core assembly and encapsidation of genomic RNA. *J Virol* **82**(9)**,** 4461-70.

Weaver, S. C., Ferro, C., Barrera, R., Boshell, J., and Navarro, J. C. (2004). Venezuelan equine encephalitis. *Annu Rev Entomol* **49,** 141-74.

Weaver, S. C., Salas, R., Rico-Hesse, R., Ludwig, G. V., Oberste, M. S., Boshell, J., and Tesh, R. B. (1996). Re-emergence of epidemic Venezuelan equine encephalomyelitis in South America. VEE Study Group. *Lancet* **348**(9025)**,** 436-40.

White, L. J., Wang, J. G., Davis, N. L., and Johnston, R. E. (2001). Role of alpha/beta interferon in Venezuelan equine encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region. *J Virol* **75**(8)**,** 3706-18.

Wielgosz, M. M., Raju, R., and Huang, H. V. (2001). Sequence requirements for Sindbis virus subgenomic mRNA promoter function in cultured cells. *J Virol* **75**(8)**,** 3509-19.

Wilkinson, T. A., Tellinghuisen, T. L., Kuhn, R. J., and Post, C. B. (2005). Association of sindbis virus capsid protein with phospholipid membranes and the E2 glycoprotein: implications for alphavirus assembly. *Biochemistry* **44**(8)**,** 2800-10.

Wu, B., and White, K. A. (2007). Uncoupling RNA virus replication from transcription via the polymerase: functional and evolutionary insights. *Embo J* **26**(24)**,** 5120-30.

Yu, H., Isken, O., Grassmann, C. W., and Behrens, S. E. (2000). A stem-loop motif formed by the immediate 5' terminus of the bovine viral diarrhea virus genome modulates translation as well as replication of the viral RNA. *J Virol* **74**(13)**,** 5825-35.

Zuker, M. (2003). Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* **31**(13)**,** 3406-15.

Vita

Raghavendran Kulasegaran Shylini was born in Chennai, India on July 17, 1983. Following the completion of his high school education at Balalok Matriculation Higher Secondary School, Raghavendran enrolled in the Industrial Biotechnology undergraduate program at Anna University, Chennai in 2000. After graduating with a degree of Bachelor of Technology in Industrial Biotechnology in 2004, he joined the Biophysical, Structural and Computational Biology Ph. D program at the University of Texas Medical Branch at Galvestion. He began his doctoral research in 2005, in the labs of Dr. David Gorenstein, in the Department of Biochemistry and Molecular Biology, and Dr. Ilya Frolov, in the department of Microbiology and Immunology.

While at UTMB, Raghavendran received the McLaughlin Predoctoral Fellowship in 2007. He was awarded the Biological Chemistry Student Organization Student Award for Service in 2007, and the Barbara Bowman Memorial Award for Research Excellence in 2008. He was also nominated to Who's Who in American Universities & Colleges in 2008. In addition, he received student travel awards from ENC to present a poster at the 47th Experimental Nuclear Magnetic Resonance Conference (ENC) in 2006 and from the RNA Society to present a poster at the 12th Annual RNA Society Meeting in 2007. Furthermore, he has also presented posters at the Annual Sealy Center for Structural Biology Symposium in 2005 and 2008, and the Annual McLaughlin Colloquium in 2007 and 2008.