

The Development of New Tools to Investigate Alphavirus Replication Kinetics

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ABSTRACT

Members of the alphavirus genus pose a serious or potential threat to public health in many areas of the world. Nearly all alphaviruses are maintained in nature by transmission cycles that involve alternating replication in a susceptible vertebrate and invertebrate host. The maintenance of this transmission cycle depends on the establishment of a life-long persistent infection in the invertebrate vector host. Although alphavirus replication has been extensively studied in vertebrate models, the strand-specific replication kinetics of alphaviruses during persistent infections of the invertebrate host have not been reported. We investigated the strand-specific replication of different alphavirus genotypes in invertebrate cells.

By comparing different detection strategies and chemistries, we identified an optimal ssqPCR assay design for strand-specific quantification of viral RNAs in infected cells and tissues. We found that primer sets incorporating the use of a non-target tag sequence were able to avoid real-time PCR detection of amplicons that were falsely-primed during reverse-transcription. We also determined that DNA hydrolysis probes increased the sensitivity of ssqPCR assays when compared to a double-stranded DNA-specific dye, SYBR Green.

Using this information, we determined the replication kinetics of two different genotypes of o'nyong nyong virus (ONNV) and chikungunya virus (CHIKV) in infected mosquito cells. We found that (-) strand viral RNAs persisted in invertebrate cells for up to 21 days after infection. We also found that significantly less (-) strand RNA was present in cells infected with opal variants of both ONNV and CHIKV than sense variants at several time points post infection, suggesting that the opal codon has a functional role in (-) strand RNA regulation. We also report the development of an ONNV replicon expression system.

In total, the tools we developed for this report will facilitate future replication studies in the mosquito that may shed light on questions regarding the regulatory role of the opal codon and the persistence of (-) strand RNAs during long-term infections. The strand-specific replication kinetics of ONNV and CHIKV genotypes reported here will serve as a foundation for such investigations.

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Chapter 1

LITERATURE REVIEW

Public health impact of vector-borne diseases

Emerging infectious diseases threaten the public health on a global scale. In particular, vector-borne viral and parasitic diseases are major economic and public health concerns. A large portion of these pathogens are mosquito-transmitted and afflict hundreds of millions of people every year. According to the World Health Organization (WHO), over 200 million cases of malaria occurred in 2006 alone, and almost one million people died as a result (WHO, 2008). While malaria is considered the most deadly vector-borne disease, mosquito-borne viral diseases can be equally morbid and debilitating. Dengue fever (DF) and dengue hemorrhagic fever (DHF), caused by dengue viruses, have been reported in over 100 countries around the world, mostly in Africa, Southern Asia, and Latin and South America (WHO, 2009). Approximately 50-100 million cases of DF occur annually, along with 250,000-500,000 annual cases of DHF, the more fatal dengue disease (Clarke, 2002; WHO, 2009). Yellow fever virus (YFV) also remains prominent in many underdeveloped countries in Africa, Central America, and South America. Despite the availability of an effective vaccine, an estimated 200,000 cases occur every year with approximately 30,000 deaths (Vainio and Cutts, 1998; WHO, 2003). The resurgence of YFV was facilitated by the reemergence of its vector, the *Aedes aegypti* mosquito, and an increase in the urban population in sub-Saharan Africa and South America (Barrett and Higgs, 2007).

Although disease control and prevention practices have been implemented, the development of a permanent, large-scale solution to mosquito-borne disease transmission still evades us. By early to mid-20th century, programs were created to control the vector population which included breeding ground eradication and insecticide usage (Gubler, 1998). Vaccine development was also initially successful, as was evident during the early to mid 1900's (Barrett and Higgs, 2007). However, the combination of these efforts ultimately proved unsuccessful as many life threatening vector-borne diseases have reemerged over the last several decades (Gubler, 1998). To date, we still face the formidable challenge of eradicating numerous vector-borne diseases that devastate our expanding global population.

Several factors have contributed to the recent emergence of several vector-borne diseases. Firstly, significant demographic changes over the last several decades are thought to be

directly related to disease emergence. Almost half of the entire population of sub-Saharan Africa currently lives in urban areas, while 50 years ago, only 15% of the population lived in an urban setting (Robert et al., 2003). Mosquito-borne disease transmission occurs in many developing countries partly due to poor sanitary conditions that accompany swift, unprecedented urbanization (Gubler, 1998). These conditions are conducive to high-density propagation of mosquito vectors, which enables vector-borne pathogens to easily resurge and establish themselves in new and old territories (Gratz, 1999). Also contributing to mosquito-borne disease epidemics are significant changes in public health policy. Because control and prevention programs were successful in the early to mid-20th century, the threat of disease dramatically decreased. This led to limitations of resources for prevention and control so that only emergency response planning and massive insecticide usage was employed to manage vector-borne disease transmission. This later proved to be detrimental, which is exemplified by the fact that the *Aedes aegypti* population is now more widely dispersed throughout Central and South America than it was when original eradication programs were implemented decades ago (Gubler, 2004). Additionally, pathogen genome evolution during the last several decades, combined with drug and insecticide resistance, exacerbated the ineffectiveness of disease control, resulting in the recent reemergence of many arthropod-borne diseases (Gubler, 1998).

Virus-host interactions and vector competence

The typical transmission cycle of a mosquito-borne virus involves a hematophagous mosquito vector and a susceptible vertebrate host (Calisher, 1994). Many factors are responsible for maintaining this cycle; the most critical of these factors may be the biological interaction between the virus and the mosquito host. Vector competence, which is the ability of an arthropod vector to become infected by, sustain, and transmit a pathogen, is influenced by several elements (Beerntsen, James, and Christensen, 2000). Viral genetic determinants, which govern the infective, replicative, and pathogenic characteristics of a virus, majorly influence viral transmissibility, and thus vector competence. On the other side, host genetic determinants also affect vector competence by regulating the vector's immune system in response to viral infection. Both viral and host genetic determinants interact with each other to form a highly-evolved, complex relationship that influences the transmissibility of disease (Beerntsen, James, and Christensen, 2000). The specific events and mechanisms through which this relationship

functions is not yet fully understood on a genetic level, but the elucidation of this would be highly beneficial to the development of novel, effective control interventions. Reducing the global threat of vector-borne disease is a daunting task; however, obtaining a detailed understanding of virus-host interactions during arboviral infection is a crucial step in this direction.

The *Alphavirus* genus

Members of the *alphavirus* genus (Family, *Togaviridae*) include species that are responsible for a wide range of disease in humans. The Old World alphaviruses include, among others, the type alphavirus Sindbis virus (SINV), Semliki Forest virus (SFV), o'nyong nyong virus (ONNV), chikungunya virus (CHIKV), and Ross River virus (RRV). Although asymptomatic infections are common, these viruses have been primarily associated with fever, polyarthritis, and/or prolonged arthralgia in infected humans (Mathiot et al., 1990; Niklasson, 1988; Niklasson et al., 1984; Strauss and Strauss, 1994). Old World alphaviruses are generally distributed throughout Africa, Asia, Australia, and South America (Calisher and Karabatsos, 1988), although a recent CHIKV outbreak in Italy has demonstrated the ominous potential of these Old World viruses to flourish in the industrialized world (Angelini et al., 2007). Notable New World alphaviruses include Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV), and Venezuelan Equine Encephalitis virus (VEEV), which are associated with numerous encephalitis-related deaths annually (PAHO, 2008). These viruses are generally located in North, Central, and South America (Calisher and Karabatsos, 1988).

Because the alphaviruses ONNV and CHIKV are associated with large epidemics and disease, their recent reemergence is significant. ONNV was first isolated in 1959 in northern Uganda during an epidemic that afflicted over 2 million people (Williams et al., 1965). This outbreak was one of the most widespread arboviral epidemics in recorded history, spreading throughout East African countries such as Kenya, Zaire, Tanzania, Mozambique, and Malawi. Despite this virus's manifestation across this region in the 1960's, ONNV isolations from humans were not obtained again until 1996. At that time, an epidemic occurred yet again in Uganda, extending throughout the southern region of the country (Lanciotti et al., 1998; Lutwama et al., 1999; Rwaguma et al., 1997). Interestingly, ONNV's primary epidemic vector appears to be *Anopheline* mosquitoes, which is unique to other alphaviruses. Specifically, ONNV

is vectored by *Anopheles gambiae*, an important vector for human malaria, and *An. funestus* (Corbet, Williams, and Gillett, 1961; Williams and Woodall, 1961; Williams et al., 1965). Besides humans, other vertebrate reservoirs for ONNV have not been identified.

Since the 1950's, frequent CHIKV epidemics have occurred in several tropical and subtropical regions, mostly in Africa and South East Asia. CHIKV's recent resurgence was first noticed in 1986 and since, has caused several massive epidemics, including a notable Indian Ocean island epidemic involving Seychelles, Mauritius, Madagascar, and Réunion islands (reviewed in Powers and Logue, 2007). During this outbreak, Réunion island alone totaled hundreds of thousands of CHIKV-infected individuals, which was over one third of the island's population (Ravi, 2006; Saxena et al., 2006). Also of special mention is the recent emergence of CHIKV in Europe, particularly in a small Italian village in mid-2007 (Angelini et al., 2007). The Italian outbreak quickly reminded the global community of CHIKV's potential to establish itself in new territories. *Aedes* species mosquitoes are the primary vector of CHIKV. Interestingly, while *Aedes aegypti* appears to be the most common vector species in urban Asia and Africa (Jupp, 1988), it was recently reported that *Aedes albopictus* was responsible for transmission during the Réunion island epidemic (Reiter, Fontenille, and Paupy, 2006). *Aedes albopictus* has a wide geographic distribution, and this fact has influenced speculation that widespread CHIKV emergence, including epidemics in other European countries and parts of the United States, could be imminent (Powers and Logue, 2007).

Like many arthropod-borne viruses, the alphavirus transmission cycle involves vertebrates and invertebrates. More than one mosquito species is responsible for alphavirus transmission; most commonly, competent vectors for clinically relevant alphaviruses are *Aedes*, *Culex*, and *Anopheline* species (Calisher, 1995; Corbet, Williams, and Gillett, 1961; Krauss, 2003). Typically, the virus is transmitted to an avian host for amplification, but transmission also occurs to other "dead-end" vertebrate hosts such as humans and equine animals (Calisher, 1995) (Figure 1.1).

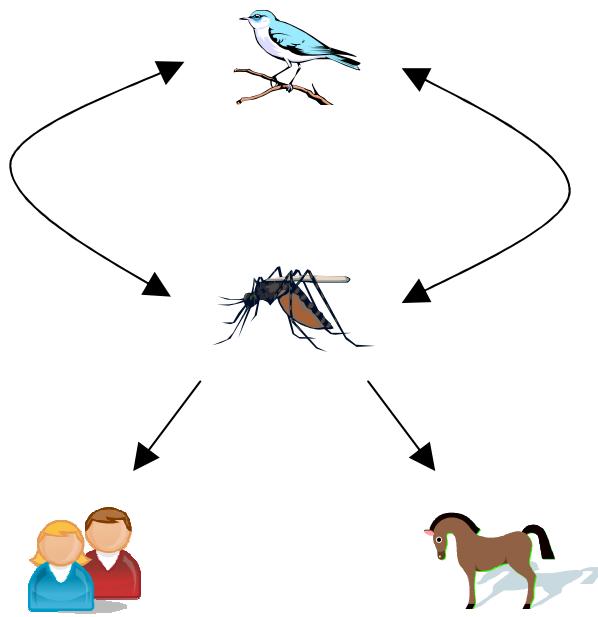


Figure 1.1. The alphavirus transmission cycle involves both vertebrates and invertebrates.
Generally, alphaviruses are vectored through mosquitoes to avian, equine, and human hosts. Avian species often serve as amplification hosts, while humans and equines serve as dead-end hosts in the cycle.

The infected vertebrate host experiences an acute infection often accompanied by disease, while the alphavirus-infected mosquito undergoes a long-term, persistent infection that lasts for the duration of the mosquito's life (Bowers, Abell, and Brown, 1995; Calisher, 1995). While some degree of pathology may be observed in alphavirus-infected mosquitoes (Weaver, Lorenz, and Scott, 1992; Weaver et al., 1988), mosquito infections are generally asymptomatic. The establishment of a non-pathogenic, persistent infection in the mosquito host is essential to the maintenance of the alphavirus transmission cycle, and thus, the propagation of the virus in nature.

A shared feature between alphavirus-infected vertebrate and invertebrate cells is heterologous interference, also called superinfection exclusion. This is where alphavirus-infected cells cannot be subsequently infected with the same or other closely related virus (Johnston, Wan, and Bose, 1974). This phenomenon is not well understood as multiple studies have shown

different results regarding the exclusion of other alphaviruses in SINV infected mosquito cells (Eaton, 1979; Stollar and Shenk, 1973).

The alphavirus genome and replication

Alphaviruses have a non-segmented, positive-sense RNA genome that is single stranded and approximately 12,000 nucleotides in length. Alphavirus genomes resemble cellular mRNAs as they are capped at the 5' terminus and have a 3' polyadenylated tail. The genome is arranged into two distinct regions: the 5' two-thirds of the genome encodes four nonstructural proteins (nsP1, nsP2, nsP3, and nsP4), while the 3' one third of the genome encodes the structural proteins. Immediately after cell entry and uncoating of the virion, the nonstructural proteins are directly translated from the 49S genomic RNA. During replication, a full-length minus (-) strand RNA intermediate is transcribed from the genomic RNA. Because an internal promoter is present in the (-) strand RNA copy, this strand functions not only as a template for new genome copies but also as a template for a 26S subgenomic RNA from which the structural proteins are translated. The structural proteins encoded are the capsid protein, the envelope proteins E3 and E2, the transmembrane protein 6K, and lastly the envelope protein E1 (reviewed in Strauss and Strauss, 1994) (Figure 1.2).

Although the function of the four alphavirus nonstructural proteins has been well studied, a complete understanding of all four proteins has not yet been ascertained. Each individual nonstructural protein actively functions as part of a polyprotein immediately after translation, and also as a mature protein later in the replication cycle. The first nonstructural protein, nsP1, is thought to be responsible for initiation of replication, particularly of (-) strand RNA synthesis (Hahn et al., 1989; Sawicki et al., 1981; Wang, Sawicki, and Sawicki, 1991). This protein also plays a role in capping genomic and subgenomic RNA (Durbin and Stollar, 1985) and modulating the protease activity of nsP2 by limiting nsP2 and nsP3 cleavage in the polyprotein (de Groot et al., 1990). nsP2 appears to have multiple roles as well, as the N-terminal of the protein functions as an RNA helicase (Gorbalya et al., 1988; Gorbalya, Koonin, and Wolf, 1990; Hodgman, 1988) and the C-terminal acts as a nonstructural proteinase (Hahn, Strauss, and Strauss, 1989). Also, nsP2 is required for synthesis of subgenomic mRNA (Hahn, Strauss, and Strauss, 1989; Sawicki and Sawicki, 1985; Sawicki and Sawicki, 1993).

Of all four proteins, the least may be understood about nsP3; however, it has been discovered through deletion studies that it does play some role in (-) strand RNA synthesis (Lemm and Rice, 1993b; Lemm et al., 1994; Shirako and Strauss, 1994) and it does affect cleavage specificity of the nsP2 proteinase (de Groot et al., 1990). Notably, two forms of nsP3 are produced depending on the presence of an opal codon or a sense codon at the nsP3-nsP4 junction; the presence of the opal termination codon results in an nsP3 that is 7 residues shorter than the nsP3 resulting from readthrough.

Finally, nsP4 functions as the viral RNA polymerase. The alphavirus nsP4 contains a conserved motif found in other RNA polymerases (Kamer and Argos, 1984), and experimental studies are consistent with this role.

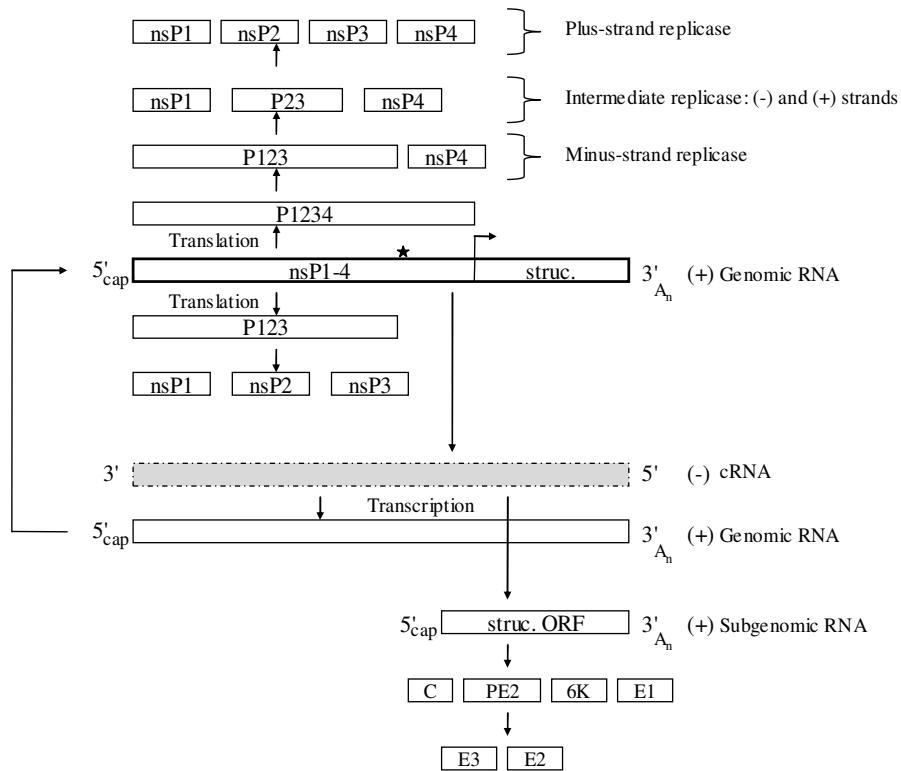


Figure 1.2. Alphavirus genome and replication. Polyprotein P123 and/or P1234 are immediately translated from the 5' 2/3 of the single-stranded, positive-strand 49S genomic RNA and are proteolytically processed to form strand-specific replicase complexes. A full-length (-) strand intermediate is transcribed that serves as a template for new genomic RNAs as well as a 26S genomic RNA that encodes the structural proteins. The black star indicates the position of an opal termination codon between nsP3 and nsP4 in the alphavirus genome.

Summary and Goals

The resurgence of vector-borne viral diseases in many countries stresses the need for a successful strategy to protect global health. Population growth and rapid urbanization, changes in public health policy, and insecticide resistance are major contributors to the downfall of previous control strategies. Now, a significant portion of the global community currently suffers from, or is threatened by, arboviral disease. It is apparent that future control and prevention strategies may be more successful if a clear understanding of virus transmission on a molecular level is first ascertained.

The alphavirus genus includes viruses that are epidemiologically relevant, and their replication and gene expression has been well studied. Genetic manipulation of the alphavirus genome is also possible, making this group of viruses an excellent model for use in virus-host interaction studies. By developing new tools such as an ONNV replicon expression vector and ssqPCR assays, we aim to elucidate the strand-specific kinetics of alphavirus replication in the invertebrate host. The alphaviruses ONN and CHIK will be used as models to research alphavirus (-) strand RNA replication during persistent infection in mosquito cells. We anticipate that this research will provide a useful foundation for future studies investigating the means by which virus-host interactions influence arboviral disease transmission.

Chapter 2

DEVELOPMENT OF AN O'NYONG NYONG VIRUS REPLICON EXPRESSION SYSTEM TO FACILITATE STUDIES OF ALPHAVIRUS REPLICATION

INTRODUCTION

The ability to genetically manipulate RNA viruses has been especially useful for the expression of heterologous proteins and RNAs in host cells. Several different types of expression vectors have been configured from alphavirus genomes (Berglund, Tubulekas, and Liljestrom, 1996; Bredenbeek, 1992; Hahn et al., 1992; Olkkonen et al., 1994; Piper et al., 1994; Schlesinger, 1993). These include replication-competent, but packaging-deficient RNA replicons (Agapov et al., 1998; Bredenbeek et al., 1993; Geigenmuller-Gnirke et al., 1991; Liljestrom and Garoff, 1991; Liljestrom et al., 1991; Pushko et al., 1997; Xiong et al., 1989). Alphavirus replicon vectors contain the genetic elements necessary for replication and expression of heterologous transcripts but lack some or all of the genes required for packaging the RNA genome (Agapov et al., 1998; Liljestrom and Garoff, 1991; Pushko et al., 1997; Xiong et al., 1989). Alphavirus replicons are therefore single cycle vectors incapable of subsequent cycles of infection (Frolov et al., 1996; Rayner, Dryga, and Kamrud, 2002).

Although alphavirus replicons do not express functional structural proteins, RNA replicons can be packaged in the presence of a defective helper RNA (DHRNA). The DHRNA molecule retains *cis*-acting genetic elements required for replication, as well as the subgenomic promoter from which the structural proteins of the virus are expressed (Frolov et al., 1996; Rayner, Dryga, and Kamrud, 2002; Xiong et al., 1989). Co-transfection of cells with the replicon and DHRNA leads to efficient packaging of RNA replicons when the transcription and replication machinery from the expression vector is supplied in *trans* to the helper, resulting in the synthesis and translation of the structural proteins encoded in the helper RNA (Figure 2.1).

Packaging of alphavirus replicons requires a specific encapsidation signal. The packaging signals of several alphaviruses have been defined (Frolova, Frolov, and Schlesinger, 1997; Geigenmuller-Gnirke et al., 1991; Lehtovaara et al., 1981; Lehtovaara et al., 1982; Weiss et al., 1989). While these studies have identified nucleotide sequences specifically required for highly efficient packaging, the presence or absence of other regions as well as the length of the RNA also appear to influence packaging efficiency. RNA lacking a previously defined functional

encapsidation sequence has also been shown to be packaged, albeit with much lower efficiency, illustrating the complexity of the process (Bredenbeek et al., 1993).

A functional packaging signal has been identified in the nsP1 coding region between nucleotides (nts) 945 and 1076 of the Sindbis virus (SINV) genome (Weiss, Geigenmuller-Gnirke, and Schlesinger, 1994; Weiss et al., 1989). However, other regions in the SINV genome also appear to promote RNA packaging in the absence of this 945-1076 nt sequence, but at lower efficiency (Bredenbeek et al., 1993). Packaging signals have also been characterized in Semliki Forest virus (SFV), Ross River virus (RRV) and Aura virus. Although there is evidence that alphavirus packaging signals are related to one another, these signals do not always appear to be present in the same location of the genome. Studies suggest the packaging signals of SFV and RRV are located in the nsP2 coding region (Frolova, Frolov, and Schlesinger, 1997; Geigenmuller-Gnirke et al., 1991; Lehtovaara et al., 1981; Lehtovaara et al., 1982), while a packaging signal for Aura virus appears to be present in the 26S region of the genome (Rumenapf, Strauss, and Strauss, 1994).

Several features of alphavirus replicon vectors are desirable for expression of heterologous proteins and sequences. Replicon vectors have a large cloning capacity, permitting efficient packaging of replicons containing more than 4 kb of heterologous sequence and expression levels reportedly as high as 10^8 polypeptides per cell and 20-25% of total cell protein (Frolov et al., 1996; Xiong et al., 1989). Incorporation of a translational enhancer sequence that facilitates heterologous gene expression equivalent to wild-type capsid protein production can in some cases increase expression further (Frolov and Schlesinger, 1994; Sjoberg, Suomalainen, and Garoff, 1994).

While replicons have been constructed from the alphaviruses SIN, SF, and Venezuelan Equine Encephalitis (VEE), the development of an o'nyong nyong virus (ONNV) replicon has not been reported to date. An ONNV replicon would facilitate high levels of foreign gene expression in Anopheline mosquitoes, a primary epidemic vector of ONNV and *Plasmodium falciparum*, the causative agent of malaria. Current methodologies for expressing foreign genes in this important vector species are limited, and transgenic *Anopheline* mosquitoes are difficult to engineer (Rasgon, 2008). An ONNV replicon could also facilitate studies of viral replication kinetics. This chapter describes the development of an ONNV replicon as well as several DHRNA RNAs. One ONNV replicon RNA and three ONNV DHRNAs were constructed from

the ONNV SG650 genome. The replicon vector was demonstrated to be functional by expressing green fluorescent protein (GFP) in cell culture. Also, we determined that the replicon was unable to be efficiently packaged by any of the three DHRNAs constructed, and the experiments performed to test this are described here.

MATERIALS AND METHODS

Genetic analysis

The ONNV, SINV, and SFV amino acid sequences were aligned using the Clustal W. method with the MegAlign program from Lasergene (v.7.0; DNASTAR, Inc., Madison, WI). The ONNV SG650 strain (Lanciotti et al., 1998, GenBank accession number AF079456), SINV AR339 (NC001547), and the prototype SFV strain (NC003215) were used in the alignments. The sequences of the SFV replicons pSFV1-3 and DHRNA pSFV-Helper2 (Invitrogen, Carlsbad, CA) were also compared to the SFV and ONNV genomes in order to design ONN replicon and DHRNA deletions.

ONNV Replicon and Defective Helper Plasmid Construction

pONNRepSP6. A fragment containing nucleotides (nts) 8024-12283 (7591-11850 in SG650 genome) was excised from the plasmid pONN.AP3 (Brault et al., 2004) using restriction enzymes *Apa*I and *Not*I (Figure 2.2 A). This was performed on two different pONN.AP3 plasmids, one containing an opal (Op) codon (UGA) at the nsP3-nsP4 junction in the nonstructural region, and the other containing an arginine (R) codon (CGA) at this position. A 1015 nt fragment was synthesized (Top Gene Technologies, Quebec, Canada) and ligated into the restriction enzyme sites *Apa*I and *Not*I in pONN.AP3. The synthesized fragment consisted of a portion of the capsid (C) protein coding sequence including the 5' terminus, a portion of the E1 protein coding sequence including the 3' terminus, the 3' untranslated region (UTR), and a multiple cloning site (MCS) (Figure 2.2 B). In total, nts 8444-11690 in pONN.AP3 (8011- 11257 in SG650 genome) were deleted from the pONNRepSP6 structural region. Two different plasmids, pONNRepSP6(Op) and pONNRepSP6(R), were generated by these methods.

pONNRepSP6+GFP. The gene encoding AcGFP (the green fluorescent protein from *Aequorea coerulescens*) was amplified from the plasmid pAcGFP-Hyg-C1 (ClonTech Laboratories, Inc., Mountain View, CA) using the proofreading DNA polymerase *Pfx* (Invitrogen, Carlsbad, CA) and primers 5'-AAAAAGGCGGCCACCATGGTGAGCAAGGG-3' (AcGFP5' AscI) and 5'-TTTTTTAATTAAATTACTTGTACAGCTCATCCATGCCGGGGT-3' (AcGFP3' PacI). PCR was performed on a DNA Engine Peltier Thermal Cycler (BioRad, Hercules, CA), and thermal conditions were as follows: (1) 94°C, 2 min, (2) 94°C, 15 sec, (3) 70°C, 30 sec, (4) 68°C, 1:30

min, (5) go to step 2 10 X, (6) 94°C, 15 sec, (7) 70°C, 30 sec, (8) 68°C 1:30 min + 5 sec/cycle, (9) go to step 6 25 X, (10) 68°C 7:00 min. After restriction enzyme digestion, the AcGFP gene product was ligated into *Asc*I and *Pac*I restriction sites in the multiple cloning sites of both pONNVRepSP6(Op) and (R) (Figure 2.2 C).

pONNHelp1. Deletions were made in the plasmid pONN.AP3 using restriction enzymes *Nhe*I and *Psh*AI. A fragment consisting of nts 410-7349 was excised in pONN.AP3 (*Psh*AI cuts at nt 6916 in SG650 genome, and *Nhe*I is only present in pONN.AP3) (Figure 2.3 A). A fragment (Top Gene Technologies, Quebec, Canada) consisting of nts 410-704 in pONN.AP3 was synthesized and cloned directly into pONN.AP3 (nt 6675 in SG650 genome) at restriction sites *Nhe*I and *Psh*AI. The final deletion of ONNV nonstructural genes *pONNHelp1* included nucleotides 705-7108 in pONN.AP3 (272-6674 in SG650 genome) (Figure 2.3 B).

pONNHelpΔ5435. Restriction enzymes *Bsp*EI and *Age*I were used to delete nucleotides 850-6285 in the plasmid pONN.AP3. After restriction enzyme digestion, the resulting compatible ends were ligated to generate the 9,300 nucleotide sequence (Figure 2.3 C).

pONNHelpΔ2322. Restriction enzymes *Bsp*EI and *Xma*I were used to delete nucleotides 850-3172 in pONN.AP3. After restriction enzyme digestion, the resulting compatible ends were ligated to generate a 12,500 nucleotide sequence (Figure 2.3 D).

In vitro Transcription

Plasmid DNA templates for ONNV replicon and ONNV DHRNAs were linearized with a 3'-terminal *Not*I site. Digested DNA was phenol/chloroform extracted, chloroform extracted, and ethanol precipitated. RNA transcripts for each construct (ONNRepSP6, ONNRepSP6+GFP, ONNHelp1, ONNHelpΔ5435, and ONNHelpΔ2322) were generated *in vitro* from 1 µg of purified template DNA. Transcription reactions also contained 1X RNA polymerase reaction buffer (New England Biolabs, Ipswich, MA); 100 µg/ml acetylated BSA (Promega, Madison, WI); 1mM rNTP nucleotide mix (New England Biolabs); 1mM m⁷G(5')ppp(5')G RNA cap analog (New England Biolabs); 5mM DTT (Promega), 60 units of RNAsin Plus RNase inhibitor (Promega), and 20 units of SP6 polymerase (New England Biolabs). RNase-free water was

added to bring the final reaction volume to 50 µl. Reactions were incubated at 39°C for 1 hour and stored at -80°C until further use.

Cell Culture Maintenance

All cell lines were maintained in Dulbecco's modified eagle medium (DMEM) and supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 1% penicillin-streptomycin solution, and 1% L-glutamine. *Aedes albopictus* cells (C6/36) were maintained at 5% CO₂ at 28°C. C6/36 cells were subcultured by removing medium, washing with phosphate buffered saline (PBS), scraping in medium, and split at a ratio of 1:4. *Mesocricetus auratus* Syrian baby golden hamster kidney cells (BHK-21) were cultured in DMEM and maintained at 5% CO₂ at 37°C. BHK-21 cells were subcultured by removing medium, washing with PBS, and trypsinizing to remove cells from the bottom of the flask. An equal volume of medium was added, and the cell/medium mixture was centrifuged at 1000 × g for 5 minutes at 4°C. After removing the trypsin and medium, cells were subsequently resuspended in fresh medium and split at a ratio of 1:10.

Rescue of ONNV Replicon and Defective Helper RNA

BHK-21 cells were grown to 80% confluence in 150 cm² flasks, trypsinized, centrifuged and washed three times with PBS. Cells were counted with a haemocytometer and resuspended in PBS at a final concentration of 1 × 10⁷ cells/ml. RNA transcripts for ONNVRepSP6+GFP and ONNV DHRNA (ONNHelp1, ONNHelpΔ5435, or ONNHelpΔ2322) were mixed with 400 µl of the resuspended cells and electroporated twice with a BTX ElectroCell Manipulator (BTX Instrument Division, Harvard Apparatus, Inc.) at 450V, 725Ω, and 75 µF. Untransfected control cells were also electroporated twice as described. After electroporation, cells and RNA were transferred to a 25 cm² flask and 5 ml of medium was added. Cells were observed for GFP expression at 24, 36, and 48 hours post transfection on a Zeiss Axiovert 200 fluorescent microscope with a Cannon Powershot A260 camera. At 48 hours post electroporation, the supernatant in the flask was harvested, centrifuged at 1000 × g for five minutes, aliquoted, and stored at -80°C until further use.

Endpoint Dilution Assays

The amount of packaged replicon was determined by seeding C6/36 cells into 96-well plates and infecting the cells with 10-fold serial dilutions (10^0 - 10^{-7} , 250 μ l/ well) of harvested replicon/DMEM fluid. Cells were maintained in DMEM (10% FBS) for up to 72 hours post infection. Endpoints were determined at 24 and 48 hours post infection by GFP expression at each dilution.

Immunofluorescence Assay (IFA)

BHK-21 cells were electroporated as described above with RNA transcripts generated from pTE3'2J (Hahn et al., 1992), pONN.AP3 (Brault et al., 2004), and pONNRepSP6. Cells were co-transfected with RNA transcripts generated from pONNRepSP6 and pONNHelp1. Cells were then seeded on glass coverslips in 12 well plates. At 24 hours post transfection, cells were washed with PBS and fixed with chilled acetone for 15 minutes at -20°C. Acetone was removed, and cells washed twice by immersing coverslips in PBS. Cells were then incubated with 75 μ l of mouse anti-SIN E1 Mab30.11a (Chanas et al., 1982) (1:200) in a humidified chamber at 37°C for 40 minutes. Cells were again washed by immersion in PBS as described above, and then incubated with 75 μ l of biotinylated sheep anti-mouse antibody (Amersham Biosciences, Pittsburgh, Pennsylvania) (1:200) at 37°C for 40 minutes. Cells were again washed in PBS and incubated with 75 μ l of FITC-conjugated streptavidin (Amersham Biosciences) (1:200, 5% Evans Blue counterstain) at 37°C for 10 minutes. Cells were again washed in PBS, with deionized water. Coverslips were mounted on glass slides with a drop of approximately 10 μ l of glycerol/PBS (9:1). Cells were examined on a Zeiss Axiovert 200 fluorescent microscope with a Cannon Powershot A260 camera.

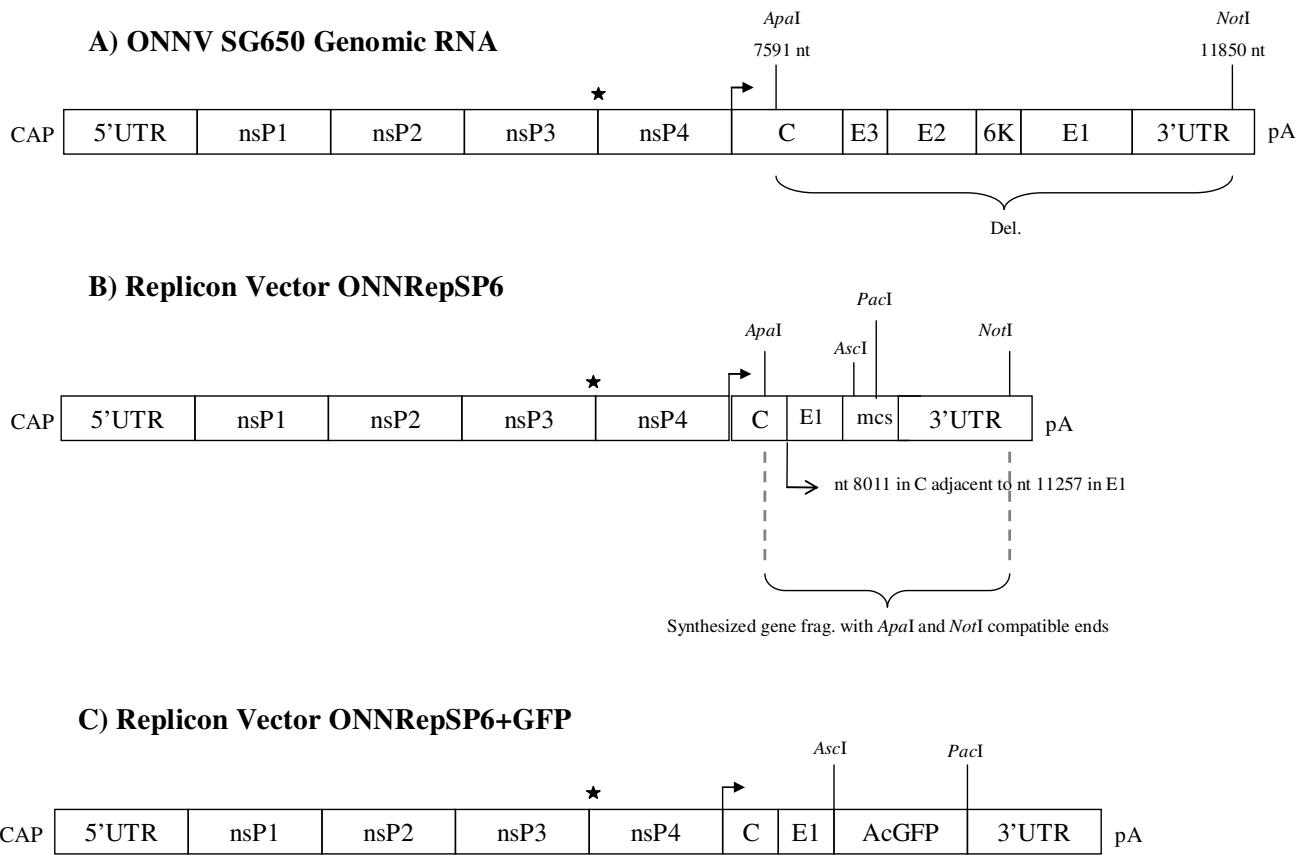
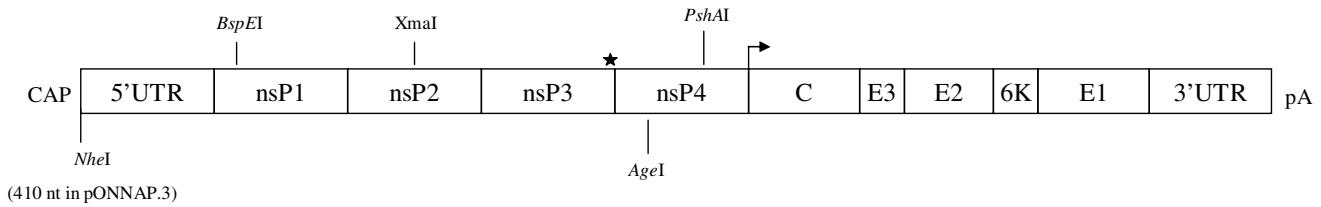
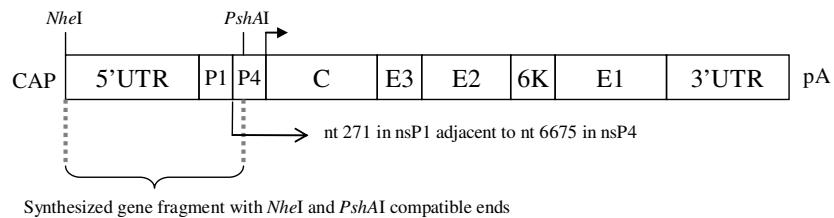


Figure 2.1. Construction of ONNRepSP6 and ONNRepSP6+GFP. (A) A portion of the structural genes in the ONNV genome were deleted using restriction enzymes *Apa*I and *Not*I. (B) A synthesized fragment was ligated into the *Apa*I and *Not*I sites. This fragment includes a multiple cloning site (mcs). (C) ONNRepSP6+GFP was constructed by cloning AcGFP into the *Asc*I and *Pac*I sites of the mcs in ONNRepSP6. The black arrow signifies the internal subgenomic promoter, while the black star represents the position at which the opal (Op) or arginine (R) codon is located. Two genotypes, Op and R, were constructed for each replicon, ONNRepSP6 and ONNRepSP6+GFP.

A) ONNV SG650 Genomic RNA



B) ONNHelp1



C) ONNHelpΔ5435



D) ONNHelpΔ2322

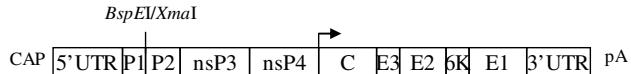


Figure 2.2. Construction of ONNV Defective Helper RNAs. (A) Restriction enzyme sites used to construct the DHRNAs, ONNHelp1, ONNHelpΔ5435 and ONNHelpΔ2322. (B) A large portion of the nonstructural gene region was deleted from the genome using the restriction enzymes *NheI* and *PshAI*. A gene fragment was synthesized containing the 5'UTR and portions of the nsP1 and nsP4 nonstructural genes, and inserted into the *NheI* and *PshAI* sites. (C) A deletion in the nonstructural region was made to construct ONNHelpΔ5434. Restriction enzymes *BspE1* and *AgeI* were used to excise the deleted region and the resulting compatible ends were ligated together. (D) To construct ONNHelpΔ2322, a shorter deletion was made in the nonstructural region using enzymes *BspE1* and *XmaI*, which also produced compatible ends that were subsequently ligated together. ONNHelpΔ2322 lacks most of nsP1 and nsP2 but retains nsP3 and nsP4.

RESULTS

ONNV, SFV, and SINV amino acid alignments

Replicon expression vectors have been constructed from the alphaviruses SINV, SFV, and VEEV. Previous amino acid alignments indicated 62.6% identity between ONNV and VEE in the nonstructural protein region and 46.8 % identity in the structural region. ONNV and SINV were previously reported to have 62.5% identity in the nonstructural region and 44.6% identity in the structural region. ONNV had the highest percentage of identity with SFV, with 73.4% and 62.4% identity in the nonstructural and structural region, respectively (Strauss and Strauss, 1994). Other phylogenetic studies also support that ONNV and SFV are more similar in their nonstructural and structural regions than are SINV and VEE with ONNV (Strauss and Strauss, 1994; Weaver et al., 1993; Levinson et al., 1990, Figure 2.4).

We analyzed the homology of the SFV and SINV genomes with the strain of ONNV used in the present study (SG650). Our analysis determined that SFV and ONNV are 69.7% and 57.4% identical in the nonstructural and structural regions, respectively.. Our amino acid alignment of SINV and ONNV determined that these genomes were 70.6% identical in their nonstructural regions and 41.4% identical in their structural regions. Based upon the results of these alignments and previously reported studies, we initially based the construction of our ONNV replicon and DHRNA on previously described SFV replicons and DHRNAs (Liljestrom and Garoff, 1991).

Sequences and maps for the previously reported SFV replicons (pSFV1-3) and DHRNA (pSFV-Helper2) were compared to the complete genomes of SFV and ONNV SG650. Liljestrom and Garoff (1991) deleted nucleotides (nts) 7783-11033 from the structural region of the SFV cDNA clone pSP6-SFV4 (equivalent to nts 7403- 10653 in the SFV genome) to produce SFV replicons pSFV1-3. Amino acid alignments indicated that the deleted SFV sequence corresponded to nts 8444–11690 in the infectious clone pONN.AP3 (8011-11257 in the ONNV SG650 genome). The DHRNA pSFV-Helper2 contained a deletion in the nonstructural region corresponding to nts 308-6398 of the SFV genome, which corresponded to nts 299-6647 in pONN.AP3 (732-7107 in the ONNV SG650 genome).

GFP Expression from ONNV replicon vector in BHK-21 cells

A plasmid, designated pONNRepSP6+GFP, was constructed by replacing nucleotides 8024-12283 in the structural protein region of pONN.AP3 with a GFP reporter gene (Figure 2.2; Materials and Methods). Expression of GFP was observed in BHK-21 cells electrotransfected with ONNRepSP6+GFP RNA transcripts, confirming replication and expression of a heterologous protein by the replicon vector. At 24 hours post-transfection, ~80% of the cells expressed GFP (Figure 2.4 A).

Inefficient packaging of ONNV replicon by ONNHelp1

The first DHRNA plasmid, pONNHelp1, was generated through a specific deletion in the nonstructural protein region of pONN.AP3 (Figure 2.3, Materials and Methods). RNA was *in vitro* transcribed from pONNRepSP6+GFP and pONNHelp1, and co-transfected into BHK-21 cells. Electroporated cells were observed at 24 and 48 hours post transfection. At 24 hours post-transfection, ~80% of the cells again expressed GFP (Figure 2.5 B).

An endpoint dilution assay was performed to determine if packaged replicon particles were present in supernatant harvested from cells co-transfected with ONNRepSP6+GFP and ONNHelp1 RNA transcripts. C6/36 cells were infected with ten-fold dilutions of the harvested virus/medium (10^0 - 10^{-7}). GFP expression was not observed in C6/36 cells at 24, 48, or 72 hours post infection, suggesting that packaged replicon virus particles were not present (data not shown).

IFA to Detect the E1 Structural Protein in Transfected BHK-21 Cells

Because GFP was not observed after co-transfection of ONNRepSP6+GFP and ONNHelp1, an indirect immunofluorescence assay (IFA) was performed, with an antibody specific to the alphavirus E1 glycoprotein (anti-SIN E1 Mab30.11a), on BHK-21 cells co-transfected with these RNA molecules in order to determine if the structural proteins from the DHRNA were being expressed. Positive control IFAs were performed using BHK-21 cells transfected with RNA generated from the SINV expression system TE/3'2J (Hahn et al., 1992) and pONN.AP3 (Brault et al., 2004). Fluorescence was observed in cells transfected with both of the control RNAs (Figure 2.5 A, B), indicating the anti-SIN E1 antibody was able to bind the E1 glycoprotein of both SINV and ONNV. However, no fluorescence was observed in cells co-

transfected with ONNRepSP6+GFP and ONNHelp1 RNA suggesting the ONNV E1 glycoprotein was not present in these cells. Fluorescence was also absent in the negative control IFA (transfection of ONNRepSP6 [no GFP insert]) (Figure 2.5 C, D).

Inefficient packaging of ONNV replicon by ONNHelpΔ5435 and ONNHelpΔ2322

Two additional DHRNA plasmids were generated by deleting the nonstructural protein region of pONN.AP3 (Figure 2.3, Materials and Methods). RNA generated from the plasmids pONNHelpΔ5435 or pONNHelpΔ2322 was electroporated into BHK-21 cells with the ONNRepSP6+GFP RNA. Cells were observed at 24 and 48 hours post transfection. Although both co-transfections resulted in ~70% of cells expressing GFP at 24 hours (Figure 2.7), GFP expression was not observed in endpoint dilution assays using the harvested cell supernatent. These results confirm that the ONNV replicon vector is replication-competent and capable of expressing the heterologous protein GFP cloned downstream of its subgenomic promoter, but suggest the replicon is unable to be packaged by ONNHelpΔ5435 and pONNHelpΔ2322.

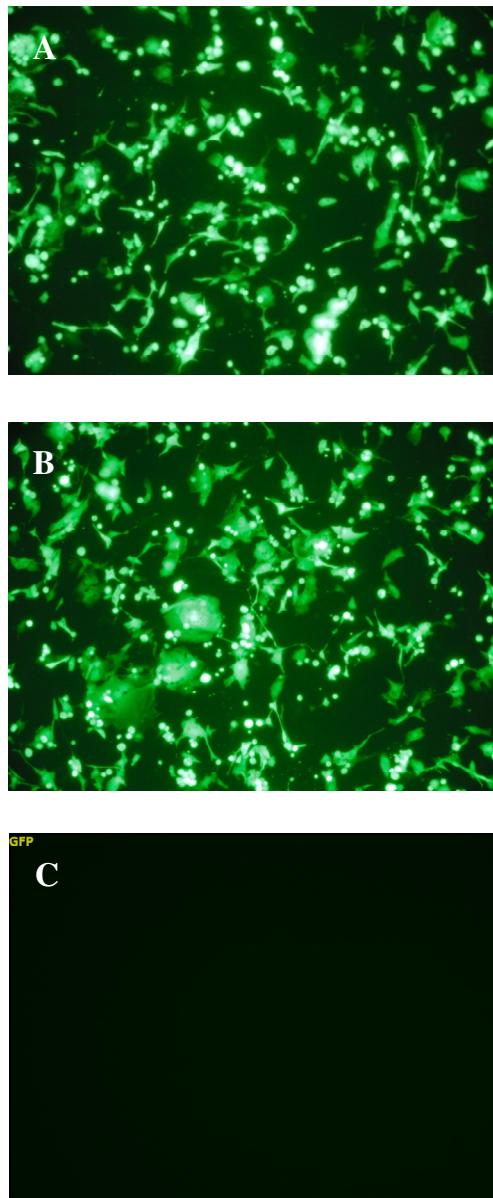


Figure 2.3. GFP Expression in BHK-21 cells transfected with ONNV replicon and DHRNA ONNHelp1 at 24 hours. (A) Cells transfected with ONNRepSP6+GFP RNA. (B) Cells co-transfected with ONNRepSP6+GFP and ONNHelp1 RNA. (C) Untransfected control cells. Approximately 80% of transfected cells expressed GFP in panels A and B.

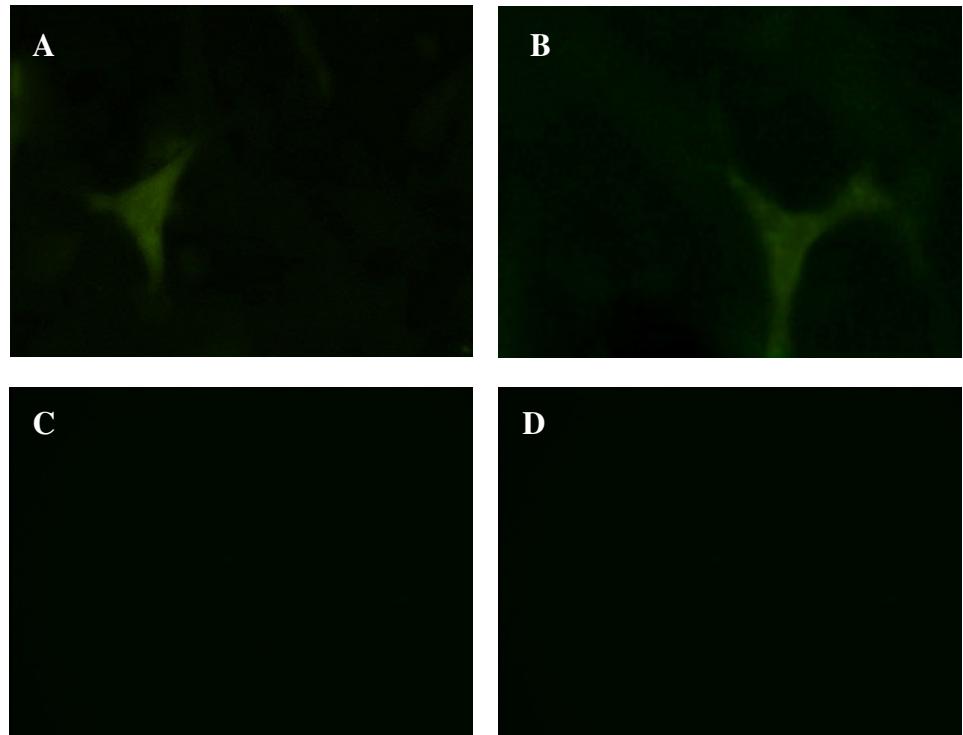


Figure 2.4. Detection of the E1 glycoprotein in BHK-21 cells. Cells transfected with RNA generated from pTE/3'2J (A) (Hahn et al., 1992), pONN.AP3 (B) (Brault et al., 2004). Cells co-transfected with RNA generated from pONNRepSP6 and pONNHelp1(C), pONNRepSP6 (D).

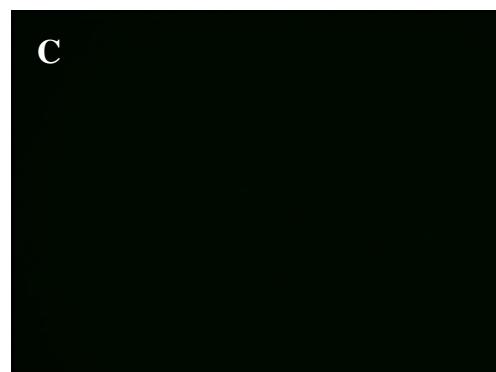
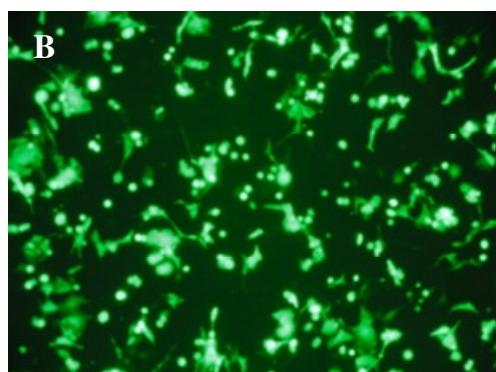
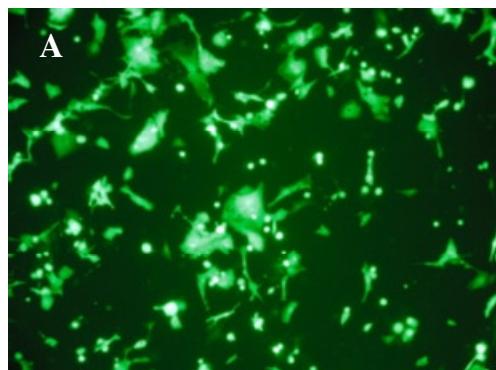


Figure 2.5. GFP Expression in BHK-21 cells co-transfected with ONNV replicon and ONNHelp Δ 5435 or ONNHelp Δ 2322 at 24 hours. A) Cells co-transfected with ONNRepSP6+GFP and ONNHelp Δ 5435 RNA. B) Cells co-transfected with ONNRepSP6+GFP and ONNHelp Δ 2322 RNA. C) Untransfected control cells. Approximately 70% of co-transfected cells expressed GFP in panels A and B.

DISCUSSION

This chapter reports the development of an ONNV replicon expression system. Other alphavirus replicons have been developed for SINV, SFV, and VEE for a variety of research applications (Agapov et al., 1998; Bredenbeek et al., 1993; Frolov et al., 1999; Frolov et al., 1996; Liljestrom and Garoff, 1991; Xiong et al., 1989). Based on the results of previously reported studies of alphavirus homology as well as our own alignments with a previously described SFV replicon expression system (Liljestrom and Garoff, 1991), we engineered an ONNV replicon and several ONNV defective helper RNAs (DHRNAs). It was determined that the replicon engineered, ONNRepSP6+GFP, was replication competent and capable of expressing the heterologous protein GFP in BHK-21 cells (Figure 2.5). However, similar GFP expression levels were observed in BHK-21 cells co-transfected with all three combinations of ONNV replicon and DHRNAs, as were observed in cells transfected with only the ONNV replicon, indicating the absence of a complete infectious cycle after transfection. In these experiments, transfection efficiencies were ~70-80%, leaving at least 20-30% of the remaining cells susceptible to viral entry, and subsequent replication, by packaged replicon particles. If the ONNV replicon was efficiently packaged by one of the DHRNAs, we would expect the percentage of cells expressing GFP to be higher when co-transfected with replicon and DHRNA than in cells transfected with only replicon RNA. However, this is not what was observed in our transfection experiments. We also analyzed the packaging efficiency of our DHRNAs with endpoint dilution assays. No GFP was seen in the endpoint dilution assays when any of the DHRNAs were co-transfected with ONNRepSP6+GFP.

There are several possibilities to explain the apparent lack of ONNV replicon packaging by any of the DHRNAs tried. It is possible that replicase activity is not being supplied in *trans* by the ONNV replicon facilitating transcription of the structural proteins encoded in the DHRNA molecules. Alternatively, translation may be inhibited following successful transcription. Our IFA results indicate that the DHRNA E1 glycoprotein is not being expressed, but further investigation will be required to identify the specific cause of the apparent packaging deficiency. Northern blot analysis to detect subgenomic RNA after co-transfection of the replicon and one of the three DHRNAs would be helpful in determining if the structural genes were being transcribed from each of the helper constructs. Additional IFAs and/or western blot analysis would also be helpful in confirming the presence or absence of structural proteins. Taken

together, these experiments might provide some insight into future DHRNA design strategies for successfully packaging the ONNV replicon we have constructed.

While our results indicate that ONNHelp1 is unable to express the structural protein, E1, it remains unclear if the ONNV replicase is functioning in *trans*. IFA may not be sensitive enough to detect low level trans-complementation of DHRNAs by the ONNV replicase. Indeed, replication and transcription from a *trans*- acting viral replicase is certain to be less robust than from a *cis*-acting replicase. As mentioned previously, the successful encapsidation and assembly of replicon particles also depends on the presence of an efficient packaging signal in the replicon molecule. To date, the identification of efficient packaging signals in the ONNV genome has not been reported. Therefore, the location of these sequences in ONNV can only be speculated on from a comparison with other alphaviruses. It has been reported that the location of efficient packaging signals in alphavirus genomes varies (Strauss and Strauss, 1994). An efficient packaging signal for SFV was identified in the nsP2 region of the genome (Frolova, Frolov, and Schlesinger, 1997; Lehtovaara et al., 1981; Lehtovaara et al., 1982; Thomson and Dimmock, 1994; Weiss et al., 1989); however, it remains unclear if the packaging signal of ONNV is located in a similar location. Therefore, it is possible that the ONNV replicon constructed for this study lacks an efficient packaging signal. Efficient packaging of the ONNV replicon may not be attainable without a fuller understanding of the fundamental differences between ONNV and other alphaviruses. Future investigations to elucidate these differences will be beneficial in the design of effective packaging systems for ONNV, thus facilitating future gene expression and viral replication investigations in anopheline mosquitoes.

CHAPTER 3

ACCURATE STRAND-SPECIFIC QUANTIFICATION OF VIRAL RNA

INTRODUCTION

Although the genomes of RNA viruses occur in a variety of confirmations, all must be efficiently copied within the infected cell. These copies are essential to the production of messenger RNA (mRNA) that can be translated by host ribosomes, and as a source of genomic RNA for packaging into mature progeny virions. The alphaviruses are a group of enveloped, positive (+) strand RNA viruses in the family Togaviridae (Strauss and Strauss, 1994). The synthesis of genomic (49S) RNA, as well as a subgenomic (26S) mRNA that encodes the virus structural proteins, depends on the synthesis of a genomic-length minus (-) strand copy. Alphaviruses are thought to synthesize (-) strand RNAs only for a short time early in the infection, although the production of (+) strand 26S and 49S RNA continues for much longer (Sawicki and Sawicki, 1980).

Members of the alphavirus genus pose a serious or potential threat to public health in many areas of the world. Nearly all alphaviruses are maintained in nature by transmission cycles that involve alternating replication in a susceptible vertebrate and invertebrate host. Because infection of the vertebrate host is acute and often associated with disease, continual transmission depends on life-long persistent infection of the invertebrate vector host, for many alphaviruses a mosquito. It is presently unclear how persistent alphavirus infections are maintained in the vector host after (-) strand synthesis terminates in the infected cells. One of the difficulties addressing this question has been the limitations of methodologies for detecting and measuring (-) strand RNA in infected cells. Competition between viral (+) strands and labeled probe makes nuclease protection assays problematic for the detection of (-) strand RNA, particularly late in the infection when (+) strands are much more abundant (Kim et al., 2004). In addition, nuclease protection assays are only semi-quantitative. Assays based on reverse transcription and PCR of cDNA derived from viral (-) strands increase sensitivity at later times after infection, but are also semi-quantitative (Shirako and Strauss, 1994). This weakness can be overcome with quantitative real-time PCR (qPCR), but accurate quantification depends on the specificity of the assay for a particular strand of viral RNA.

Although a variety of strand-specific quantitative real-time PCR (ssqPCR) assays have been reported (Anderson and Rico-Hesse, 2006; Anwar, August, and Too, 2006; Bartolome et al., 2007; Campbell et al., 2008; Castillo et al., 2006; Gu et al., 2007; Hashimoto and Valles, 2008; Komurian-Pradel et al., 2004; Purcell et al., 2006; Richardson et al., 2006; Wang et al., 2002; Yuki et al., 2006), no study has yet determined if the specificity, accuracy, and sensitivity of each is equivalent. Here we report on the development and validation of new ssqPCR assays for the alphaviruses o'nyong nyong (ONNV) and chikungunya (CHIKV). Although the assays developed are specific for CHIKV and ONNV, different assay designs, detection strategies and chemistries were evaluated during the development process and those results are also reported here. We show that accurate quantification of a specific strand of viral RNA, in the presence of relatively higher levels of cDNAs generated from the complimentary strand, required incorporation of a unique tag sequence into cDNA generated during the RT step and the use of a tag-specific primer during the qPCR step. Our results also indicate a greater dynamic range for tagged ssqPCR assays using DNA hydrolysis probes in the quantification of low copy templates. These findings should be useful in informing the design of future ssqPCR assays for detecting and accurately measuring replicating viral RNA in infected cells and tissues.

MATERIALS AND METHODS

Infection of C6/36 cells with ONNV

C6/36 cells were maintained as previously described. Cell monolayers were grown to 80% confluence in 12-well plates, washed twice with PBS, and infected with ONNV at a multiplicity of infection (MOI) of 5. Virus was combined with Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech, Inc., Manassas, VA) for a total volume of .5 mL/well and then incubated at 4°C for one hour. After incubation, fresh medium was added to the wells to bring the total medium-virus volume up to 1 mL, and infected C6/36 cells were placed at 28°C for 1 hour.

Isolation of Total RNA from Infected Cells

Total RNA was isolated from ONNV-infected cells by using TRI Reagent RT at 1 hour post infection. Virus-medium supernatant was removed from wells and infected cells were washed with PBS three times before adding .5 mL of TRI Reagent RT (MRC, Inc., Cincinnati, OH) per one well to lyse the cells. After transferring TRI Reagent RT-cell homogenate to a 1.5 mL centrifuge tube, 25 µl of bromoanisole was added, the mixture was shaken vigorously for 15 seconds, and then was centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase containing the RNA was removed and placed in another 1.5 mL centrifuge tube. RNA was precipitated by adding an equal volume of isopropyl alcohol and centrifugation of 12,000 × g at 4°C for 5 minutes after sitting at room temperature (rt) for 5-10 minutes. The supernatant was removed and the RNA pellet was washed with 75 µl of 70% ethanol and centrifuged at 6000 × g for 5 minutes at 4°C. The ethanol wash was removed and the RNA pellets were dried at rt for 2-5 minutes. Pellets were resuspended in nuclease free water and stored at -80°C until further use.

Generating in vitro RNA transcripts

To generate strand-specific standard curves for ssqPCR, (+) and (-) strand RNA was transcribed *in vitro* from a plasmid containing a portion of the nsP1 gene from either ONNV or CHIKV. The plasmids pblue-nsP1 (ONNV) and pblue-nsP1 (CHIKV) were produced by cloning the 5' terminal 853 and 669 nucleotides of the respective viral nsP1 gene into pBluescript II SK (-) (Stratagene, La Jolla, CA). Minus strand RNA was synthesized with T7 RNA polymerase from *Hind*III digested plasmid templates in a standard *in vitro* transcription reaction. Positive strand

RNA was synthesized with SP6 RNA polymerase from *KpnI* digested plasmid templates in a standard in vitro transcription reaction. The RNA generated during the in vitro transcription reactions was isolated with Tri Reagent RT, as described above. The absence of template DNA was confirmed through PCR. The concentration of RNA transcripts was determined by NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA). The cloned ONNV nsP1 gene fragment has a molecular weight of 273,545 g/mol, while the cloned CHIKV nsP1 gene fragment has a molecular weight of 214,574 g/mol. One μ g of RNA transcribed from pblue-nsP1 (ONNV) equals approximately 2.2×10^{12} molecules of the nsP1 gene fragment, while one μ g of RNA transcribed from pblue-nsP1 (CHIKV) equals approximately 2.8×10^{12} molecules of the nsP1 gene fragment.

Reverse Transcriptase-PCR

cDNA of both polarities was transcribed with primers containing a 5' tag sequence (Komurian-Pradel et al., 2004; Lin, Fevery, and Hiem Yap, 2002), or with primers lacking the 5' tag sequence (Table 1). Forward primers were used to transcribe cDNA from (-) strand RNA, while reverse primers were used to transcribe cDNA from (+) strand RNA. Primers and RNA were incubated at 70 °C for 5 min and then placed on ice for 2 min. Primer was added to the reverse transcription reaction at a final concentration of 500 nM. cDNA was synthesized with Superscript II® (Invitrogen) at 50 °C for 30 min, and then heat inactivated at 95 °C for 15 min. Unincorporated primers present in heat inactivated reverse transcription reactions were digested with exonuclease I (New England Biolabs). cDNA used in the generation of standard curves was serially diluted ($\approx 10^{10} - 10^2$ gene copies/ μ l) and stored at -20 °C until further use. To facilitate the generation of only falsely-primed RT products, separate RT reactions were performed as described above, but with no forward or reverse primer. cDNA was generated from 4.5 μ g of total RNA for the false-priming RT reactions.

Strand Specific Quantitative Real-Time PCR

TaqMan® Assays

To increase fluorescent signal strength during ssqPCR reactions, an AT-rich 12-nucleotide flap sequence (5'AATAAAATCATAA 3') was added to the 5' end of tagged primers (Afonina et al., 2007). ssqPCR was performed with the appropriate combination of primers and

TaqMan® probe (Table 1). ssqPCR was performed using the ABI 7300 System. Each reaction contained 12.5 µl of 1X ABI Gene Expression Master Mix, TaqMan® probe at a final concentration of 250 nM, forward and reverse primers, each at a final concentration of 900 nM, and 2 µl of diluted cDNA. Samples were run in triplicate. The standard cycling conditions were 50° C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 61°C for 1 min. Data collection occurred during the 61°C extension step.

SYBR® Green Assays

ssqPCR was performed with the appropriate forward or reverse and tag-specific primer pair (Table 1). When a tag sequence was not present in the cDNA, ssqPCR was performed using only nsP1-specific forward and reverse primer pairs (Table 1). Reactions contained 10 µl of 1X Power SYBR® Green Master Mix, forward and reverse primers, each at a final concentration of 800 nM, and 2 µl of cDNA. The standard cycling conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 56 °C for 30 sec, 72 °C for 30 sec, and to monitor potential non-specific amplification one cycle of 95 °C for 15 sec, 60 °C for 1 min, and 95 °C for 15 sec. Data collection occurred during the 72 °C extension step.

TABLE 1: Sequences of oligonucleotides used for either reverse transcription (RT) or quantitative PCR (qPCR).

Oligonucleotide Name	Purpose	Nucleotide sequence (5'-3') ^{a,b}
SYBR Green Assays		
<i>ONNV (-) strand detection</i>		
ONNV F S tag S	RT	GGCCGTATGGTGGCGAATAATACCAACCAGGCATCAAGGAGTC
ONNV R S	qPCR	aataaatcataaAACACTCGGTGCGATGGCTTCAAT
Tag S	qPCR	aataaatcataa GGCCGTATGGTGGCGAATAA
<i>ONNV (+) strand detection</i>		
ONNV R S tag S	RT	GGCCGTATGGTGGCGAATAAAACACTCGGTGCGATGGCTTCAAT
ONNV F S	qPCR	aataaatcataaTACCACCAGGCATCAAGGAGTC
Tag S	qPCR	(see sequence listed above)
TaqMan Assays		
<i>ONNV (-) strand detection</i>		
ONNV F T tag T	RT	GGCAGTATCGTGAATTGATGCACCGAGAAAACTTGCATCA
ONNV R T	qPCR	aataaatcataaTTTTCCGGAGATGTTTTATCTGT
Tag T	qPCR	aataaatcataa GGCAGTATCGTGAATTGATGC
ONNV probe	qPCR	CCGCTGGAAAGGT
<i>ONNV (+) strand detection</i>		
ONNV R T tag T	RT	GGCAGTATCGTGAATTGATGCTTTCCGGAGATGTTTTATCTGT
ONNV F T	qPCR	aataaatcataaACCGAGAAAACTTGCATCA
Tag T	qPCR	(see sequence listed above)
ONNV probe	qPCR	(see sequence listed above)
<i>CHIKV (-) strand detection</i>		
CHIKV F T tag T	RT	GGCAGTATCGTGAATTGATGCGACGCAGAACGCCACATT
CHIKV R T	qPCR	aataaatcataaGTCCGCCCTTGTCTACATGA
Tag T	qPCR	(see sequence listed above)
CHIKV probe	qPCR	TGCTTGCACACTGACGT
<i>CHIKV (+) strand detection</i>		
CHIKV R T tag T	RT	GGCAGTATCGTGAATTGATGCGTCCGCCCTTGTCTACATGA
CHIK F T	qPCR	aataaatcataaGACGCAGAACGCCACATT
Tag T	qPCR	(see sequence listed above)
CHIKV probe	qPCR	(see sequence listed above)

^aThe non-alphavirus tag sequences are shown in boldface.

^bThe AT-rich flap sequences (Afonina et al. 2007) are shown in lowercase.

RESULTS

Accurate quantification of specific viral RNA strands depends on the presence of a tag sequence in the cDNA.

The ability of ssqPCR assays that employ a tagged primer system to detect and quantify viral RNA of a specific polarity has been well demonstrated (Gu et al., 2007; Komurian-Pradel et al., 2004; Purcell et al., 2006). These assays incorporate a unique tag sequence into cDNA synthesized from a specific strand of viral RNA. A tag-specific primer used during PCR amplification ensures that only cDNA possessing the unique tag sequence is detected and quantified. Standard qPCR assays that employ unmodified primers have also been used to quantify specific strands of viral RNA (Anderson and Rico-Hesse, 2006; Campbell et al., 2008; Hashimoto and Valles, 2008; Richardson et al., 2006; Wang et al., 2002; Yuki et al., 2006), but the accuracy of these measurements remains unclear. To directly compare the accuracy of commonly used qPCR strategies for measuring specific strands of viral RNA, total RNA was extracted from ONNV- infected mosquito cells and reverse transcribed using either an ONNV-specific forward primer possessing a unique 5' tag sequence, or an unmodified ONNV-specific forward primer (Table 1). The respective cDNAs, with or without tag sequence, were qPCR amplified in the presence of SYBR® Green dye using a primer set containing a tag-specific forward primer and an ONNV-specific reverse primer (Table 1), or with a primer set containing only ONNV-specific primers (Table 1). The amount of ONNV (-) strand RNA in the unknown sample was calculated from standard curves (Fig. 3.1; A and B). The standard curve generated (see materials and methods for details) for the assay using unmodified ONNV-specific primers had a slope of -3.4, coefficient of determination (R^2) of 0.994, and amplification efficiency (Eff%) of 96.8%. The standard curve (see materials and methods for details) values for the assay using a combination of tag-specific and ONNV-specific primers had a slope of -3.4, an R^2 of 0.999, and Eff% of 96.8. Although both assays gave values for the quantity of ONNV (-) strand RNA that were within the acceptable ranges of their respective standard curves, 8.0×10^5 copies of (-) strand RNA/ μ g of total RNA for the ssqPCR assay using unmodified primers and 1.1×10^5 copies of (-) strand RNA/ μ g of total RNA for the ssqPCR assay using a tagged primer system (Fig. 3.1; A and B), the values differed significantly from each other ($P < 0.001$; one-way ANOVA).

Accurate quantification of specific viral RNA strands with a ssqPCR assay depends on the supposition that the amount of cDNA quantified by qPCR closely approximates amounts of specific strands of viral RNA in the RT reaction. We hypothesized that the observed variation in the experimental results obtained with our two ssqPCR assays was occurring during qPCR amplification. More specifically, it has been demonstrated that cDNA can be transcribed from RNA during an RT reaction in the absence of any specific primer (Gunji et al., 1994; Peyrefitte et al., 2003; Timofeeva and Skrypina, 2001). By design, ssqPCR assays using unmodified primers depend on the RT reaction (where only a single virus-specific primer is present) for strand-specificity. After the RT step, cDNA from either strand can be amplified with virus-specific forward and reverse primers. A combination of virus-specific and tag-specific primers used during qPCR should limit amplification of dsRNA from falsely-primed cDNAs, as these would lack the unique tag sequences added during the RT step. Thus ssqPCR assays using tag-specific primers should be able to better distinguish cDNAs transcribed from a specific strand of viral RNA from other cDNAs present. To test this, an RT reaction was performed with the same total RNA (from ONNV-infected mosquito cells) used in the experiments described above, but in the absence of any specific primer. The resultant cDNA was then used in qPCR reactions with the same primer sets used in the previously described experiments, a tag-specific forward primer and an ONNV-specific reverse primer, or unmodified ONNV-specific primers (Table 1). Amplification of dsDNA products was undetectable in qPCR reactions containing the unprimed cDNA when the tag-specific primer set was used; however, a value of 7.4×10^6 copies of ONNV RNA/ μg of total RNA (the polarity of the RNA is unclear) was determined by standard curve when a primer set containing only ONNV-specific primers was used (Fig. 3.1; A and B).

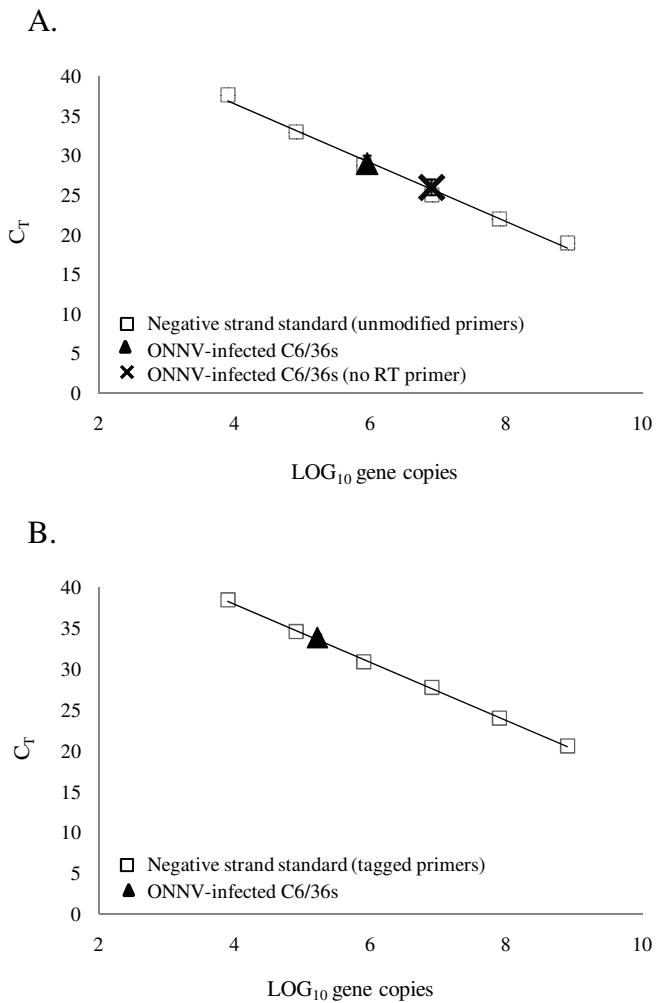


Figure 3.1. Detection of ONNV RNA with ssqPCR assays using unmodified and tagged primer systems. Quantification of ONNV (-) strand RNA with a ssqPCR assay using unmodified primers (A), and with an assay using a tagged primer system (B) produces significantly different values ($P<0.001$). Amplification of cDNAs generated in an unprimed RT reaction with unmodified ONNV-specific primers (A). Undetectable amplification of dsDNA products in qPCR reactions containing unprimed cDNAs using a tag-specific primer set (B).

To evaluate the strand specificity of qPCR with virus-specific primers in the presence of cDNAs transcribed from a competing strand of viral RNA, we generated standard curves (as outlined in materials and methods) with our ONNV (-) strand RNA ssqPCR assays in the presence or absence of a fixed amount of cDNA corresponding to a ONNV (+) strand RNA sequence (see materials and methods for details). Standard curves generated with the combination of tag-specific and ONNV-specific primers had similar values when compared (Fig. 1D); a slope of -3.6, R^2 of 0.995, and Eff% of 89.6 in the absence of ONNV (+) strand cDNA, and a slope of -3.7, R^2 of 0.990, and Eff% of 86.3 in the presence of ONNV (+) strand cDNA. However, standard curves produced with the unmodified primers had very different values depending on whether or not cDNA corresponding to the ONNV (+) strand RNA sequence was present during qPCR (Fig. 3.2A); a slope of -3.6, R^2 of 0.990, and Eff% of 89.6 in the absence of ONNV (+) strand cDNA, and a slope of -1.4, R^2 of 0.809, and Eff% of 417.9 in the presence of ONNV (+) strand cDNA. Only at dilutions in which ONNV (-) strand cDNA was present in excess of ONNV (+) strand cDNA were C_T values comparable between standard curves generated with unmodified ONNV-specific primers (Fig. 3.2A). Higher levels of cDNA from the competing (+) strands generally resulted in lower C_T values when compared with reactions that did not contain cDNA from the (+) strand, indicating dsDNA amplification from cDNA generated from the competing strand. These results suggest that a higher relative level of competing (+) strand cDNAs during qPCR specifically inhibits accurate quantification of ONNV (-) strand RNA with unmodified virus-specific primers. However, accurate quantification of ONNV (-) strand RNA using a combination of tag-specific and virus-specific primers was unaffected by the presence of higher levels of ONNV (+) strand cDNA, as evidenced by the reproducibility of standard curves (Fig. 3.2B)

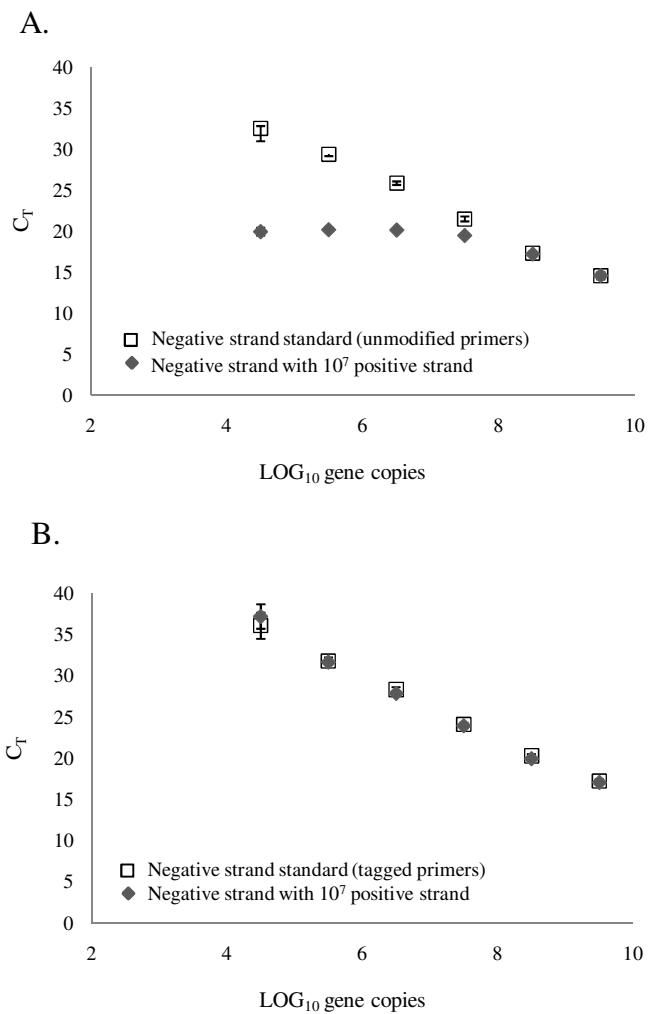


Figure 3.2. Strand-specificity of ssqPCR assays using unmodified and tagged primer systems.
 Standard curves generated in both the presence and absence of a fixed amount of competing strand cDNAs with a ssqPCR assay using unmodified primers (A) or with an assay using a tagged primer system (B).

DNA hydrolysis probes increase the sensitivity of qPCR with a tag-specific primer.

SYBR ® Green emits a strong fluorescence signal upon binding to dsDNA. Because the intensity of this fluorescent signal increases with the amount of dsDNA present, the dye can be used to detect and measure the accumulation of qPCR amplicons. However, amplification of nonspecific templates after many PCR cycles can result in additional fluorescence unrelated to any specific target. This nonspecific fluorescence has been shown to limit the sensitivity of dsDNA specific dyes for the detection and quantification of low-copy number targets (Higuchi et al., 1993; Wittwer et al., 1997). Because in alphavirus-infected cells (-) strand RNA synthesis ceases early in infection, concentrations of (-) strand RNA are likely to diminish with time (Sawicki and Sawicki, 1980). Thus, SYBR ® Green may not represent an ideal chemistry for detecting and measuring alphavirus (-) strand RNA in infected cells, particularly later in infection when (-) strand RNA is less abundant. Previous work suggested DNA hydrolysis probes, which are sequence-specific, might provide increased sensitivity when used with ssqPCR assays (Wittwer et al., 1997). In this case, the detection and measurement of amplification during qPCR is achieved by the fluorescent signal generated by a fluorophore released from a dual-labeled oligonucleotide probe. A fluorescent reporter dye is released from the 5' end of the probe by the exonuclease activity of Taq polymerase, reducing proximity to a quencher dye at the 3' end of the probe. Thus in contrast to dsDNA dyes, no fluorescence is generated from amplification of nonspecific templates because the fluorescent signal is dependent on hydrolysis of the probe following hybridization to a specific target sequence.

To determine if DNA hydrolysis probes increase the sensitivity of qPCR with a tag-specific primer, RNAs corresponding to ONNV (+) or (-) strands were synthesized in an *in vitro* transcription reaction (see materials and methods for details). Following reverse transcription with a tagged forward or reverse primer (Table 1), ONNV (+) and (-) strand cDNAs were serially diluted and used in the generation of standard curves. Two standard curves were generated with each 10-fold dilution series of ONNV (+) or (-) strand cDNAs, one with SYBR ® Green dye and the other with an ONNV-specific TaqMan® probe (Table 1). New specific primer sets were designed for use with the TaqMan-based detection strategy and chemistry (Table 1). While the lowest amount of either cDNA strand (+ or -) that could be detected by qPCR with SYBR ® Green 8×10^3 copies of ONNV cDNA/reaction (Fig. 3.3; A and C), the TaqMan®-based assay was able to detect 8×10^2 copies of ONNV cDNA/reaction (Fig. 3.3; B and D).

These results indicate a greater dynamic range for the assays using DNA hydrolysis probes in the quantification of low copy templates.

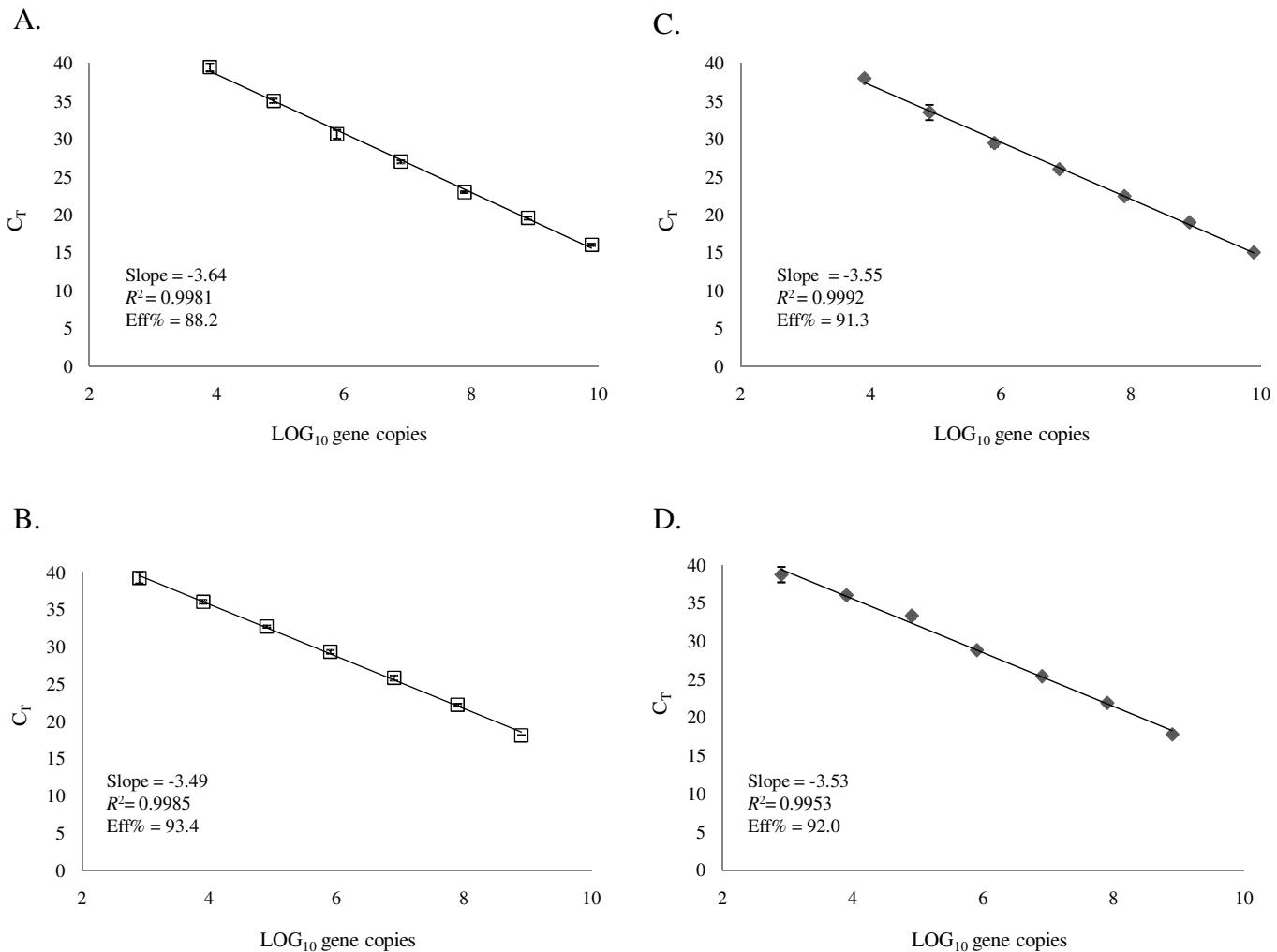


Figure 3.3. Sensitivity of ssqPCR assays using TaqMan® and SYBR Green®. Serial dilutions of ONNV (-) strand cDNAs quantified with tagged ssqPCR assays using SYBR Green® (A) or TaqMan® (B). Serial dilutions of ONNV (+) strand cDNAs quantified with tagged ssqPCR assays using SYBR Green® (C) or TaqMan® (D).

Development and validation of ssqPCR assays for CHIKV.

We next applied the information garnered in the previously described experiments to the design of ssqPCR assays for CHIKV, the cause of recent large scale outbreaks of debilitating disease in India and islands in the Indian Ocean (Enserink, 2008; Powers and Logue, 2007). Primer sets and TaqMan® probe sequences are listed in Table 1. Standard curves were generated (as outlined in materials and methods) and are shown in figure 3.4 (A and B). The lowest amount of either cDNA strand (+) or (-) that could be detected with the CHIKV ssqPCR assays was 1×10^3 copies of CHIKV cDNA/reaction (Fig. 3.4; A and B). Reactions containing 100 copies of either cDNA strand did not consistently generate threshold crossing fluorescence in less than 40 cycles, indicating that this template concentration was outside the dynamic range of the assays. The specificity of the (-) strand assay was unaffected by the presence of cDNAs transcribed from competing (+) strand viral RNAs (Fig. 3.4C); standard curves generated with the primer set containing the tag-specific primer had a slope of -3.3, R^2 of 0.987, and Eff% of 100.9 in the absence of ONNV (+) strand cDNA, and a slope of -3.5, R^2 of 0.973, and Eff% of 93.07 in the presence of ONNV (+) strand cDNA. As an additional test of the strand specificity of our CHIKV assays, cDNA dilutions used in the generation of standard curves were used in qPCR reactions with the primer set for the opposite strand. Amplification of (-) strand cDNA was undetectable with the (+) strand primer set, and conversely amplification of (+) strand cDNA was undetectable with the (-) strand primer set (data not shown), confirming a high level of fidelity for the intended target strand.

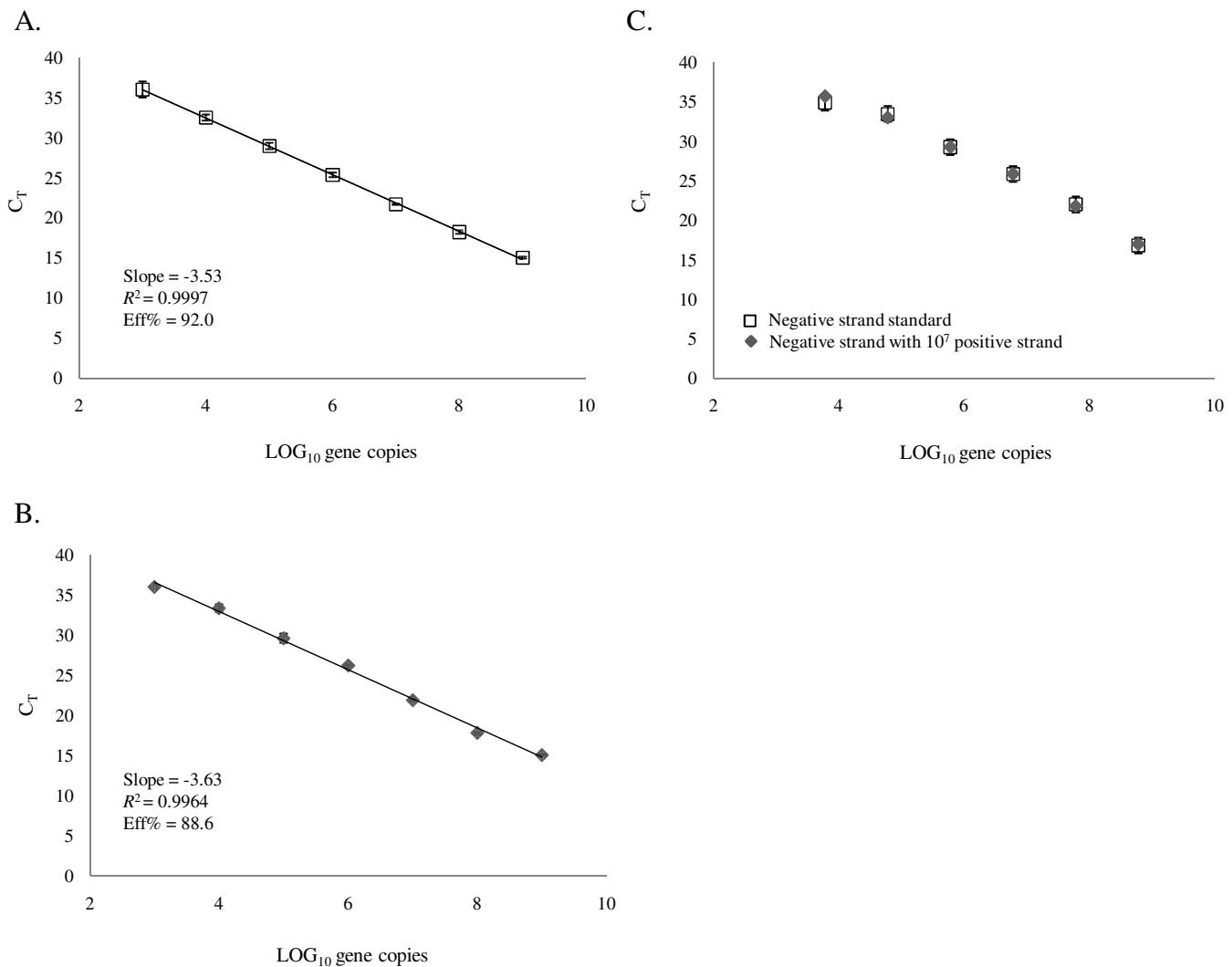


Figure 3.4. Strand specificity and sensitivity of CHIKV ssqPCR assays. Quantification of CHIKV cDNAs with tagged ssqPCR (-) strand assay (A) and with (+) strand assay (B). Standard curves generated both in the presence and absence of a fixed amount of competing strand cDNAs with the tagged CHIKV ssqPCR (-) strand assay (C).

DISCUSSION

Because RNA virus genomes must be efficiently copied in an infected cell, ssqPCR assays are valuable tools for the detection and quantification of replicating virus. However, the amount of cDNA quantified during qPCR must accurately reflect amounts of a specific viral RNA strand in the RT reaction. To determine how best to accomplish this, we developed several different ssqPCR assays for ONNV and then compared various parameters of those assays with each other.

We have shown that accurate quantification of (-) strand RNA in mosquito cells infected with ONNV is inhibited in the presence of relatively higher levels of cDNA generated from the competing (+) strand RNA, when standard unmodified ONNV-specific primers were used during reverse transcription and qPCR (Fig. 3.2A). Falsely-primed cDNAs have previously been demonstrated following reverse transcription of viral RNAs from dengue virus- and hepatitis C virus-infected cells and in vitro transcribed dengue RNAs in the absence of any specific primer (Gunji et al., 1994; Peyrefitte et al., 2003). Several mechanisms have been proposed to explain how false-priming may occur during the RT step, including self-priming of the reverse transcriptase from secondary hairpin structures present in highly folded viral RNA and random-priming by short endogenous or exogenous nucleic acids (Gunji et al., 1994; Peyrefitte et al., 2003; Timofeeva and Skrypina, 2001). Regardless of mechanism, we also demonstrated false priming of viral RNA during reverse transcription of RNA extracted from ONNV infected cells in the absence of a primer (Fig. 3.1A). Although it was not possible to quantify the amounts of falsely-primed cDNAs that were generated from specific strands of viral RNA, it is reasonable to presume that the ratios of falsely-primed products approximate ratios of (+) and (-) strand RNAs in the infected cell. Because imbalances in the synthesis of genomic RNAs and their full-length complements are common during RNA virus infections, false priming of the more abundant strand during the RT step of the assay is likely to inhibit accurate quantification of the less abundant strand when standard unmodified virus-specific primers are used for reverse transcription and qPCR. However, we have shown that inhibitory effects of falsely-primed cDNAs on the accuracy of ssqPCR assays can be eliminated. When a unique tag sequence was added to cDNA generated from ONNV (-) strands during reverse transcription, accurate quantification of ONNV (-) strand RNA was possible with a tag-specific primer in the presence of higher levels of ONNV (+) strand cDNA (Fig. 1D). Amplification of falsely-primed cDNAs

generated from the competing (+) strand RNAs were also undetectable during qPCR, as the tag-specific primer could not anneal to falsely-primed products lacking a complimentary sequence (Fig. 3.1; A and B).

The sensitivity of our ONNV (-) strand ssqPCR tag-specific assays was determined with serial dilutions of cDNA generated from in vitro transcribed ONNV RNAs, and was between 8000 and 800 copies per reaction when the dsDNA-specific dye, SYBR ® Green, was used. However, the limit of low-copy number detection decreased to between 800 and 80 copies per reaction when a TaqMan® DNA hydrolysis probe was used to monitor amplification. The increased sensitivity likely resulted from eliminating the non-specific amplification of dsDNA products that is common when monitoring the generic fluorescence emitted by dsDNA-binding dyes. The sensitivities of our ONNV (+) strand ssqPCR tag-specific assays were determined to be identical to those of the (-) strand assays, with both detection strategies and chemistries. In the case of alphaviruses, the increased sensitivity of assays incorporating DNA hydrolysis probes will be desirable for quantifying the much less abundant (-) strand RNAs.

Finally, using the information obtained from directly comparing various parameters of multiple ONNV-specific ssqPCR assays, we developed and validated assays to detect and quantify CHIKV (-) and (+) strand RNAs. Serial dilutions of cDNA generated from in vitro transcribed CHIKV RNAs were used to confirm strand-specificity and determine sensitivity. Amplification of dsDNA was undetectable when the (+) strand primer set was used with cDNA derived from the (-) strand at all concentrations tested. The reverse was also found to be true using the (-) strand primer set and cDNA generated from (+) strand RNA. The accuracy of the CHIKV (-) strand assay was confirmed in the presence of cDNAs transcribed from competing (+) strand RNAs (Fig. 3.4C). The sensitivity of our CHIKV ssqPCR assays was determined to be between 1000 and 100 copies per reaction.

In summary, we have developed and validated two new ssqPCR assays for the medically important alphaviruses, CHIKV and ONNV. The assays, reported here, will be useful in studies to determine how persistent alphavirus infections are maintained in the vector host, and in the detection and quantification of replicating virus from clinical specimens and potential reservoir hosts. In the course of developing these assays, we have shown that accurate quantification of cDNAs generated from specific strands of viral RNA, in the presence of higher levels of falsely-

primed cDNA products generated from competing RNA strands, requires incorporation of a unique tag sequence during reverse transcription, which in combination with a tag-specific primer can be used to specifically amplify cDNAs corresponding to the intended target strand during qPCR. While it was also possible to quantify specific strands of viral RNA with assays employing unmodified virus-specific primers, the accuracy of these measurements depends on conditions in which lower relative levels of cDNA generated from the competing strand are present during qPCR. Therefore, previously reported results obtained with assays of this type should be interpreted with caution, particularly when the assay in question has been used to measure amounts of anti-genomic strands, which are typically less abundant than genomic RNAs in cells and tissues infected with RNA viruses.

Chapter 4

INVESTIGATING STRAND-SPECIFIC ALPHAVIRUS REPLICATION KINETICS IN VERTEBRATE AND INVERTEBRATE CELL CULTURE USING REAL-TIME PCR

INTRODUCTION

The mosquito-borne alphaviruses in the family Togaviridae have a single-stranded, non-segmented, positive (+) strand RNA genome approximately 12 kb in length. There are four nonstructural proteins (nsP1-4) encoded in the 5' two thirds of the genome, and the structural genes are encoded in the 3' one third of the genome. Depending on the virus genotype, the polyprotein(s) P123 and/or P1234 are translated after entry into the cell. The first polyprotein, P123, is produced in the presence of an opal termination codon between nsP3 and nsP4, (Strauss, Rice, and Strauss, 1983) and P1234 is translated if readthrough of the stop codon occurs (de Groot et al., 1990; Shirako and Strauss, 1994), or if a sense codon exists at the nsP3-nsP4 junction. The genomic (49S) RNA is replicated through a full-length, minus (-) strand RNA intermediate. Because an internal promoter is present in the (-) strand copy, this copy also serves as a template for a subgenomic (26S) RNA from which the structural proteins are translated (reviewed in Strauss and Strauss, 1994).

The kinetics of (-) strand RNA synthesis by both mutant and wild-type Sindbis (SINV) and Semliki Forest viruses (SFV) has been extensively studied in chicken cells, baby hamster kidney cells (BHK-21s) and mouse embryos (Fata, Sawicki, and Sawicki, 2002; Kim et al., 2004; Sawicki et al., 2006; Sawicki and Sawicki, 1993; Sawicki et al., 2003; Shirako and Strauss, 1994; Wang, Sawicki, and Sawicki, 1994). Previous studies of SINV and SFV RNA synthesis have contributed to the current model of alphavirus RNA replication, which suggests that replication occurs in two discreet stages. These stages include an early, transient phase where replicase complexes are formed that synthesize both (+) and (-) strand RNA, and a later phase where the replicase complexes formed synthesize only (+) strand RNA. Previous studies suggest that (-) strand synthesis occurs only during the first several hours post infection (Sawicki et al., 2006; Sawicki and Sawicki, 1980; Sawicki et al., 1981), while (+) strand RNA synthesis continues for much longer (Sawicki and Sawicki, 1980; Strauss and Strauss, 1986). Proteolytic processing of alphavirus polyproteins generates strand-specific replicase complexes at different times during infection (de Groot et al., 1990; Hardy and Strauss, 1989). Uncleaved P123 and

mature nsP4 function together to form the (-) strand RNA replicase complex (Hardy and Strauss, 1989; Lemm and Rice, 1993a; Lemm and Rice, 1993b; Lemm et al., 1994; Shirako and Strauss, 1994; Strauss et al., 1992). However, this complex is unstable as P123 can be further cleaved in a bi-molecular reaction to produce nsP1, P23 and nsP4, components of another replicase capable of synthesizing both (-) and (+) strand RNA. Fully cleaved polyproteins (thus, mature nsP1, nsP2, nsP3 and nsP4) function as the (+) strand RNA replicase complex (Shirako and Strauss, 1994). Late in infection, high levels of P123 in the cell cleave nascent polyproteins between nsP1 and nsP2 so rapidly, that new translation of P123 is prevented. Thus, only (+) strand RNA replicases are formed at later times in infection (Shirako and Strauss, 1994).

The transmission cycles of most alphaviruses require alternating replication in both a vertebrate host and a mosquito vector. The pathogenic effects of alphavirus infection differ in infected vertebrate and mosquito hosts. While infection of the vertebrate host is acute and often accompanied by symptoms (Calisher, 1994), a persistent, life-long infection is established in the invertebrate host that is generally asymptomatic (Bowers, Abell, and Brown, 1995). The establishment of a persistent infection in the invertebrate host is essential to the maintenance of the alphavirus transmission cycle. Cultured cells infected with alphaviruses appear to be a good model for pathogenic effects in whole animals. Cytopathology is typically observed as soon as 24 hours after infection of several vertebrate cell lines with alphaviruses, while infection of cultured mosquito cells generally causes little to no cytopathology. Large quantities of virus are shed from infected mosquito cells during an early stage of infection, which is then followed by an extended persistent late phase of infection, where replication occurs at much lower levels (Brown, 1984; Igarashi, Koo, and Stollar, 1977; Riedel and Brown, 1977).

Several alphaviruses have been reported to contain either an opal stop codon (UGA) or a sense codon near the nsP3/nsP4 junction in the nonstructural protein region (reviewed in Strauss and Strauss, 1994). Sequence information initially indicated that the termination codon before nsP4 had been replaced with an arginine codon (CGA) in ONNV. However, passaging of ONNV prior to sequencing may have determined the codon at this particular locus. Consensus sequences obtained from later, lower passage, isolates indicate that ONNV possess both sense and stop codons at this position. Further, when ONNV isolates found to have an opal termination codon are passaged in vertebrate cells (Vero), they rapidly acquire in its place an arginine codon. Previous studies also indicate *An. gambiae* mosquitoes are more frequently infected with opal

virus variants of ONNV (Myles et al., 2006). These results suggest that alphavirus quasispecies populations include both stop and sense codons in equilibrium at this locus, and that both sequences are somehow required for the survival of these viruses in nature. Host specific replication of alphaviruses (in either vertebrate or arthropod hosts) may determine the predominance of the codon at this particular locus in the quasispecies population.

Although the exact role of the opal codon remains unclear, translational termination between nsP3 and nsP4 should result in a more rapid build-up of the *trans*-acting protease P123 in the infected cell, accelerating the transition from the production of (-) strand replicase to the production of (+) strand replicase. It has been proposed that the mechanism used by alphaviruses for regulating minus-strand RNA synthesis evolved from the necessity of these viruses to control pathogenicity in the invertebrate host and that this is necessary for the establishment of a persistently infected state. Additional regulation of (-) strand RNA synthesis when an opal codon is present in the alphavirus genome may be advantageous for the establishment of productive infections in the mosquito host.

In order to determine if differences exist in the strand-specific replication kinetics of alphaviruses possessing either an opal or arginine codon preceding nsP4, ssqPCR was used to measure levels of viral RNA in alphavirus-infected cell cultures.

MATERIALS AND METHODS

Cell culture maintenance

BHK-21 cells and C6/36 cells were maintained and subcultured as previously described in Chapter 2. *Aedes aegypti* cells (Aag2) were maintained in Schneider's Drosophila Medium (Lonza-BioWhittaker, Walkersville, MD), and supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 1% penicillin-streptomycin solution, and 1% L-glutamine. Aag2 cells were subcultured by removing medium, washing with PBS, scraping in medium, and split at a ratio of 1:10. Aag2 cells were maintained at 5% CO₂ at 28°C.

in vitro Transcription

The full-length cDNA clones, pONN.AP3 (Brault et al., 2004) and pCHIK37997-ic, (Vanlandingham et al., 2005) were used to generate infectious RNA transcripts. Plasmids pONN.AP3 and pCHIK37997-ic were linearized at 3'-terminal *NotI* sites. Digested DNA was phenol/chloroform extracted, chloroform extracted, and finally ethanol precipitated. RNA transcripts of two different genotypes, containing either an opal (Op) or arginine (R) codon, were generated from 1 µg of purified template. Transcription reactions also contained 1X RNA polymerase reaction buffer (New England Biolabs, Ipswich, MA); 100 µg/ml acetylated BSA (Promega, Madison, WI); 1mM rNTP nucleotide mix (New England Biolabs); 1mM m⁷G(5')ppp(5')G cap analog (pONN.AP3) or m⁷G(5')ppp(5')A cap analog (pCHIK) (New England Biolabs); 5mM DTT (Promega), 60 units of RNAsin Plus RNase inhibitor (Promega), and 20 units of SP6 polymerase (pONN.AP3) or T7 polymerase (pCHIK) (New England Biolabs). RNase-free water was added to bring the final reaction volume to 50 µl. Reactions were incubated at 39°C for 1 hour and stored at -80°C until further use.

Rescuing Virus from ONNV and CHIKV Infectious Clones

Virus working stocks were generated by electroporating *in vitro* transcribed RNAs into BHK-21 cells. BHK-21 cells were grown to 80% confluence in 150 cm² flasks, trypsinized, centrifuged and washed three times with PBS. Cells were counted with a hemocytometer and resuspended in PBS at a final concentration of 1 × 10⁷ cells/ml. Twelve microliters of transcription reaction were mixed with 600 µl of resuspended cells and electroporated twice on a BTX ElectroCell Manipulator (BTX Instrument Division, Harvard Apparatus, Inc., Holliston,

MA) at 450V, 725Ω, and 75 µF. After electroporation, cells and RNA were transferred to a 25 cm² flask, 5 ml of DMEM was added, and the flask transferred to an incubator at 37°C with 5% CO₂. When 70-90% of the BHK-21 cells exhibited cytopathic effects (CPE), typically ~48-72 hours after electroporation, the culture supernatant was harvested, centrifuged at 1000 × g for five minutes, aliquoted and then stored at -80°C.

Infecting C6/36, BHK-21, and Aag2 Cells with ONNV and CHIKV

Harvested ONNV-Op and ONNV-R stocks were used to infect C6/36 and BHK-21 cells, and harvested CHIKV-Op and CHIKV-R stocks were used to infect Aag2 cells. Two independent infections for each time point were performed as biological replicates for each virus. Cell monolayers were grown to 80% confluence in 12-well plates, washed twice with PBS, and infected at a multiplicity of infection (MOI) of 5. Virus was combined with medium, either DMEM (C6/36, BHK-21) or Drosophila medium (Aag2), for a total volume of 0.5 mL/well and then incubated at 4°C for one hour. After incubation, fresh medium was added to the wells to bring the total medium-virus volume up to 1 mL, and cells were placed at 28°C (C6/36, Aag2) or 37°C (BHK-21). During the course of infection, infected C6/36 and Aag2 cells that appeared to be overgrown were split by trypsinization (previously described in chapter 2) at a ratio of 1:4 as frequently as necessary.

Isolation of Total RNA from Infected Cells

Total RNA was isolated from infected cells by using TRI Reagent RT at timed intervals (t= 1, 5, 9, 12, and 24 hours for ONNV-infected BHK-21 cells and t= 1, 5, 9, 12, 24, 36, 60, 72, 84, 96, 144, 192, 240, 336, and 504 hours for ONNV-infected C6/36 cells and CHIKV-infected Aag2 cells). Virus-medium supernatant was removed from wells and infected cells were washed with PBS three times before adding 0.5 mL of TRI Reagent RT (MRC, Inc., Cincinnati, OH) per one well to lyse the cells. After transferring TRI Reagent RT-cell homogenate to a 1.5 mL centrifuge tube, 25 µl of bromaoanisole was added, the mixture was shaken vigorously for 15 seconds, and then was centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase containing the RNA was removed and placed in another 1.5 mL centrifuge tube. RNA was precipitated by adding an equal volume of isopropyl alcohol and centrifugation of 12,000 × g at 4°C for 5 minutes after sitting at room temperature (rt) for 5-10 minutes. The supernatant was

removed and the RNA pellet was washed with 75 µl of 70% ethanol and centrifuged at 6000 × g for 5 minutes at 4°C. The ethanol wash was removed the RNA pellets were dried at rt for 2-5 minutes. Pellets were resuspended in nuclease free water and stored at -80°C until further use.

Determination of RNA Integrity

The integrity of total RNA from infected cell culture samples was assessed using the Agilent Eukaryotic RNA 6000 Nano Assay on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Prior to running the assay, the electrodes on the bioanlayzer were decontaminated using RNase Free (CLP, San Diego, CA) for one minute and then neutralized with nuclease-free H₂O for ten seconds. The ladder and all RNA samples used were denatured for two minutes at 70°C and then placed on ice. The preparation of the nano chips and the loading of 9 µl of gel-dye mix, 5 µl of the RNA 6000 Nano Marker, 1 µl of RNA sample, and 1 µl of RNA ladder were performed via the manufacturer's instructions. Because the Agilent Eukaryotic RNA 6000 Nano Assay is designed to analyze vertebrate-derived RNA, and many of the samples used in this study were derived from invertebrates, modifications were made to the software analysis setpoints in order to achieve a reliable RIN for infected C6/36 and Aag2 samples. Under "General Assay Setpoints: RNA Intergrity Number", the fast region anomaly threshold was changed from 0.56 to 2.0, and the ribosomal ratio anomaly threshold was changed from 0.7 to 1.0.

Reverse Transcription of ONNV and CHIKV Strand-Specific cDNA

cDNA of both polarities was transcribed from total RNA extracted from ONNV- and CHIKV-infected cells. Reverse transcription (RT) reactions were performed using a forward or reverse primer containing a unique 5' tag sequence, as described in Chapter 3. Briefly, one microgram of total RNA template was added to the reaction, along with 500 nM forward primer (- strand detection) or reverse primer (+ strand detection) (Table 3.1) for 5 minutes at 70°C followed by 2 minutes on ice. Subsequently, 1mM dNTPs (Roche Molecular Diagnostics, Pleasanton, CA), 10mM DTT (Invitrogen, Carlsbad, CA), 1X Reverse Transcriptase Buffer (Invitrogen), 20 U of RNAsin (Promega, Madison, WI), and 50 U of Superscript II (Invitrogen) were added to a final reaction volume of 20 µl. Reactions were then incubated at 50°C for 30 minutes and then heat inactivated for 15 minutes at 95°C. Unincorporated primers present in heat

inactivated RT reactions were digested with exonuclease I (New England Biolabs, Ipswich, MA). The exonuclease reactions were incubated at 37°C for 30 minutes followed by 70°C for 15 minutes. Each reaction was brought up to a final volume of 100 µl and stored at -20°C.

Real-Time PCR for Viral RNA Quantitation

Primers and probes were designed using Primer Select (DNASTAR, Madison, WI) and Primer Express 2.0 (Applied Biosystems, Inc., Foster City, CA). ssqPCR was performed with the appropriate combination of a tag-specific primer pair and TaqMan probe (Table 3.1). Unknown samples of viral transcripts from either ONNV- or CHIKV-infected cells were amplified synchronously with their respective standard curves during qPCR. The generation of the standard curves is described in Chapter 3. qPCR was performed using the ABI 7300 Real-Time PCR System. The two biological replicates for each virus at each time point were run in triplicate during qPCR. Reaction tubes contained 12.5 µl 1X ABI Gene Expression Master Mix (Applied Biosystems, Inc.) with 250 nM probe, 900 nM forward primer, 900 nM reverse primer, and 10 µl (unknowns) or 2 µl (standards curve dilutions) of diluted cDNA. Nuclease-free H₂O was added to bring the final reaction volume to 25 µl. cDNA samples were run in triplicate. The standard cycling conditions were 50° C for 2 min, 95°C for 10 mins, followed by 40 cycles of 95°C for 15 s and 61°C for 1 min. Data collection occurred at the extension step at 61°C. Samples were run in triplicate. The quantities of ONNV and CHIKV NSP1-gene copies per unknown sample were determined by comparing them to the standard reference curves via the ABI 7300 system software.

RESULTS

Determination of RNA integrity of samples used for RT and qPCR

Because the quality of RNA is critical to accurate data analysis when using highly sensitive PCR methodologies, we aimed to determine the quality, or integrity, of our total RNA samples using the Agilent 2100 Bioanalyzer. An RNA Integrity Number (RIN) is a numerical value from 1-10 that describes the quality of RNA fragments. A RIN of 10 indicates the highest level of quality (in other words, the least amount of degradation) possible for a sample of RNA (Mueller, Lightfoot, and Schroeder, 2004). The assay we utilized for this purpose was the Agilent Eukaryotic RNA 6000 Nano Assay. The Agilent software automatically generates accurate RINs using the default software settings for samples isolated from vertebrate cells and tissues, but we found that it does not always automatically generate RINs for RNA samples derived from invertebrate cells and tissues. Therefore we modified the software setpoints as described in the materials and methods to obtain a RIN for total RNA samples isolated from invertebrate Aag2 and C6/36 cells. However, per manufacturer's advice, the RIN values generated after manipulation of the default settings for invertebrate RNA analysis may not reflect the true quality of the RNA sample. Thus, total RNA degradation was assessed by two factors, 1) the RNA integrity number (RIN) and 2) the visual appearance of the 18S and 28S ribosomal RNA peaks and bands in the electropherograms and gel-like images generated by the bioanalyzer software.

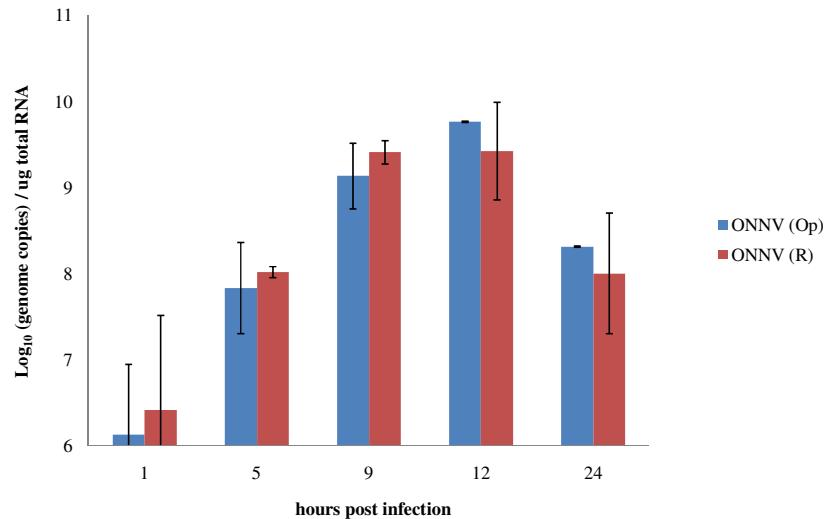
After examining RINs, electropherogram traces, and gel images, we determined that samples of total RNA from infected C6/36 cells with RIN values of 5.5 or higher appeared acceptable for use in subsequent RT and qPCR reactions and was thus the RIN range that applied to the C6/36 samples used in this study. As with the RNA samples isolated from ONNV-infected C6/36s, RINs and ribosomal peaks and bands were analyzed to determine the integrity of each RNA sample extracted from CHIKV-infected Aag2 cells. Each sample of total RNA isolated from Aag2 cells used for RT and qPCR had a RIN of 5.0 or greater. Additionally, each sample of total RNA from infected BHK-21 cells used for RT and qPCR had a RIN of 8.6 or greater, and these RINs were generated by the software using the default settings.

Quantification of (+) and (-) strand RNA in ONNV-infected BHK-21 cells

The majority of strand-specific alphavirus replication studies have been performed in SINV- and SFV-infected vertebrate models (Fata, Sawicki, and Sawicki, 2002; Kim et al., 2004; Sawicki et al., 2006; Sawicki and Sawicki, 1993; Sawicki et al., 2003; Shirako and Strauss, 1994; Wang, Sawicki, and Sawicki, 1994). Because many of the tools used for these previous studies are limited in their strand-specificity and are only semi-quantitative, we investigated the (+) and (-) strand replication kinetics of ONNV in BHK-21 cells using ONNV TaqMan ssqPCR assays (described in chapter 3). Total RNA was extracted from the infected cells at various time points post infection. RT and qPCR were subsequently performed to quantify copy number of NSP1 transcripts per microgram of total RNA for both ONNV genotypes. Unknown samples of total RNA from ONNV-infected cells were compared to a (+) strand and (-) strand standard reference curve for absolute quantification. We quantified (+) and (-) strand RNAs for both genotypes of ONNV in BHK-21 cells at several time points post infection. The quantity of (-) strand RNA for both genotypes steadily increased by several logs over the first 9-12 hours of infection (Fig. 4.1A). The peak quantities of both RNA strands (+ and -) for the two ONNV genotypes (Op and R) were detected at 12 hours post infection (h.p.i.). We observed that both (+) and (-) strand RNA levels decrease between 12 and 24 h.p.i., but because of the cytopathic effects (CPE) that occur in alphavirus-infected vertebrate cells, the strand-specific RNA quantities of ONNV beyond this time point could not be determined.

To better visualize the replication kinetics of the two ONNV genotypes during infection, the ratio of (+ and -) RNA strands was analyzed at various time points post infection in BHK-21 cells. The strand-specific RNA ratio was expressed as (-) strand RNA copies per one (+) strand copy. This ratio for ONNV-Op and ONNV-R RNAs appear to differ at several time points (Figure 4.2). However, differences in strand-specific ratios between the two genotypes were not determined as significant at any time point post infection in BHK-21 cells.

A.



B.

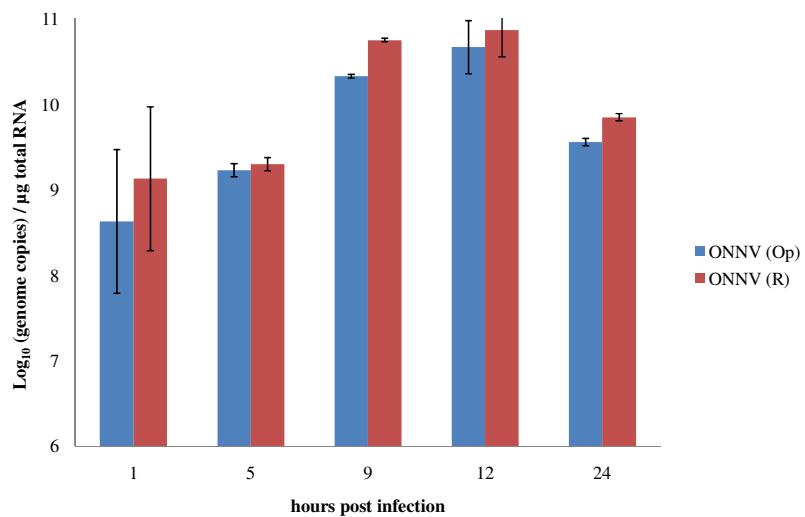


Figure 4.1. Quantities of viral minus-strand RNA and plus-strand RNA in ONNV-infected BHK-21 cells. TaqMan ssqPCR assays were used to detect ONNV (-) strand RNA (A) and (+) strand RNA (B).

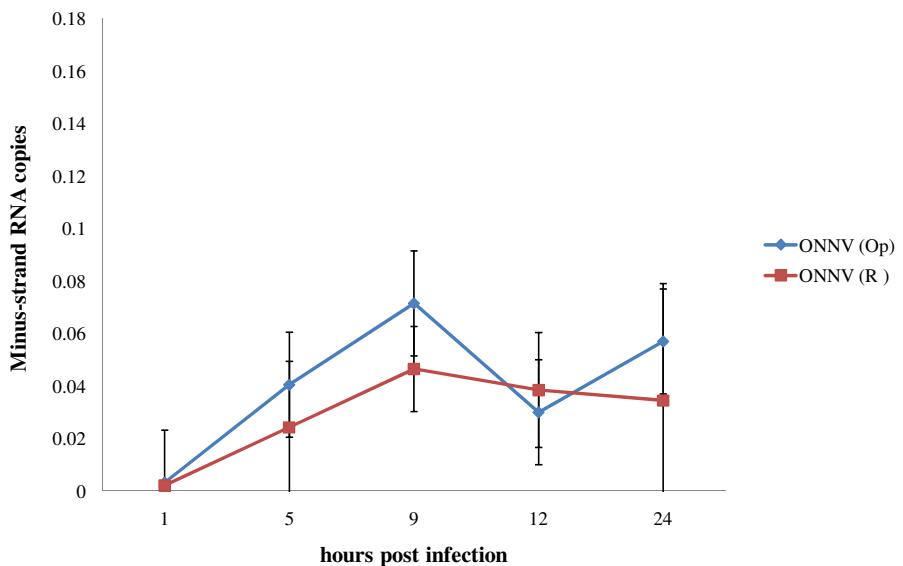


Figure 4.2. Minus-strand RNA copies per one plus-strand RNA copy in ONNV-infected BHK-21 cells. No significant differences in RNA quantities were determined between the ratios of the two genotypes.

Quantification of (+) and (-) strand RNA in ONNV-infected C6/36 cells

After using ssqPCR to determine the replication kinetics of ONNV in BHK-21 cells, we compared these findings to the (+) and (-) strand replication kinetics of ONNV in invertebrate cells. C6/36 cells were infected with ONNV-Op and ONNV-R and total RNA was extracted at specific time points post infection for 21 days (see Materials and Methods). As with the BHK-21 samples, the previously described ONNV TaqMan ssqPCR assays (see chapter 3) were used to quantify the copy number of NSP1 transcripts per microgram of total RNA for both ONNV genotypes in C6/36 cells.

Using ssqPCR, we were able to detect ONNV (-) strand RNA in C6/36 cells 21 days post infection (d.p.i) (Figure 4.3). This is the first report of the detection of (-) strand RNA at this time point, which far surpasses the time points at which (-) strand RNA has been detected in previous reports using other methods. We also determined that (-) strand RNA quantities detected for

ONNV-Op were significantly lower than that of ONNV-R at 1 and 10 d.p.i. (ANOVA, $P < 0.01$ and $P < 0.05$, respectively). ONNV (+) strand RNA levels were also quantified, and no significant differences between the two genotypes were observed during the course of infection (Figure 4.4). Thus, it was interesting to us that the (+) strand RNA quantities for ONNV-Op at 1 and 10 d.p.i. (Figure 4.4) were unaffected by the quantities of ONNV-Op (-) strand RNA at 1 and 10 d.p.i. that were significantly lower than that of the arginine genotype (Figure 4.3).

By analyzing the ratios of strand-specific RNA (+ and -) at various time points post infection, we determined that the quantity of (-) strand RNA per one (+) strand copy for ONNV-Op was significantly lower than that of ONNV-R at 1 d.p.i. (ANOVA, $P < 0.0001$) (Figure 4.5). Conversely, we found that the amount of (-) strand RNA per one (+) strand copy for ONNV-Op is significantly higher than that of ONNV-R at 3.5 d.p.i. (ANOVA, $P < 0.01$) (Fig. 4.5). In addition, we determined that the amount of (-) strand per one (+) strand copy for ONNV-R significantly increased from 12 h.p.i. to 1 d.p.i and significantly decreased from 1 d.p.i. to 1.5 d.p.i. (ANOVA, $P < 0.01$ at both time intervals) (Figure 4.5).

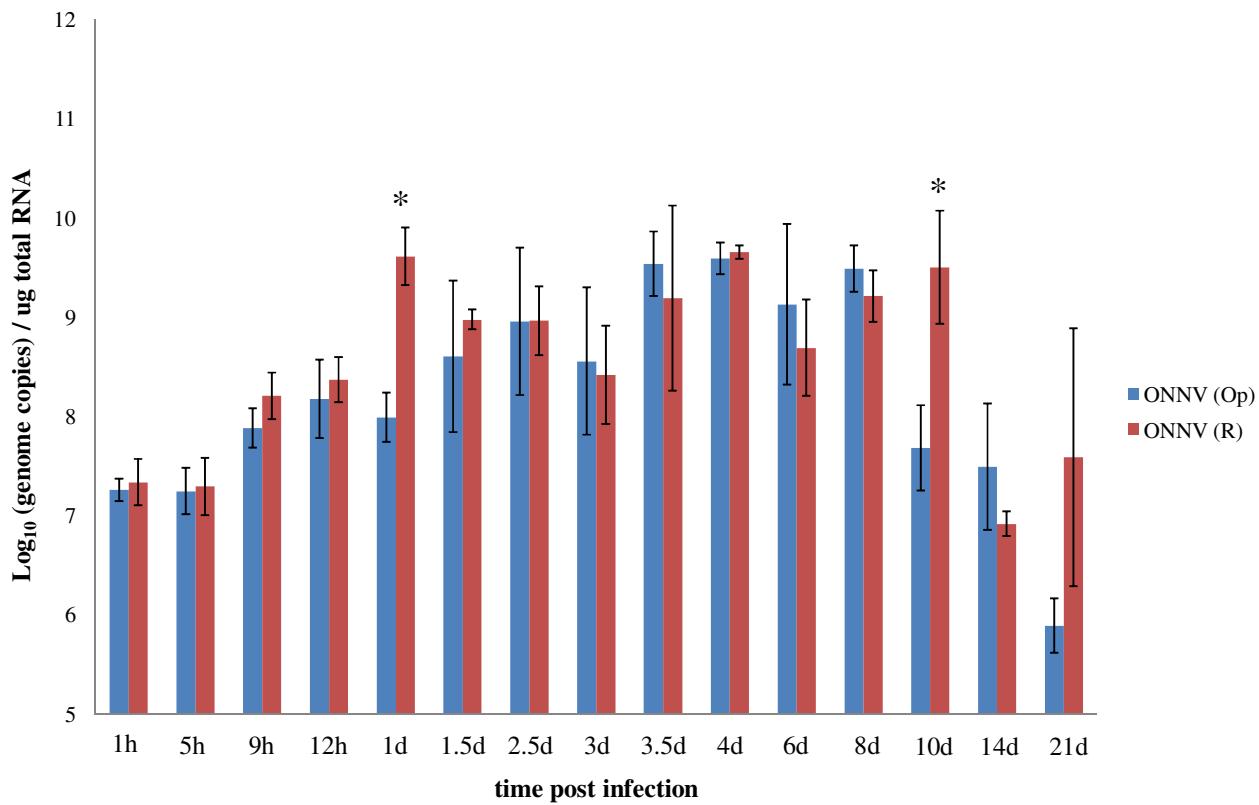


Figure 4.3. Minus-strand RNA quantities for ONNV-Op and ONNV-R in C6/36 cells. The (-) strand ssqPCR TaqMan assay was used for (-) strand RNA detection. Asterisks denote significant differences between ONNV genotypes (ANOVA, $P < 0.01$ and $P < 0.05$ at $t = 1$ and 10 d.p.i., respectively).

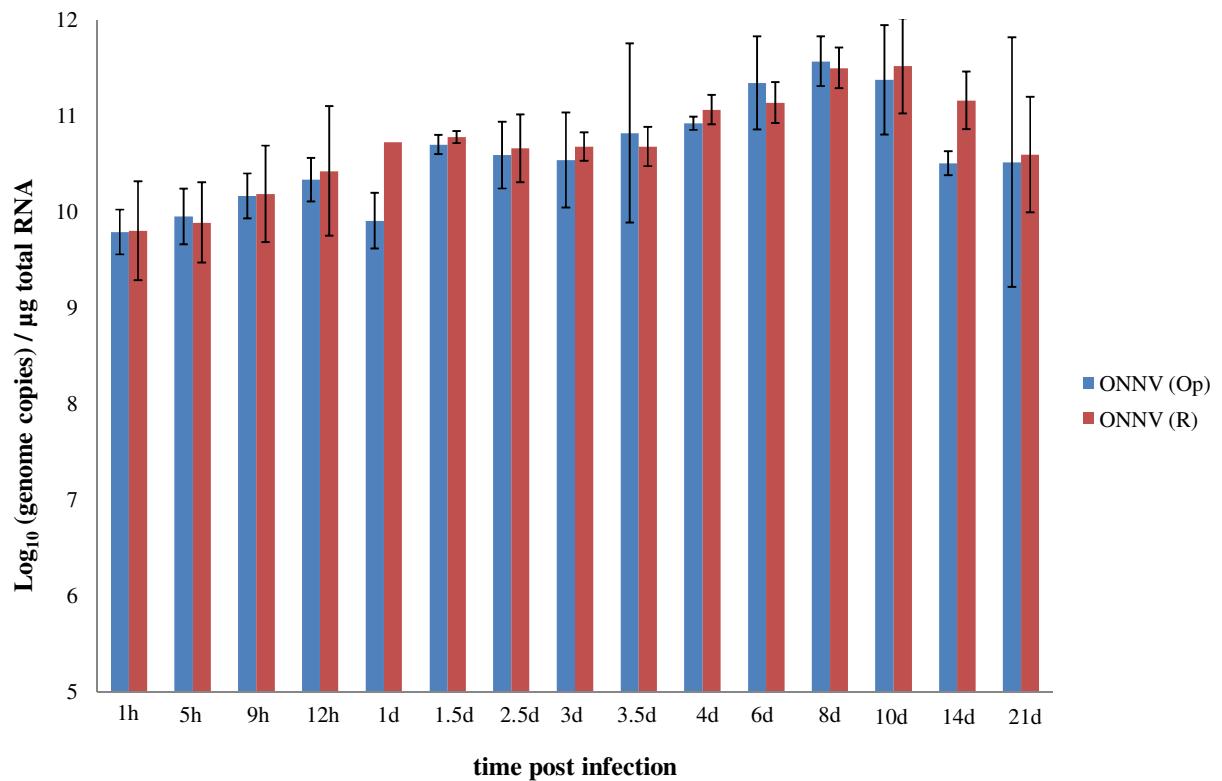


Figure 4.4. Plus-strand RNA quantities for ONNV-Op and ONNV-R in C6/36 cells. The (+) strand ssqPCR TaqMan assay was used for (+) strand RNA detection.

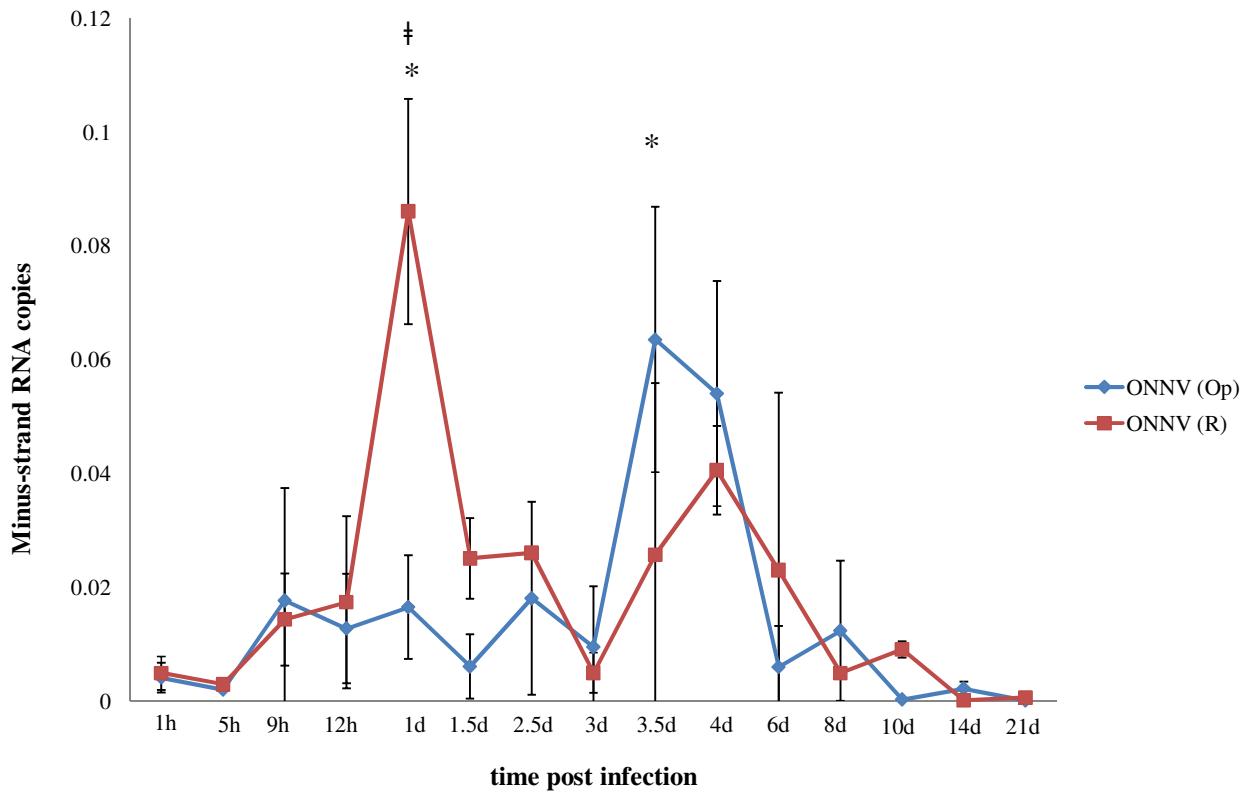


Figure 4.5. Minus-strand RNA copies per one plus-strand RNA copy in ONNV-Op and ONNV-R-infected C6/36s. Asterisks denote significant differences between ONNV genotypes (ANOVA, $P < 0.0001$ and $P < 0.01$ at $t = 1$ and 3.5 h.p.i., respectively). Cross symbol denotes the time point at which ONNV-R (-) strand RNA copies are significantly higher than the quantities determined for ONNV-R at time points immediately preceding and following it (ANOVA, $P < 0.01$ between 12 h.p.i. and 1 d.p.i. and between 1 and 1.5 d.p.i.).

Quantification of (+) and (-) strand RNA in CHIKV-infected Aag2 cells

Because of the paucity of alphavirus replication studies that have been performed in invertebrate models, we investigated the strand-specific replication kinetics of alphaviruses in persistently-infected invertebrate cells. Part of this investigation examined the replication kinetics of ONNV in invertebrate C6/36 cells; however, the species from which C6/36 cells are derived, *Aedes albopictus*, is not a natural vector of ONNV. We aimed to investigate the (+) and (-) strand replication kinetics of CHIKV in cells derived from a natural vector of the virus, *Aedes aegypti* (Jupp, 1988). *Aedes aegypti* Aag2 cells were infected with CHIKV-Op or CHIKV-R and total RNA was isolated at specific time points post infection for 21 days (see Materials and Methods for exact time points). The previously described CHIKV TaqMan ssqPCR assays (see chapter 3) were used to quantify the copy number of NSP1 transcripts per microgram of total RNA for both CHIKV genotypes in Aag2 cells.

Parallel to our findings in ONNV-infected C6/36 cells, CHIKV (-) strand RNA was detected in Aag2 cells at 21 d.p.i. (Fig. 4.6). We found that the quantities of CHIKV-Op (-) strand RNA were significantly lower than that of CHIKV-R at 6, 8, and 10 d.p.i. (ANOVA, $P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively; Fig. 4.6). This finding suggests that the opal codon in CHIKV may play a role in the regulation of (-) strand RNA during the establishment of a long-term persistent infection.

It was determined that the quantity of (+) strand RNA detected at 8 and 10 d.p.i. for CHIKV-R was significantly higher than that of the CHIKV-Op (Figs. 4.7, (ANOVA, $P < 0.01$ and $P < 0.05$, respectively). This is unlike the ONNV (+) strand replication kinetics seen in C6/36 cells where no significant differences in (+) strand RNA quantity were observed between the two ONNV genotypes at any time post infection (Fig. 4.4). Thus, it appears that the (+) strand RNA quantities for CHIKV-Op at 8 and 10 d.p.i. (Figure 4.7) may have been affected by the significantly lower quantities of CHIKV-Op (-) strand RNA at 8 and 10 d.p.i. (Figure 4.6). Interestingly, although significant differences in (-) strand RNA levels were observed between the two genotypes at 6 d.p.i., no significant differences in (+) strand RNA levels between the two viruses was observed at this time point.

It was also determined that (-) strand RNA quantities per (+) strand copy between CHIKV-Op and CHIKV-R differ significantly at multiple time points post infection. The number of CHIKV-Op (-) strand RNAs per one (+) strand RNA copy was significantly less than CHIKV-

R at 3 and 6 d.p.i. (ANOVA, $P < 0.01$ and $P < 0.001$, respectively; Fig. 4.8). This finding indicates that differences may exist between the (-) strand replication kinetics of CHIKV-Op and CHIKV-R. The amount of (-) strand RNA per one (+) strand copy for CHIKV-R significantly increased from 2.5 d.p.i. to 3 d.p.i and significantly decreased from 3 d.p.i. to 3.5 d.p.i. (ANOVA, $P < 0.01$ at both time intervals) (Figure 4.8). Similar to ONNV-Op in C6/36 cells, a significant peak in (-) strand RNA per (+) strand RNA per (+) strand for CHIKV-Op was not determined in Aag2 cells.

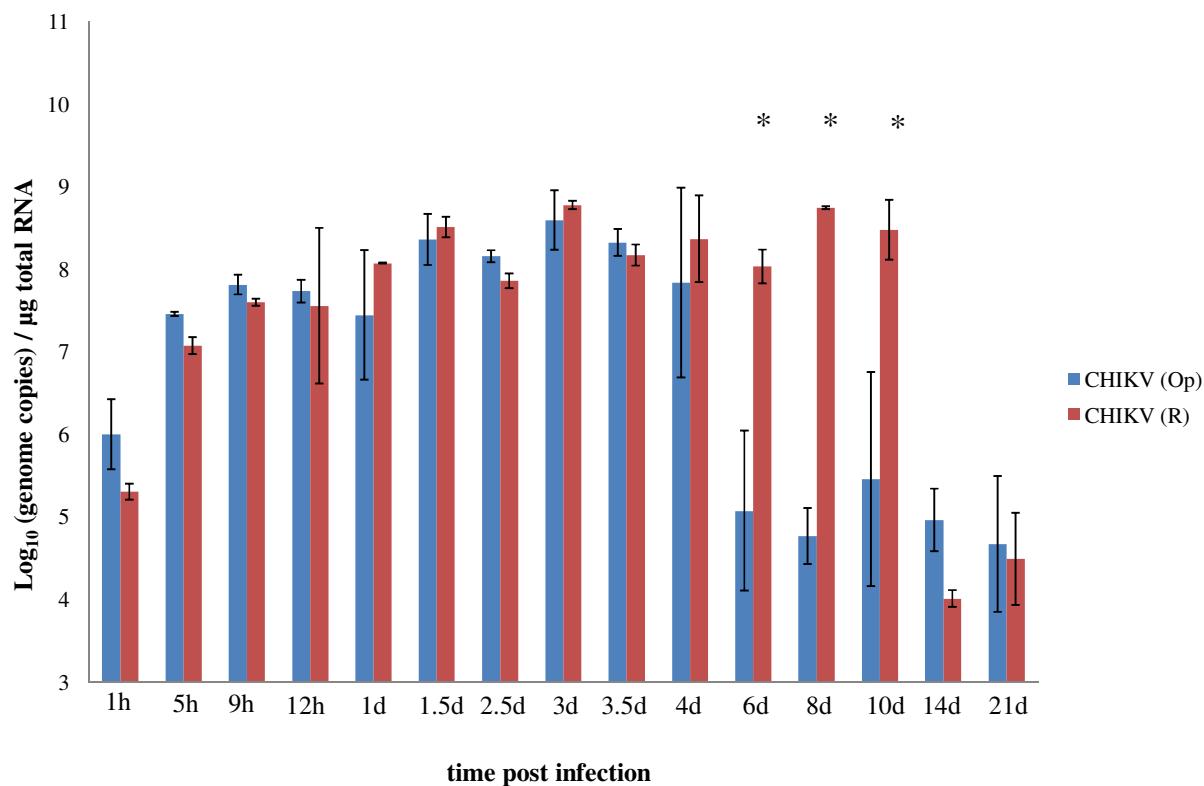


Figure 4.6. Minus-strand RNA quantities for CHIKV-Op and CHIKV-R in Aag2 cells. (-) strand CHIKV ssqPCR TaqMan assay was used for (-) strand RNA detection. Asterisks denote significant difference between CHIKV genotypes (ANOVA, $P < 0.01$, $P < 0.01$, and $P < 0.05$ at $t = 6, 8$, and 10 d.p.i., respectively).

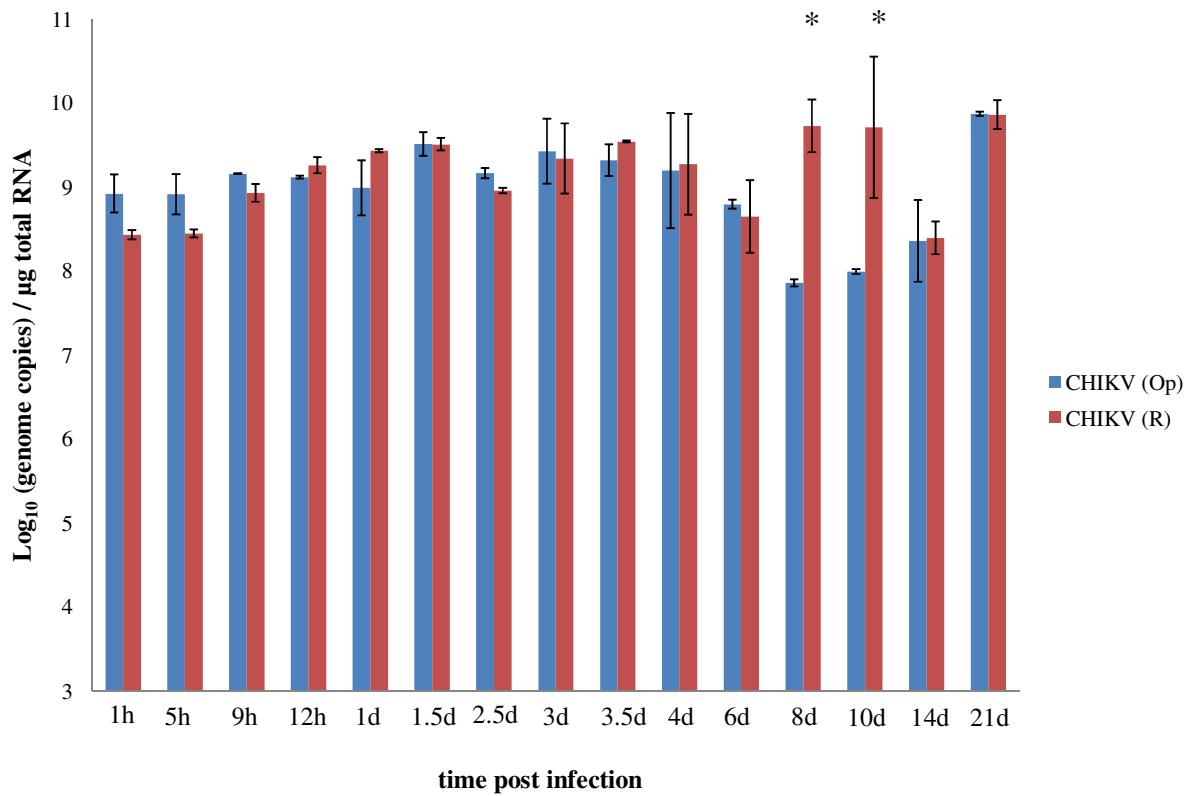


Figure 4.7. Plus-strand RNA quantities for CHIKV-Op and CHIKV-R in Aag2 cells. (+) strand CHIKV ssqPCR TaqMan assay was used for (+) strand RNA detection. Asterisks denote significant difference between CHIKV genotypes (ANOVA, $P < 0.01$ and $P < 0.05$ at $t = 8$ and 10 d.p.i., respectively).

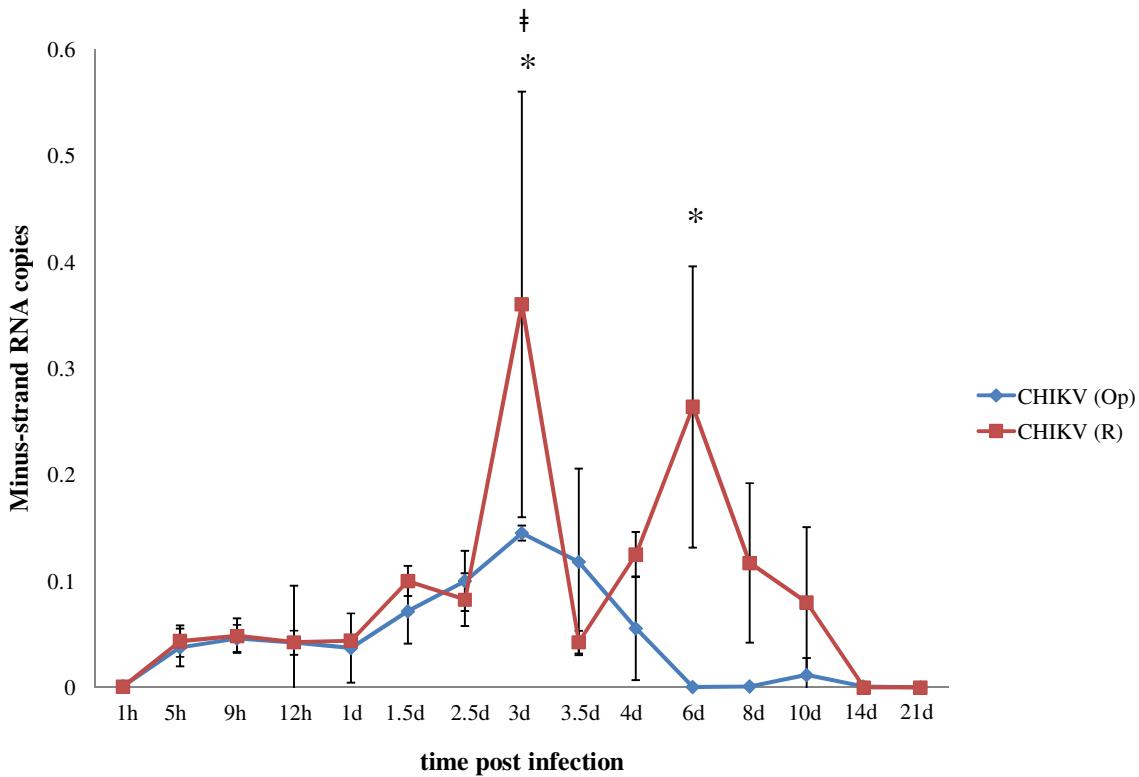


Figure 4.8. Minus-strand RNA copies per one plus-strand RNA copy in CHIKV-infected Aag2 cells. Asterisks denote significant difference between CHIKV genotypes (ANOVA, $P < 0.01$ and $P < 0.001$ at $t = 3$ and 6 d.p.i., respectively). Cross symbol denotes the time point at which ONNV-R (-) strand RNA copies are significantly higher than the quantities determined for ONNV-R at time points immediately preceding and following it (ANOVA, $P < 0.01$ between 2.5 h.p.i. and 1 d.p.i. and between 1 and 1.5 d.p.i.).

DISCUSSION

Previous studies have reported the presence of (-) strand RNA in host cells at specific time points post infection (Kim et al., 2004; Shirako and Strauss, 1994). Wild type SINV (-) strand RNA has been detected at 24 hours post infection (h.p.i.) in vertebrate cells using reverse transcription-PCR (Shirako and Strauss, 1994). Until now, that study reports the latest time point post infection at which alphavirus (-) strand RNA has been detected. Previous research also reports that alphavirus (-) strand RNA synthesis shuts off after only several hours post infection (Sawicki et al., 2006; Sawicki and Sawicki, 1980; Sawicki et al., 1981). Here, using novel ssqPCR assays, we report the detection of ONNV and CHIKV (-) strand RNA in mosquito cells 21 days post infection (d.p.i.). This study reports the first detection of alphavirus (-) strand RNA at this time point in any cell type, and this marks the first use of quantitative real-time PCR for determining strand-specific RNA kinetics during a persistent alphavirus infection.

The detection of (-) strand RNA at 21 d.p.i. suggests that (-) strand RNA plays a role in the establishment of a prolonged persistent infection in invertebrate cells. Because it is currently unclear how the establishment of a persistent infection may occur in the absence of (-) strand RNA synthesis, this finding is meaningful. However, future experiments will be required to further elucidate the molecular mechanisms involved in establishing a persistent infection. Determining the strand-specific replication kinetics of alphaviruses in live mosquitoes will facilitate these studies. The data presented here will serve as a foundation for such studies.

Multiple studies have previously suggested that the opal termination codon at the nsP3-nsP4 locus influences the rapid conversion of (-) strand RNA replicase complexes to (+) strand RNA replicase complexes (Kim et al., 2004; Li and Rice, 1989; Shirako and Strauss, 1994; Strauss and Strauss, 1994). It has also been recently observed that the opal codon confers a fitness advantage to the virus in *Anopheles gambiae* mosquitoes (Myles et al., 2006). Taken together, these previous studies influenced our hypothesis that alphaviruses containing the opal stop codon at the nsP3-nsP4 junction produce less (-) strand RNA in invertebrate cells infected with alphaviruses containing a sense codon at the same locus.

We detected significantly different amounts of (-) strand RNA for the opal and arginine genotypes of the alphaviruses ONNV and CHIKV at multiple time points post infection, indicating that these genotypes have different replicative properties in mosquito cells. At 1 and 10 d.p.i., the (-) strand RNA quantity for ONNV-Op was significantly lower than that of ONNV-

R in C6/36 cells (Fig. 4.3). We also found that the quantity of (-) strand RNA for CHIKV-Op was lower than that of CHIKV-R at 6, 8, and 10 d.p.i. in Aag2 cells (Fig. 4.6). Additionally, the quantities of (-) strand RNA per one (+) strand RNA copy differ significantly between opal and arginine genotypes of ONNV and CHIKV at multiple times points post infection in mosquito cells (Fig. 4.5, 4.8).

The majority of alphavirus replication studies to date have been performed in vertebrate cell lines, such as chicken embryo fibroblasts, BHK-21 cells, vero cells, and mouse embryo fibroblasts (Fata, Sawicki, and Sawicki, 2002; Kim et al., 2004; Sawicki et al., 2006; Sawicki and Sawicki, 1993; Sawicki et al., 2003; Shirako and Strauss, 1994; Wang, Sawicki, and Sawicki, 1994). While the alphavirus transmission cycle does involve both vertebrates and invertebrates, it can be argued that it is most critical to understand the regulation of (-) strand RNA in the invertebrate host because this is the organism in which a persistent infection occurs. In this study, we conducted ONNV infections in vertebrate cell lines from baby hamster kidneys (BHK-21 cells) and invertebrate cell lines derived from *Aedes albopictus* mosquitoes (C6/36 cells). We also conducted CHIKV infections in Aag2 cells, an *Aedes aegypti* cell line, which is a natural vector of CHIKV (Jupp, 1988). While no significant differences between alphavirus genotypes were determined in BHK-21 cells, we did detect less (-) strand RNA for viruses with the opal genotype than those with the arginine genotype in invertebrate cells. While we expected to see these results, we hypothesized that these differences would be exhibited at much earlier time points than were actually observed. Determination of alphavirus (-) strand RNA quantities in infected whole mosquitoes will supplement the cell culture experiments reported here, as will investigations examining (-) strand RNA regulation in response to invertebrate host immunity.

Chapter 5

SUMMARY

Strand-specific RNA replication of alphaviruses has been previously studied. The current model of alphavirus replication postulates that during an early phase in infection, a transient RNA replicase complex is formed that facilitates early (-) strand RNA synthesis. Later, it is thought that this complex is proteolytically processed to create the (+) strand RNA replicase complex during a late phase in infection (de Groot et al., 1990; Hardy and Strauss, 1989). It has been previously reported that (-) strand RNA synthesis terminates at this time (Sawicki et al., 2006; Sawicki and Sawicki, 1980; Sawicki et al., 1981). It has been hypothesized that an opal termination codon preceding the nsP4 gene in the alphavirus genome accelerates this shift from (-) strand RNA synthesis to (+) strand RNA synthesis more quickly than the presence of a sense codon at this same locus (Kim et al., 2004; Li and Rice, 1989; Shirako and Strauss, 1994). Although this model is widely accepted today, the majority of the research that influenced these hypotheses has been conducted in vertebrate cells. This inspired us to examine the strand-specific replication of alphaviruses in mosquito cells using new tools such as strand-specific real-time PCR assays. Using these assays we determined the kinetics of alphavirus (-) strand RNA synthesis for different alphavirus genotypes. The current study also reports the development of an ONNV replicon vector and several defective helper RNAs (DHRNA). We based our ONNV replicon and DHRNAs on the previously described SFV replicon expression system (Liljestrom and Garoff, 1991). The replicon we engineered, ONNRepSP6+GFP, was able to heterologously express a marker gene (GFP) in cultured cells. However, the three DHRNAs we constructed were unable to efficiently package the replicon.

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