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EVALUATION OF ARTHROPOD-BORNE VIRUS RECOMBINATION POTENTIAL AND PHENOTYPE: *IN VITRO* AND *IN VIVO* ANALYSIS OF FLAVIVIRUS AND ALPHAVIRUS MODEL SYSTEMS

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by
Charles Edwin McGee

Dissertation
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To my parents, who supported and encouraged me

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EVALUATION OF ARTHROPOD-BORNE VIRUS RECOMBINATION POTENTIAL AND PHENOTYPE: IN VITRO AND IN VIVO ANALYSIS OF FLAVIVIRUS AND ALPHAVIRUS MODEL SYSTEMS

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Recombination is a mechanism whereby positive sense single stranded RNA viruses exchange segments of genetic information. Recent phylogenetic analyses of naturally occurring recombinant flaviviruses have raised concerns regarding the potential for the emergence of virulent recombinants either post-vaccination or following co-infection with two distinct wild-type viruses. In order to characterize the conditions and sequences that favor RNA arthropod-borne virus recombination I designed, constructed, and evaluated various alphavirus (chikungunya virus (CHIKV) and Sindbis virus (SINV)) and flavivirus (yellow fever virus (YFV) 17D) recombinant crosses. Standard molecular cloning methodologies were used to generate CHIKV, SINV, and YFV 17D replicon/defective helper systems possessing *trans*-complementary sequences necessary and sufficient for the generation of full length viable recombinant viruses following transfection, replication, and purification in cell culture.

Alphavirus recombinant crosses were utilized to both establish standardized conditions for the reproducible generation and cell culture purification of full length recombinant viruses, and to elucidate general characteristics about the nature of RNA arbovirus recombination. The efficiency of CHIKV recombination was related to the functional constraints on the sequence participating in the event with inter-genic recombination being ~100 fold more efficient (minimum co-infection: $\sim10^3$) than intra-genic (minimum co-infection: $\sim10^5$). Contrary to previous reports full length SINV recombinants were readily and reproducibly purified in cell culture to a minimum co-infection of $\sim10^4$. Furthermore, heterotypic recombination was observed to reproducibly result in the generation of viable chimeric CHIKV/SINV and SINV/CHIKV genomes capable of replication, transcription, translation, assembly, and release.

Flavivirus recombination was evaluated via co-transfection of YFV 17D replicon genomes, containing complementary in-frame deletions of the envelope protein coding sequence. However, full length recombinant YFV 17D virus was not detected under any of the experimental conditions examined, despite achieving estimated YFV replicon co-infection levels of $\sim2.4\text{x}10^6$ in BHK-21 (vertebrate) cells and $\sim1.05\text{x}10^5$ in $C_7$10 (arthropod) cells. Additionally, to address concerns that a flavivirus vaccine/wild-type

recombinant virus might have a high mosquito infectivity and/or vertebrate virulent phenotype, the YFV backbone vaccine sequences of YFV 17D and ChimeriVax Dengue 4 were wholly replaced with the corresponding YFV wild-type sequences. The phenotype of the resulting recombinant chimeras were then evaluated in *Aedes aegypti* mosquitoes and *Cynomolgus macaques*. Although these chimeras were observed to efficiently replicate *in vitro* they were significantly attenuated with respect to mosquito infectivity and pathogenicity in non-human primates. Therefore it is concluded that even in the unlikely event of recombination between wild-type and vaccine flaviviruses virulent vaccine associated transmission cycles would not be established.

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LIST OF ABBREVIATIONS

αMEM	alpha- minimum essential medium
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
aa	amino acid
ADE	antibody dependent enhancement
ACL	arthropod containment level
Ae.	*Aedes*
ALT	alanine aminotransferase
AP	alkaline phosphatase
APTT	activate partial thromboplastin time
arbovirus	arthropod-borne virus
AST	aspartate aminotransferase
ATP	adenosine triphosphate
BGS	bovine growth serum
BHK-21	baby hamster kidney cell line
BL	basal lamina
C	capsid protein
ºC	degrees Celsius
C6/36	*Aedes albopictus* cell line
Ca^{+}	calcium
Ca:Phos	calcium phosphate ratio
C/G	Cherry and GFP
$C_7$10	*Aedes albopicuts* cell line
cDNA	complementary DNA
CHIKV	chikungunya virus
CIP	calf intestinal phosphatase
CL	colorless
cm	centimeters
CO	Cherry only
cp	cytopathic
CPE	cytopathic effect
Cs.	*Culesita*
CsCl	cesium chloride
CTP	cytidine triphosphate
Cx.	*Culex*
DAPI	4',6-diamidino-2-phenylindole
DdDp	DNA dependent DNA polymerase
DDN	degeneration and/or necrosis
DENV	dengue virus
DF	dengue fever
DHF/DSS	dengue hemorrhagic fever/dengue shock syndrome
DI	defective interfering
DMEM	Dulbecco's minimum essential medium

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
dpi	days post-infection
E	envelope glycoprotein
E. coli (MC1061)	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EEEV	eastern equine encephalitis virus
FBS	fetal bovine serum
FL	full length
FMDV	foot-and-mouth disease virus
FMDV 2A	foot-and-mouth disease virus protease
Fwd	forward
g	gravity
GFP	enhanced green fluorescent protein
GO	GFP only
GOI	gene of interest
GTP	guanosine triphosphate
gua^D	guanidine dependence
h	hour
H(#)	denotes helper genome followed by numerical identifier
HCl	hydrochloric acid
HCV	Hepatitis C virus
Help	helper
I	insert
i.c.(s)	infectious clone(s)
IFA	indirect immunofluorescence
IHC	immunohistochemistry
IL	interleukin
INF	interferon
IRES	internal ribosomal entry site
IT	intra-thoracic
JEV	Japanese encephalitis virus
KAc	potassium acetate
Kb	kilo-bases
L	liter
L-glu	L-glutmaine
LAC	LaCrosse virus
LAV	live attenuated vaccine
LiCl	lithium chloride
LMP	low melting point
LR	La Reunion
m	minutes
M	molar

MCI	mononuclear cell infiltrates
MEB	midgut escape barrier
MEM	minimum essential medium
mg	milligram
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
MEB	midgut escape barrier
MIB	midgut infection barrier
mL	milliliter
mm	millimeter
mM	millimolar
moi	multiplicity of infection
mRNA	messenger RNA
n	number of individuals examine
NaCl	sodium chloride
NaOH	sodium hydroxide
ncp	non-cytopathic
NEAA	non-essential amino acids
nfw	nuclease free water
ng	nanogram
NIH	National Institutes of Health
nm	nanometer
NS	nonstructural
nsP	alphavirus nonstructural protein
nt	nucleotide
o/n	over night
ONNV	O'nyong nyong virus
ORF	open reading frame
P1	cell culture passage #1
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pen-strep	penicillin streptomycin
pM	picomolar
PNK	polynucleotide kinase
poly-A	poly-adenosine tail
pr	primer
prM	premembrane
prep	preparation
PT	prothrombin time
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
R(#)	denotes replicon genome followed by numerical identifier
Rep	replicon
Rev	reverse
RNA	ribonucleic acid
RdRp	RNA dependent RNA polymerase

rpm	revolutions per minute
RRV	Ross River virus
RT	reverse transcription
rt	room temperature
RVFV	Rift Valley fever virus
p(#)	denotes plasmid followed by numerical identifier
PBS	phosphate buffered saline
SARS-coV	severe acute respiratory syndrome coronavirus
s	seconds
SGP	subgenomic promoter
SFV	Semliki Forest virus
SINV	Sindbis virus
SLEV	St. Louis encephalitis virus
spp.	species
SR5	SinRep5
ss	single stranded
ST	structural
Strep-AP	streptavidin alkaline phosphatase
TAE	Tris-acetic acid-EDTA buffer
TBEV	tick-borne encephalitis virus
$TCID_{50}$	tissue culture infectious dose 50%
TE	Tris-HCl-EDTA buffer
TEM	transmission electron microscopy
temp	temperature
TNF	tumor necrosis factor
TPB	tryptose phosphate broth
ts	temperature sensitive
TTP	thymidine triphosphate
U	units
UTMB	University of Texas Medical Branch
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	vector
VAPP	vaccine associated paralytic poliomyelitis
Vero	African green monkey kidney cell line
VLP	virus like particle
VPg	picornavirus virion protein genome linked
VEEV	Venezuelan equine encephalitis virus
WEEV	western equine encephalitis virus
WHO	World Health Organization
WNV	West Nile virus
wt	wild-type
YF	yellow fever
YFV	yellow fever virus

+	positive sense
-	negative sense
µg	microgram
µL	microliter
ΔE	envelope deletion
ΔFS	frame shift deletion

CHAPTER 1: INTRODUCTION

1.1 SINGLE STRANDED POSITIVE SENSE RNA VIRUS RECOMBINATION

A. Overview of single stranded positive sense RNA viruses

Positive-sense (+) single-stranded (ss) RNA viruses represent a large and diverse virus group with representatives capable of infecting humans, animals, plants, and bacteria. Within this group extreme variation in genome size can be noted with *Picornaviruses* (such as poliovirus) possessing genomes of only ~7.5 Kb whilst *Coronavirus* (such as SARS co-V) genomes can be as large as 30 Kb; one of the largest stable RNA genomes known to exist (Lai 2000, Strauss & Strauss 2002). Nevertheless, all +ssRNA viruses have the distinction of possessing genomes that are competent to function as messenger (m)RNA and as such are considered "infectious" upon introduction into a host cell. Based upon this shared coding polarity, coupled with: similarities in virion organization, conserved protein structural motifs, polymerase core sequence motifs, and nucleotide and amino acid sequence similarities, it was suggested that these diverse viruses may have arisen from a single common ancestor (Koonin & Dolja 1993, Strauss & Strauss 1988).

All +ssRNA viruses, with the exception of retroviruses whose life cycle includes genome reverse-transcription (which will not be further discussed), perform the processes of translation, replication, assembly, maturation, and release without a DNA intermediate. To achieve this life-cycle, these viruses have evolved two distinct genome organizations and can be further sub-categorized as: 1) those that produce only full-length RNA, such as members of the families Flaviviridae and Picornaviridae; and 2) those that produce both full length and subgenomic RNAs, such as the members of the family

Togaviridae (Ball 2001)(**Figure 1.1**). This dichotomy in coding strategies results in subtle but significant differences in the viral life cycle. Upon release into the cellular cytoplasm, the genomes of these viruses are recognized by host ribosomes which begin the process of viral protein translation. For non-subgenomic +ssRNA viruses (e.g. flaviviruses) the entire protein complement, including replicative and structural proteins, is expressed as a single polyprotein from a single open reading frame (ORF). Following post-translational processing of these proteins by host and viral proteases, the viral genome is replicated, encapsidated, the virion matures and is released (**Figure 1.2A**). In contrast, subgenomic +ssRNA viruses (e.g. alphaviruses) possess multiple distinct ORFs and following cellular entry only the 5' portion of the genome which codes for the

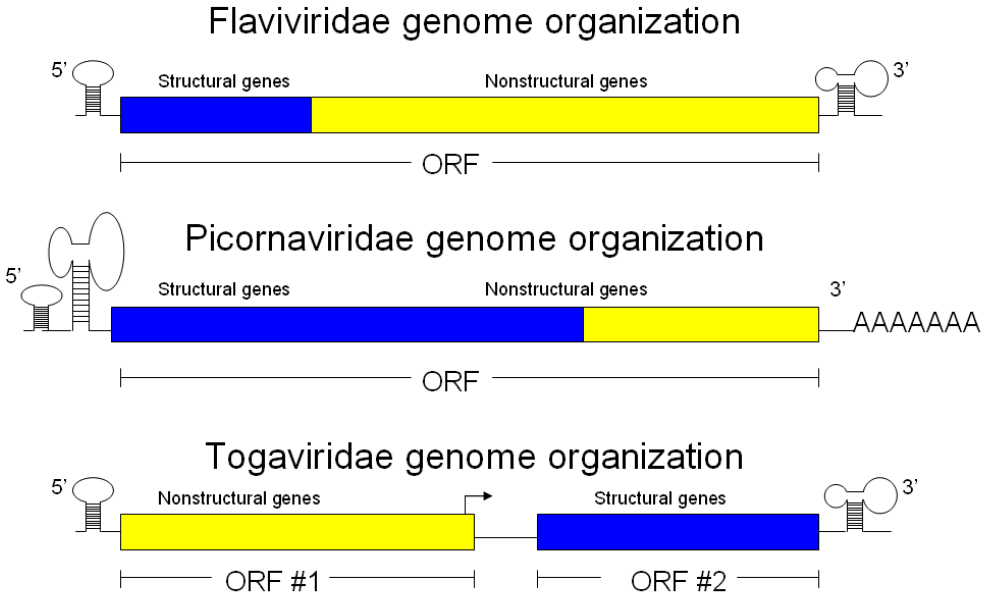


FIGURE 1.1 SCHEMATIC REPRESENTATION OF POSITIVE SINGLE STRANDED RNA VIRAL GENOME TOPOLOGIES.
ORF-open reading frame
hairpin structures denote untranslated regions, nonstructural gene coding sequence is depicted in yellow, structural gene coding sequence is depicted in blue.

replicative machinery is initially expressed. Once assembled, this replicative complex, based on conformation, is responsible for both genome replication and expression of subgenomic RNAs. These subgenomic RNA(s) are subsequently translated to form the viral structural proteins (**Figure 1.2B**). Following expression of proteins from these subgenomic transcripts, the viral genomes and structural proteins then come together and form mature virus progeny.

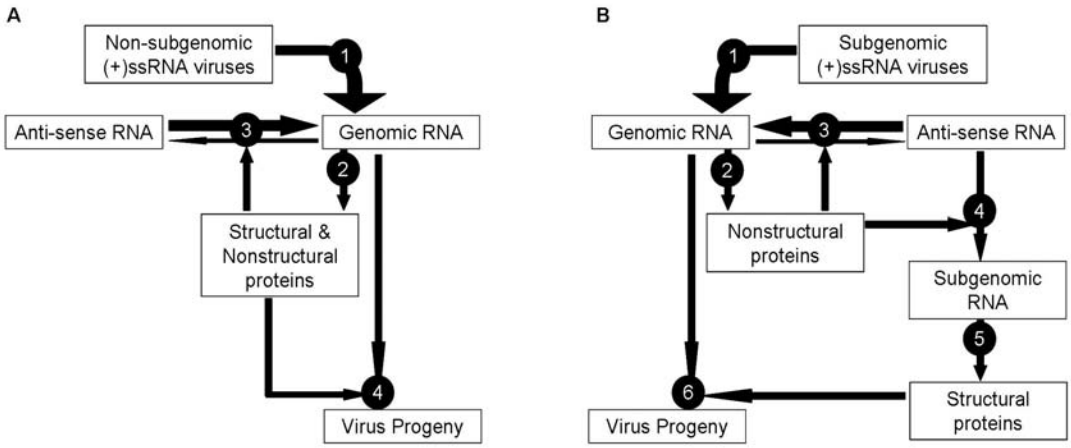


FIGURE 1.2 GENERAL REPLICATION SCHEMES OF NON-SUBGENOMIC AND SUBGENOMIC POSITIVE SENSE RNA VIRUSES.
A, Non-subgenomic +ss RNA virus life-cycle. (*Step 1*) Receptor binding, entry, and genome uncoating. (*Step 2*) Translation and post-translational processing of viral polyprotein. (*Step 3*) Asymetic synthesis of negative-sense and positive-sense (genomic) RNAs. (*Step 4*) Assembly, maturation, and release of virus progeny. B, Subgenomic +ss RNA virus life-cycle. (*Step 1*) Receptor binding, entry, and genome uncoating. (*Step 2*) Translation and post-translational processing of viral non-structural polyprotein. (*Step 3*) Asymetic synthesis of negative-sense and positive-sense (genomic) RNAs. (*Step 4*) Synthesis of subgenomic RNA(s). (*Step 5*) Translation and post-translational processing of viral structural proteins. (*Step 6*) Assembly, maturation, and release of virus progeny. (Adapted from Ball 2001).

B. Evolutionary mechanisms of single stranded positive-sense RNA viruses

Evolution of non-segmented +ssRNA viruses occurs via two mechanisms: 1) clonal accumulation of selectively advantageous point mutations via replicative error, and/or 2) recombination between related but disparate genomes replicating in the same

cell. A model of viral evolution, known as "modular evolution," has been proposed which unifies these two mechanisms of RNA virus evolution. In this model, individual units of genetic information that code for functionally/structurally important motifs evolve, presumably via accumulation of point-mutations under specific selective pressures, and then are re-assorted or recombined to form optimized viral genomes (Botstein 1980).

RNA dependent RNA polymerase

Regardless of the mechanism (mutation or recombination), RNA virus evolution is a directly dependent on the how the functions of the viral RNA dependent RNA polymerase (RdRp) seed genetic diversity into a population thus allowing for subsequent selection. Analyses of structural conformations of various RdRp alone and/or when complexed with template RNAs have provided significant insights into polymerase functions including: template binding and initiation of RNA synthesis (see below) (Butcher *et al.,* 2001, Kao *et al.,* 2001, Paul *et al.,* 1998, Ranjith-Kumar *et al.,* 2002, van Dijk *et al.,* 2004). The crystal structure for the RdRp for representative members of a number of virus families has been solved: Picornviridae; poliovirus, foot-and-mouth disease virus, coxackie virus, and human rhinovirus (Ferrer-Orta *et al.,* 2006, Gruez *et al.,* 2008, Hansen *et al.,* 1997, Love *et al.,* 2004, Paul *et al.,* 1998); Flaviviridae; Hepatitis C virus, dengue 3 virus, and West Nile virus (Bressanelli *et al.,* 1999, Malet *et al.,* 2007, Yap *et al.,* 2007); Calciviridae; Norwalk virus and rabbit hemorrhagic disease virus (Ng *et al.,* 2002, Ng *et al.,* 2004); and the double stranded RNA bacteriophage Φ6 (Butcher *et al.,* 2001). Comparative analysis of these polymerases has identified a number of conserved structural motifs across all RdRp, and indeed across all nucleic acid polymerases (Steitz *et al.,* 1994). The overall topology of the RdRp has been compared to a cupped or closed human right-hand, with thumb, palm, and finger domains (**Figure**

1.3). This "closed" confirmation is achieved via interactions occurring between the finger and thumb domains which create a tunnel to facilitate docking of the RNA template. A second channel, comprised of positively charged residues, facilitates recruitment of the dNTP building blocks into the catalytic site (van Dijk *et al.,* 2004). Within the palm domain exists the catalytic core comprised of two conserved aspartic acid residue-associated magnesium ions which facilitate the addition of the dNTPs to the growing nascent chain (Steitz & Steitz 1993, van Dijk *et al.,* 2004). It is thought that this structural conformation facilitates the best arrangement of the RNA template, dNTPs, and primer (if necessary) with the catalytic site to facilitate initiation and elongation of the nascent RNA strand (Ferrer-Orta *et al.,* 2006, Ferrer-Orta *et al.,* 2006).

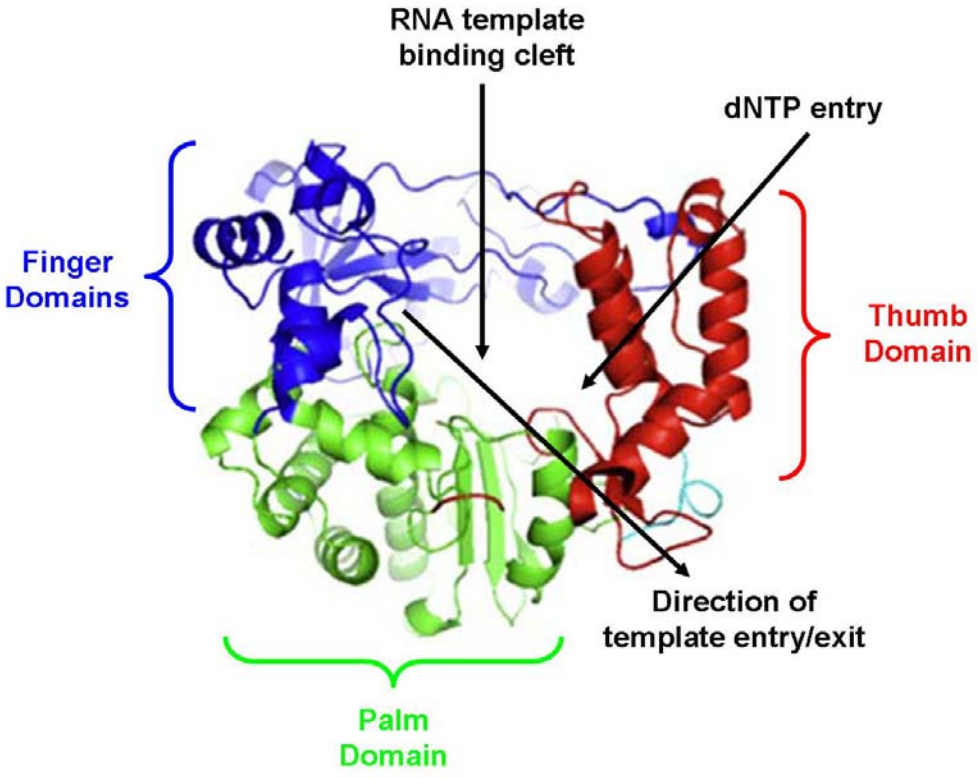


FIGURE 1.3 STRUCTURE OF THE POLIOVIRUS RNA DEPENDENT RNA POLYMERASE. Finger, palm, and thumb domains are depicted in blue, green, and red respectively (adapted from Lescar & Canard 2009).

Mutation

The primary mode of +ssRNA virus evolution is via clonal accumulation of point mutations because of replicative error. A relatively high error rate arises due to the lack of proof-reading capability inherent in all viral RdRp. The rate of mutation associated with a number of +ssRNA viruses has been estimated using direct and indirect methods to average approximately 10^{-4} (range: 10^{-3} to 10^{-6}) substitutions per nucleotide per round of replication (deFilippis & Villarreal 2001, Drake *et al.,* 1998, Smith & Inglis 1987, Steinhauer & Holland 1987, Strauss & Strauss 1988). This high mutation rate gives rise to a virus progeny population, or quasi-species, comprised of genomes which are similar but not identical to the founding parental virion (Domingo *et al.,* 1996). It is this error driven quasi-species that allows these viruses to rapidly adapt to changing environmental conditions and in turn limits genome size to a maximum of what can be synthesized with viable fidelity. However, the observed mutation frequencies for some arthropod-borne (arbo) +ssRNA viruses, such as eastern equine encephalitis virus (EEEV; family Togaviridae, genus *Alphavirus*), yellow fever virus, and the dengue viruses (YFV; DENV 1-4; family Flaviviridae, genus *Flavivirus*) have been significantly lower, on the order of 10^{-4} substitutions/site/year (Sall *et al.,* 2010, Weaver *et al.,* 1991). To reconcile these difference in quantified polymerase error rates vs. observed rates of alphavirus and flavivirus evolution, two distinct hypotheses have been proposed: 1) environmental constraints, such as the need to replicate in two phylogenetically distinct hosts (i.e. a vertebrae and an arthropod), as is the case for arboviruses, may significantly decrease the observed mutation rates in nature, and/or 2) the RdRp of flaviviruses and alphaviures may simply be capable of higher fidelity replication (Scott & Weaver 1989, Weaver 2006, Weaver *et al.,* 1993, Weaver *et al.,* 1992). Furthermore, the specific constraints on

individual +ssRNA arbovirus mutation driven evolution may be species specific. Interestingly, while EEEV appears to exhibit polymerase error rates of ~10^{-4} substitutions per nucleotide per round of replication (Weaver *et al.,* 1993), the RdRP of YFV has been shown to replicate with ~1000-fold higher fidelity (~10^{-7} per nucleotide per round of replication) (Pugachev *et al.,* 2004). As such, it appears that the relative stability of alphavirus and flavivirus genomes in nature may result from the combined forces of selection and increased polymerase fidelity.

Recombination

RNA virus recombination is defined here as: *the exchange of genetic material between RNA viruses resulting in the generation of a viable covalently-linked chimeric genome with sequences derived from both parental genomes coincident with an identifiable break point*. Recombination of +ssRNA viruses was first reported in the 1960's coincident with the observation that co-infection of cell culture with picornaviruses with disparate phenotypes resulted in generation of progeny virus containing phenotypes derived from both parental viruses (Hirst 1962, Ledinko 1963, Ledinko & Hirst 1961). Subsequent to these initial observations, recombination has been observed in studies of several other +ssRNA virus groups including coronaviruses, and togaviruses as well as several plant RNA viruses (as reviewed by Agol 2006, Alejska *et al.,* 2001, Koonin & Dolja 1993, Lai 1992, Lukashev 2005, Meyers & Thiel 1996, Noueiry & Ahlquist 2003, Strauss & Strauss 1988, Worobey & Holmes 1999). Picornavirus recombination is arguably the most well-characterized and most significant in terms of human disease. Therefore, the general mechanistic basis of recombination, including site selection, will be discussed here using picornaviruses as the prototypical example with observations from other virus groups mentioned where appropriate.

The single most important requirement for recombination to occur is co-infection of the same cell (McCahon *et al.,* 1977, Tolskaya *et al.,* 1983, Twiddy & Holmes 2003, Worobey *et al.,* 1999), that is to say both parental genomes must be spatially and temporally associated to serve as templates for the generation of a chimeric recombinant progeny genome. Additionally, it has been suggested that high levels of sequence homology/identity favor recombination potential while viruses with very divergent sequence have a decreased likelihood of recombination (McCahon *et al.,* 1977, Tolskaya *et al.,* 1983, Twiddy & Holmes 2003, Worobey *et al.,* 1999). Although these requirements are necessary to allow for recombination to occur, they are not sufficient to generate a viable recombinant genome. A viable recombinant genome can only result from a template switch that occurs in such a way that it gives rise to a functional viral genome, capable of autogenous replication, which ideally can be purified from the parental population (compiled from (Twiddy & Holmes 2003). Furthermore, it is logical to assume that for a recombinant virus to be selected for the resultant genome topology would likely have to exhibit comparable or better fitness than the parental genomes. The ability to detect naturally occurring recombinant genomes is dependent on at least three criteria: 1) the recombinant sequence must be long enough to allow for phylogenetic analysis, 2) the sequence must be divergent enough to allow unambiguous identification of both parental genomes, and 3) the recombinant genotype must be maintained during post-recombination evolution (Tolou *et al.,* 2001). Finally, to confirm the presence of true recombination either naturally occurring or laboratory generated the recombinant virus must be clonally isolated and the sequence verified in a single PCR amplicon following cloning to ensure it occurs as a single covalently linked molecule (Tolou *et al.,* 2001, Weaver & Vasilakis 2009).

Mechanism(s) of RNA virus recombination

Cooper *et al.,* (1974), following systematic analysis of the characteristics of picornavirus recombinants, proposed the following mechanism of RNA virus recombination: "We suggest the possibility that template dissociation *in vivo* could be appreciable. Thus poliovirus recombinants could well arise by reassortment of nascent chains plus replicase between templates, followed by precise realignment and continued replicase action on the new template. This is a form of copy choice (Copper *et al.,* 1974)." This model was selected from several potential mechanisms due to it being consistent with the observed phenotypic characteristic of putative recombinants namely: that recombinant virus retained parental-like genome (non-segmented single strand of approximately 7.5 Kb) and ability to reproducibly generate mutant loci maps. However, nonreplicative RNA recombination of poliovirus (family Picornaviridae, genus *Enterovirus*) has been documented. In this instance trans-esterification reactions and/or cryptic rybozyme activity were implicated as potential mechanisms (Gmyl *et al.,* 1999). Nevertheless, it is widely accepted that the majority of +ssRNA virus recombinants are generated when the RdRp "jumps" from one template to another during negative strand synthesis (**Figure 1.4**) (Jarvis & Kirkegaard 1992, Kirkegaard & Baltimore 1986, Romanova *et al.,* 1986, Tolskaya *et al.,* 1987). It is thought that the RdRp can pause during nascent strand synthesis and that during such an arrest in replication the RdRp-nascent RNA complex can disassociate from the primary template. Several analyses have identified "hot-spots" for recombination located in sequences capable of forming stem-loop structures (Romanova *et al.,* 1986, Tolskaya *et al.,* 1987, Wilson *et al.,* 1988). Inherently this seems logical as it would be expected that areas of strong secondary structure would have to be unwound, to be replicated, and as such would likely cause slowing or pausing of the replicative complex. If this model is true sequence homology

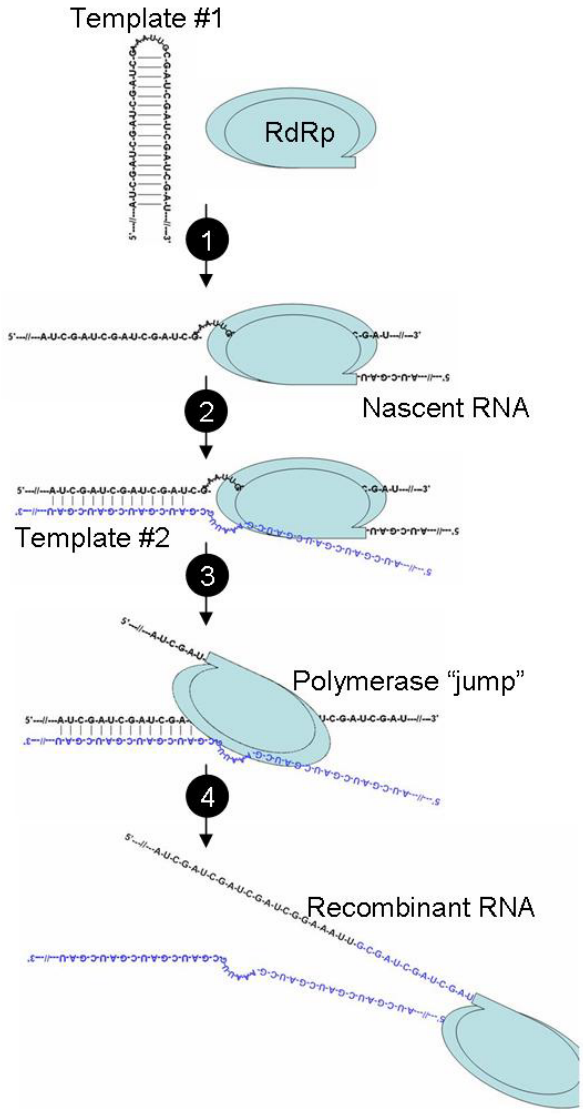


FIGURE 1.4 SCHEMATIC REPRESENTATION OF RNA RECOMBINATION BY A COPY-CHOICE MECHANISM.
(*Step 1*) The RdRp initiates negative strand synthesis on a positive template followed by pausing of the replicative complex due to the need to unwind an RNA stem-loop. (*Step 2*) During this pause in synthesis it a secondary template associates with the primary template-RdRp-nascent strand complex. (*Step 3*) Disassociation of the replicative complex from the primary template and re-association with the secondary template. (*Step 4*) Re-initiation of RNA synthesis on the secondary template and generation of a chimeric recombinant daughter strand.

RdRp-RNA dependent RNA polymerase

of the stem-loop sequence could allow for a second template RNA to align itself based on reverse complementarity (**Figure 1.4 #3**). This type of donor template-acceptor template interaction could facilitate association of the replicase complex with the secondary template. Replication then re-initiates, and a chimeric daughter RNA be generated (Romanova *et al.,* 1986).

Interestingly, sequence analysis of poliovirus inter-typic recombinants indicated that recombination could occur at random positions of very low homology (Kirkegaard & Baltimore 1986). Furthermore, polymerase chain reaction (PCR)-based analysis of coronavirus recombination, under non-selective conditions, provided direct evidence that recombination appears to occur completely randomly with cross-overs uniformly distributed throughout the examined gene sequence (Banner & Lai 1991). However, cell culture passage resulted in rapid loss of this heterogenecity and the clustering of cross-over regions. Given these contradicting reports, Lai (1992) proposed a revised model of RNA virus recombination whereby RdRp pausing may result from encountering stem-loop elements resulting in disassociation. Following primary template release, the RdRp-nascent RNA complex is redirected to a secondary template by hybridization of the nascent RNA and secondary template driven by sequences upstream of the cross-over site. The RdRp may then bind the secondary template and re-initiate at either homologous or non-homologous sites. To explain the apparent lack of stem-loops at cross-over points, it was proposed that processing or cleavage of the 3' end of the nascent RNA could occur during this process by some, as of yet, unidentified factors (Lai 1992).

The random nature of primary template release could also be explained by premature physical displacement of the replicative complex. It has been proposed that the RdRp-nascent RNA complex may be dislodged during negative strand synthesis as a result of colliding with actively translating ribosomes (Barton *et al.,* 1999, Charini *et al.,*

1994). If this were to occur it could happen at any position along the length of the genome. As such, this mechanism of template disassociation could potentially reconcile the contradictory reports of the presence and absence of stem-loop structures at cross-over points, without the need for additional enzymatic activities.

Types of RNA virus recombination

RNA virus recombination events, either occurring within coding sequence (intra-genic) or noncoding sequences (inter-genic), can be classified into three distinct categories: homologous, aberrant homologous, and non-homologous/illegitimate (as proposed by (Lai 1992). Homologous recombination occurs when two parental viral RNAs, with a high degree of sequence identity, align in such a way that template switching of the replicative complex results in the generation of a recombinant RNA with sequence topology that is identical or comparable to that of the parental RNAs. Homologous recombination is characteristic of picornavirus and coronavirus recombination and is believed to be the mechanism responsible for the generation of the recombinant alphavirus western equine encephalitis virus (WEEV) (Hahn *et al.,* 1988). Aberrant homologous recombination occurs between two parental viral RNAs, with high sequence homology, but at sights that are not dictated by that homology. This results in the generation of a recombinant with sequence abnormalities such as deletions, duplications, and insertions. SINV and Venezuelan equine encephalitis virus (VEEV) replicon/defective helper systems have been observed to generate full length viruses, containing sequence abnormalities, via aberrant homologous recombination (Pushko *et al.,* 2000, Vasilakis *et al.,* 2003, Weiss & Schlesinger 1991). Non-homologous or illegitimate recombination occurs when two RNAs with no regions of sequence homology undergo recombination. The classic example of non-homologous

recombination is the incorporation of host mRNA sequences into the bovine viral diarrhea virus genome (Meyers *et al.,* 1989)

C. Recombination of picornaviruses

Picornaviruses are perhaps the most well characterized +ssRNA animal viruses with respect to recombination. The picornaviruses (family Picornaviridae), which include such significant human and veterinary pathogens as poliovirus and foot-and-mouth disease virus (FMDV), are a group of non-enveloped, +ssRNA viruses. The picornavirus virion is ~30 nm and is comprised of the RNA genome (~7.5-8 Kb) surround by the protein coat. The picornavirus genome (**Figure 1.1**) encodes a single ORF flanked by long 5' and 3' untranslated regions (UTRs) that are in-turn flanked by a 5' covalently linked protein (VPg) and a 3' poly-adenosine tail. One unique characteristic of the picornavirus genome is the long 5' UTR that forms a highly organized secondary structure known as an internal ribosomal entry site (IRES). The specific function of this IRES sequence is to drive cap-independent translation of the viral poly protein following the shut off of host protein synthesis (compiled from Racaniello 2001).

The first studies that suggested +ssRNA viruses could undergo recombination were performed in the 1960's. It was observed by Hirst (1961) and Ledinko (1962) that co-infection of cell cultures with picornaviruses with disparate characteristics (sero-specificity, temperature sensitivity, guanidine sensitivity, etc.) resulted in the generation of progeny virus containing phenotypes derived from both parental viruses (Hirst 1962, Ledinko 1963, Ledinko & Hirst 1961). The general strategy for generation and detection of viable picornavirus recombinants in these studies is depicted in **Figure 1.5**. Briefly, these experiments employed the use of viruses with distinct phenotypic properties, one having two sets of mutations conveying specific growth characteristics (examples indicated above) and the other being wild-type (wt). Cell culture monolayers were

inoculated with a mixture of these two viruses (in various ratios) at high multiplicity of infection (moi) to achieve a high level co-infection. These infections were then permitted to proceed without selective pressure. Specific selective pressure was not employed during the first passage in cell culture because the efficiency of mutation driven adaptation may be higher than the potential for recombination. Virus progeny was then harvested from these mixed infection supernatants and re-passaged in cells under dual selective pressure. As such, only those recombinant progeny possessing both traits required for replication should be detected. However, the definitive evidence of picornavirus recombination required molecular and biochemical analysis to verify that putative recombinant picornaviruses had indeed inherited genetic sequences and protein products from both distinct parental viruses (King *et al.,* 1982, Romanova *et al.,* 1980).

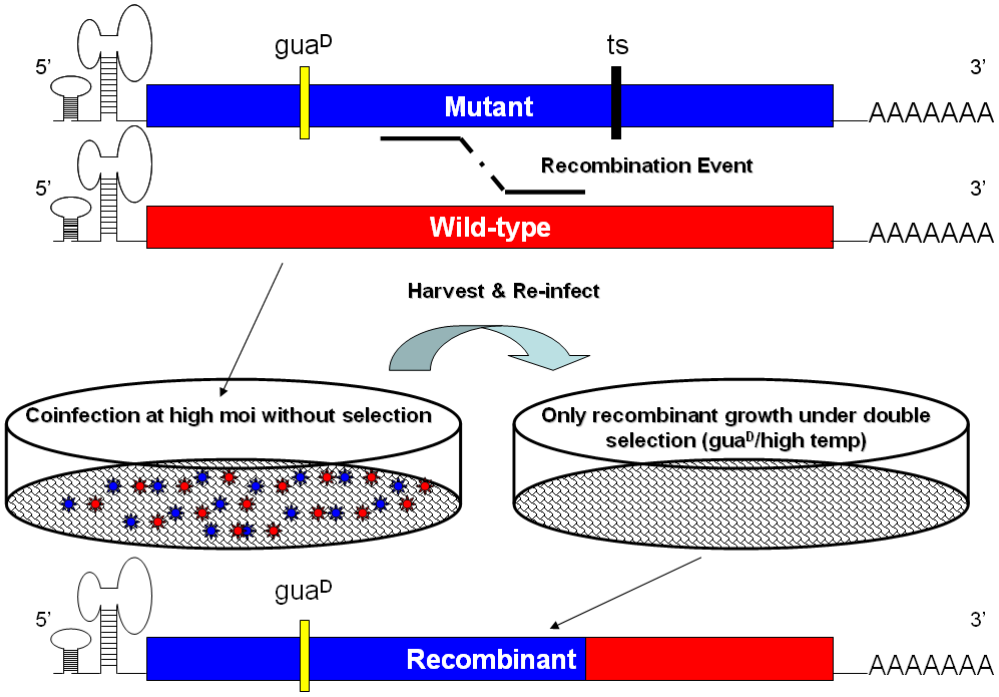


FIGURE 1.5 EXPERIMENTAL DESIGN FOR THE DETECTION OF PICORNAVIRUS RECOMBINANTS.
gua^D-guanidine dependence loci, ts-temperature sensitivity loci, moi-multiplicity of infection, temp-temperature.

The efficiency of recombination in studies of this type was calculated as: recombination frequency = (yield of recombinant virus under selective growth/sum of yield of parental viruses under non-selective growth) (Copper *et al.,* 1974, Hirst 1962, Jarvis & Kirkegaard 1992, Kirkegaard & Baltimore 1986, Ledinko & Hirst 1961). The ability to calculate picornavirus recombination efficiencies, assuming a direct relationship between recombination efficiency and distance between mutant loci, allowed for the generation of genetic maps of ts markers (Copper *et al.,* 1974, Lake *et al.,* 1975, McCahon *et al.,* 1977). Extrapolations, assuming that cross-over sites were uniformly distributed throughout the viral genome, suggested that the genome wide efficiency of picornavirus recombination could be as high as 10-20% (King 1988, Kirkegaard & Baltimore 1986, McCahon *et al.,* 1977). Interestingly, the frequency of picornavirus recombination appears to be related to sequence homology between the two parental viruses since the efficiency of inter-typic recombination was observed to be >100-fold lower than for the corresponding intra-typic cross (Kirkegaard & Baltimore 1986). These observations raise the question: "Is there some type of sequence specificity which dictates recombination efficiency?" In this regard, several contradictory reports exist. It has been suggested by some that site selection is specific and associated with stem-loop structures (Romanova *et al.,* 1986, Tolskaya *et al.,* 1987), however, others have observed a seemingly random selection of cross-overs scattered throughout the genome (King 1988, Kirkegaard & Baltimore 1986). In an attempt to reconcile these data, King (1988) conducted a systematic analysis of all the available recombinant picornavirus sequences, available at that time. These bioinformatic analyses suggested that: 1) picornavirus recombination predominantly occurred during negative strand synthesis, as had been previously suggested by Kirkegaarad and Baltimore (1986), 2) site selection was neither

random nor was it dependent upon specific sequences or motifs, 3) cross-overs occured at sequences that required low free energy to facilitate disassociation and resulted in the generation of viable chimeras, and 4) re-initiation was likely to occur between sequences of reasonably high homology.

Despite its lower efficiency, picornavirus inter-typic recombination has significant implications in terms of human morbidity, specifically with regard to vaccine safety. The oral polio vaccine is administered as a tri-valiant formulation of live attenuated poliovirus vaccine viruses representing all three poliovirus serotypes (Sabin & Boulger 1973). Between 1:50,000 and 1:1,200,000 poliovirus vaccinees experience vaccine-related adverse events known as vaccine associated paralytic poliomyelitis (VAPP; Nkowane *et al.,* 1987). The use of a vaccine formulation comprised of multiple distinct replication competent viruses may facilitate *in vivo* recombination. Indeed, analysis of stool and CNS isolates from patients with VAPP have identified heterotypic vaccine/vaccine and vaccine/non-vaccine recombinants (Furione *et al.,* 1993, Georgescu *et al.,* 1994, Kew *et al.,* 1981). The repeated generation of virulent recombinant polioviruses post-vaccination serves to underscore the importance of recombination as a mechanism of genetic exchange, not only in the laboratory but in nature, which may result in evolution towards human virulence.

D. Recombination of alphaviruses and flaviviruses

The accumulated literature on alphavirus and flavivirus recombination will be extensively reviewed and discussed in Chapters 2 and 3, respectively.

1.2 INTRODUCTION TO ARTHROPOD-BORNE VIRUSES

> "Arthropod-borne (arbo)viruses are viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous (blood-feeding) arthropods; they multiply and produce viremia in the vertebrates, multiply in the tissues of arthropods, and are transmitted to new vertebrates by the bites of arthropods after

a period of extrinsic incubation (World Health Organization definition 1967; Karabatsos 1985)."

These viruses possess the distinct characteristic that transmission is facilitated by an arthropod, following a period of replication and/or development within the tissues of the vector (extrinsic incubation) and not simply via inoculation from contaminated mouthparts (Higgs & Beaty 2005). The vast majority of arboviruses are RNA viruses, in fact African swine fever virus (family Asfarviridae, genus *Asfarvirus*) which has a DNA genome is the only known exception (Calisher & Karabatsos 1988, Karabatsos 1985, Weaver 2006). However, within the world of RNA arboviruses several genetically diverse groups are represented including: +ssRNA viruses (family Togaviridae, genus *Alphavirus* and family Flaviviridae, genus *Flavivirus*), negative sense (-) ssRNA viruses (family Rhabdoviridae, genus *Vesiculovirus*), negative sense segmented RNA viruses (family Orthomyxoviridae, genus *Thogotovirus*), negative/ambi- sense segmented RNA viruses (family Bunyaviridae, genera *Bunyavirus*, *Nairovirus*, and *Phlebovirus*), and double stranded segmented RNA viruses (family Reoviridae, genus *Orbivirus*) (Calisher & Karabatsos 1988, Karabatsos 1985).

The remainder of this section will focus specifically on the interactions between mosquito-borne viruses and their respective vectors. Several distinct and overlapping transmission cycles have been described for mosquito-borne viruses including: sylvatic/endemic maintenance cycles which can result in incidental human and agricultural animal infections, rural/savannah cycles which can be associated with emergence of endemic arboviruses, and urban/epidemic cycles supported by peridomestic mosquito-borne transmission between viremic humans (**Figure 1.6**). Additionally, transovarial transmission whereby viruses are passed vertically from the infected adult female to her progeny has also been described. Transovarial transmission was first

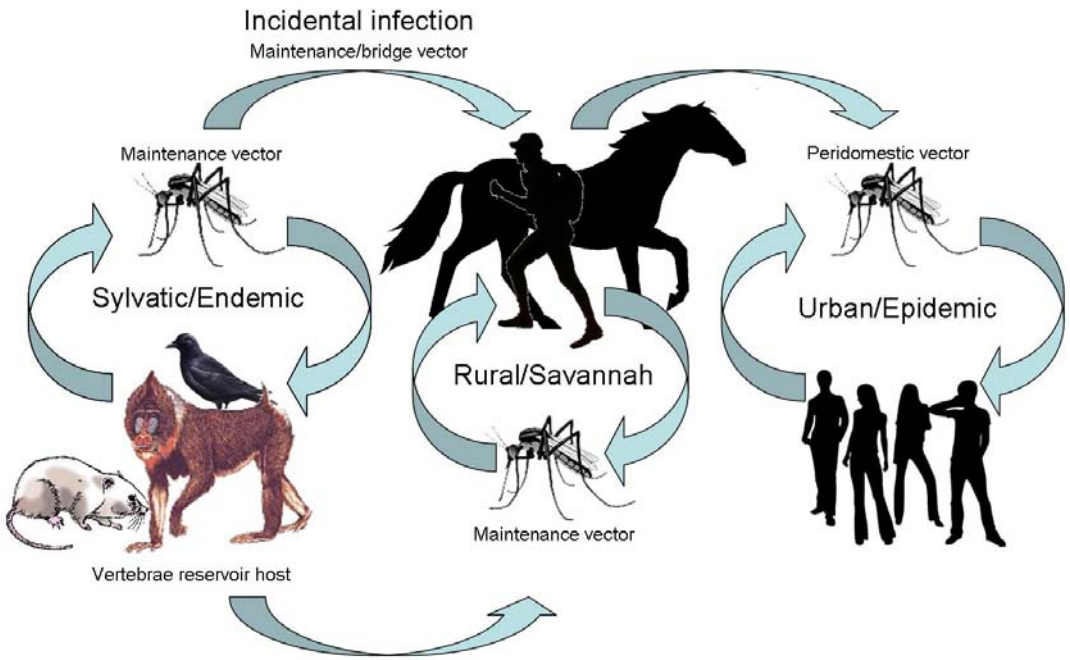


FIGURE 1.6 MOSQUITO-BORNE VIRUS TRANSMISSION CYCLES

described and has been best characterized for LaCrosse virus infection of *Aedes triseriatus* mosquitoes (Watts & Eldridge 1975, Watts *et al.,* 1973, Watts *et al.,* 1975) where infection rates in progeny can reach ≥80%. Transovarial transmission has now been demonstrated for a number of arboviruses to various efficiencies. Successful arbovirus transmission cycles require that viruses infect, replicate in, and escape from midgut epithelial cells into the hemocoel. Subsequently virus must disseminate to and amplify in secondary tissues (e.g. fat, nervous, and/or muscle), culminating in infection of salivary gland tissue. Infectious virions must then be released into the saliva and secreted into the vertebrate host during blood feeding. For basic anatomical organization of the internal anatomy of the mosquito see **Figure 1.7 A**. The ability of arboviruses to infect and escape from the midgut is controlled by a combination of viral factors and

mosquito barriers to infection and dissemination (Horzinek & Mussgay 1971, Mussgay 1964).

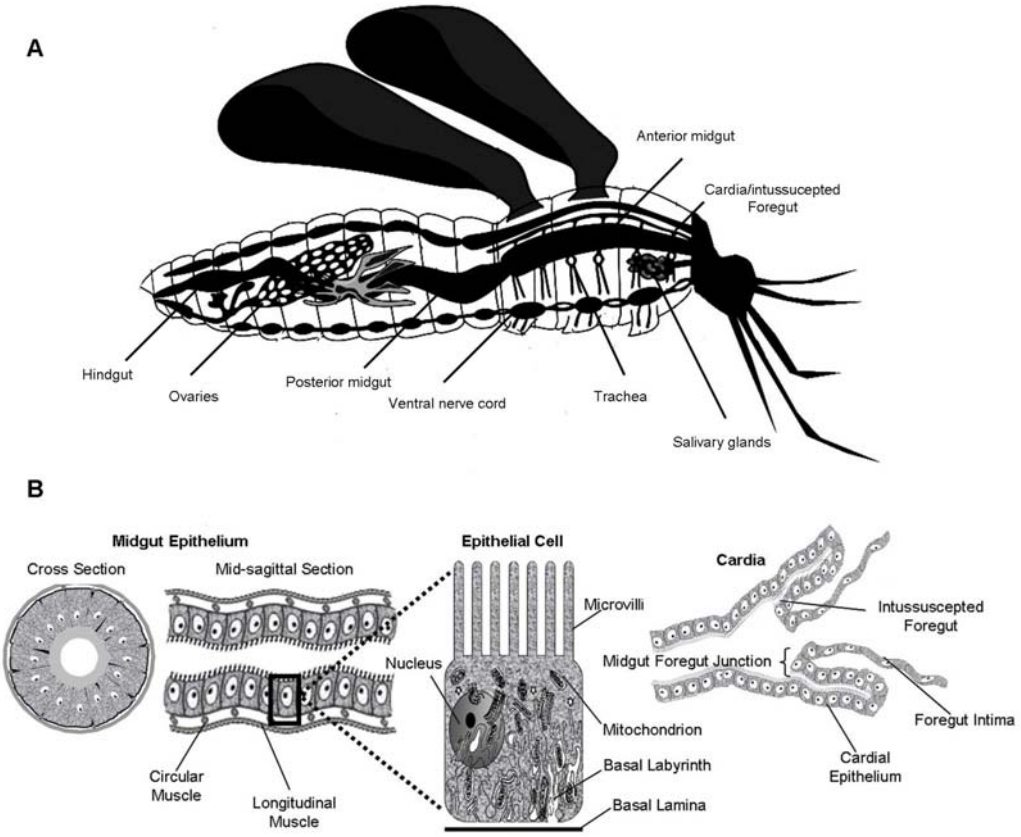


FIGURE 1.7 INTERNAL ANATOMY OF THE MOSQUITO WITH SPECIAL ATTENTION TO MIDGUT MORPHOLOGY.
A, Macroscopic organization of important organ systems. B, Microscopic details of midgut morphology.

An arthropod vector is defined as exhibiting the innate ability to become infected with and successfully potentiate biological transmission of a virulent agent (Higgs & Beaty 2005). For an arthropod species to be competent for transmission of a specific arbovirus it should: exhibit a low titer threshold for midgut infection, support permissive replication/amplification in midgut epithelial cells, allow for dissemination through the midgut basal lamina, support infection and replication in secondary organs, and facilitate productive infection, replication, and release from salivary gland tissue (Higgs & Beaty

2005). The shorter the extrinsic incubation, temporal period required for the successful completion of these events, the more successful the vector. There are a number of intra-arthropod obstacles to vector competence including midgut infection and escape as well as salivary gland infection and escape barriers (**Figure 1.8**). Intra-vector infection, dissemination, and transmission barriers are believed to be the result of intra-vector genetic factors. Therefore, any genetic polymorphisms resulting in the absence of critical vector components for viral replication will present as infection/dissemination barriers and directly reduce or eliminate vector competence (Black & Severson 2005). Additionally, vector competence may be influenced by the stimulation/activation of the arthropod immune response, such as the RNA interference pathway, subsequent to infection by an arthropod-borne disease agent (Adelman *et al.,* 2001, Blair *et al.,* 2000, Campbell *et al.,* 2008, Franz *et al.,* 2006, Olson *et al.,* 2002, Olson *et al.,* 1996, Travanty *et al.,* 2004).

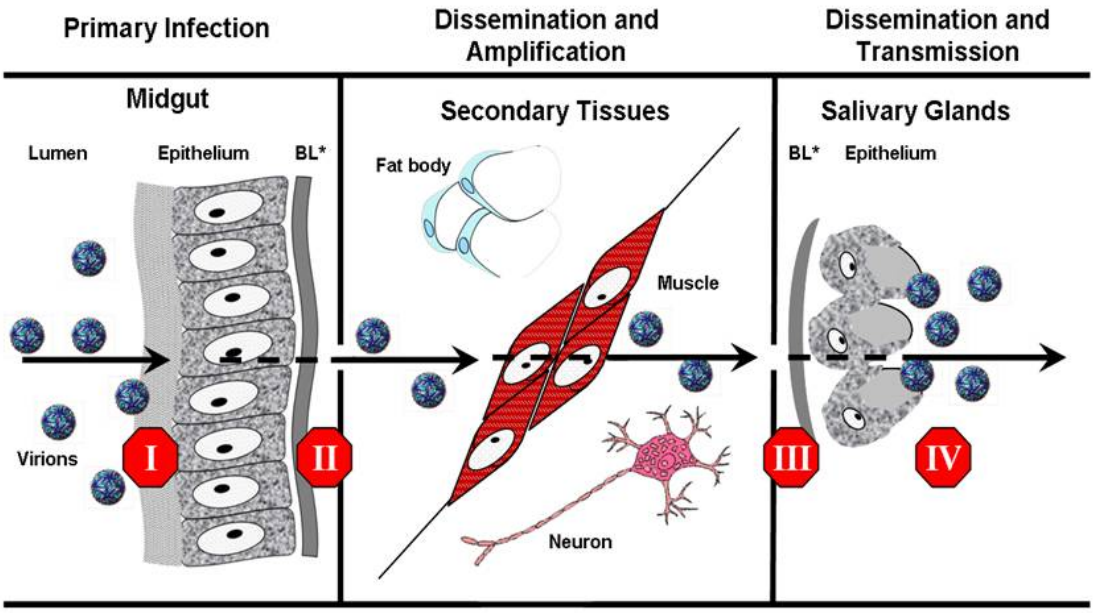


FIGURE 1.8 SCHEMATIC REPRESENTATION OF THE PROGRESSION OF AN ARBOVIRUS THROUGH THE MOSQUITO VECTOR.
(I) Midgut infection barrier, (II) Midgut escape barrier, (III) Salivary gland infection barrier, and (IV) Salivary gland escape barrier. *BL-Basal lamina

A. Midgut infection and escape barriers

Passage of a virus through the midgut may involve several steps. Following entry into the midgut, the virions must bind to the midgut epithelial cells, presumably via a midgut receptor (Germi *et al.,* 2002, Ludwig *et al.,* 1996, Yazi Mendoza *et al.,* 2002). Virus envelope structural proteins must interface with the lumenal surface of the midgut epithelial cells. The first potential barrier to infection is therefore the midgut infection barrier (MIB), if virus is unable to infect these cells subsequent biological transmission cannot occur. An inability to infect cells is thought to reflect the lack of appropriate cell receptors for viral proteins in the mosquito midgut. Observations of a dose-dependent MIB for some viruses suggest that a threshold concentration of virus may be required for midgut infection to occur (Chamberlain & Sudia 1961) likely due to low concentrations of receptors and/or low affinity interactions between viral and cellular proteins. Studies of WEEV, conducted by Houk *et al.,* (1990), found that WEEV could bind to brush border fragments that were isolated from both refractory and susceptible mosquito midgut epithelial cells (Houk *et al.,* 1990). These data suggested that establishment of midgut infection may require more than just the presence of the appropriate cell surface receptor. In support of this conclusion recent studies of chikungunya virus (CHIKV), suggest that vector specific differences in midgut infection my arise not from differences in binding but from differences in membrane fusion as it relates to lipid composition (Tsetsarkin 2009, Tsetsarkin *et al.,* 2007).

The second impediment to successful arbovirus transmission is the midgut escape barrier (MEB). A major obstacle to understanding the mechanism or nature of the MEB is the lack of knowledge of how certain viruses successfully breach the midgut and disseminate to secondary tissues. Several routes of viral midgut escape have been suggested:

"Leaky" midgut

The detection of arboviruses in the hemolymph of mosquitoes within hours of engorgement (Boorman 1960, Miles *et al.,* 1973, Weaver 1986) coupled with observations of midgut lesions following blood feeding (Weaver *et al.*, 1991) have lead to the hypothesis of rapid dissemination of these viruses due to a "leaky midgut." This leakage was thought to be caused by passive seepage of virions through intercellular spaces and/or physical disruptions in the mosquito midgut. Houk *et al.,* (1981) suggested the possibility that physical distension or biochemical stress could alter the structure of the basal lamina, provided it possessed sufficient plasticity (Houk *et al.,* 1981). However, transmission electron microscopic (TEM) analysis of the permeability limits of the midgut basal lamina have indicated that, although hemoglobin and latex particles (90nm in diameter) have been observed between epithelial cells (Hardy *et al.,* 1983), no particulate matter greater than 8nm can traverse the basal lamina (Houk *et al.,* 1981). Additionally, in all instances when a "leaky midgut" was observed, breach of the midgut integrity as a result of experimental manipulations could not be excluded. Many arboviruses, including midgut-restricted vaccine strains such as YFV 17D will replicate in mosquito tissues if introduced directly into the hemocoel by intra-thoracic (IT) inoculation. Not only does this technique bypass the MEB, but it also often negates normal species barriers, and even males and non-hematophagous mosquitoes such as *Toxorhynchites sp.* can be infected in this manner. If midgut lesions resulting from physical distension during blood feeding plays any role in viral dissemination, then we should observe disseminated infections for all mosquito-borne viruses including non-disseminating viruses, such as YFV 17D, at some basal level as a byproduct of this mechanical delivery to the hemocoel. Since this is not the case, dissemination from the midgut likely results from an alternative mechanism.

Cytopathological replication

Midgut escape via active cytopathologic replication in the vector has been demonstrated by Weaver and colleagues (1988) using an alphavirus-mosquito model system (Weaver *et al.,* 1988). It is generally accepted that it is disadvantageous for an arbovirus to cause deleterious effects that result in decreased vector fitness and survival (Higgs & Beaty 2005). This is because any negative effects imposed upon the vector, by viral replication, will likely decrease the potential for transmission. This assumption is based on the genetic theory of co-evolution: i.e. that a parasite and its host will co-evolve resistance and attenuation respectively to facilitate the successful survival of both species (Scott & Lorenz 1998). However, oral infection of *Culiseta melanura* with EEEV was associated with severe cytopathic effect (CPE) in midgut epithelial cells including: degeneration and sloughing of infected cells, degeneration of the epithelial brush border, and breaches in the integrity of the basal lamina. These findings provided a potential explanation for the short extrinsic incubation of EEEV in *Cs. melanura,* 48-72 hours (Weaver 2001). Subsequent analysis of WEEV infection of midgut epithelial cells, in two strains (high and low susceptibility) of *Culex tarsalis* demonstrated a correlation between mosquito strain susceptibility and cytopathology (Weaver *et al.,* 1992). These data indicate that in some instances midgut escape can be facilitated via cytopathologic replication within the mosquito midgut.

Direct passage across the basement membrane

Mature arbovirus virions are typically larger than the physical permeability limits of the junctions between midgut epithelial cells and the pores of the basal lamina. However, it has been postulated that virions could enzymatically alter the basal lamina or vice versa, to facilitate midgut escape via this route (Houk *et al.,* 1981). Ultrastructural analysis of St. Louis encephalitis virus (SLEV) identified a unidirectional trafficking of

virions toward the basal surface of infected epithelial cells and direct basal lamina penetration of virions (Murphy 1975). These observations indicated that some viruses may use an unidentified mechanism for direct passage across this structure.

Infection of specific regions/tissues

Another hypothesis for midgut escape is via infection of regions of the gut in proximity to cells/structures that traverse the basal lamina and facilitate infection of secondary amplifying tissues. Romoser *et al.,* (2004) analyzed VEEV infection in the cardia/intussuscepted foregut using immunohistochemical (IHC) analysis and TEM toward identification of so-called "conduits of escape" (Romoser *et al.,* 2004). Observations of the tropisms of VEEV virus like particles (VLP) following IT inoculation and oral ingestion, identified infection of the tracheal system secondary to the cardia (proventriculus) as a potential route of virus dissemination. Additionally, infection of the cardia and intussuscepted foregut has been implicated in the dissemination of DENV 3 and EEEV in *Ae. aegypti* and *Cs. melanura*, respectively (Linthicum *et al.,* 1996, Weaver *et al.,* 1991). The physiology of the mosquito cardia, midgut, and associated structures (**Figure 1.7 B**) appear conducive to facilitating midgut escape. Direct linkage of the cardia with tracheae/tracheoles, midgut muscle fibers, and ventricular ganglia provide multiple potential routes of escape for arboviruses (Lerdthusnee *et al.,* 1995).

Recent ultrastructural analysis of *Cx. pipiens* mosquitoes indicated that midgut muscle associated tracheae/tracheoles infiltrate the posterior midgut epithelium (Romoser *et al.,* 2005). These tissues have been consistently observed to be infected and hypothesized to play a role in dissemination for various virus-vector systems including: VEEV, Rift Valley fever virus (RVFV), SINV, and various baculoviruses (Barrett *et al.,* 1998, Bowers *et al.,* 1995, Romoser *et al.,* 2005, Romoser *et al.,* 2004). Salivary gland

infection, subsequent to amplification in nervous tissue, has been observed for DENV 2 (Kuberski 1979, Sriurairatna & Bhamarapravati 1977), JEV (Leake & Johnson 1987), and Whataroa virus (Miles *et al.,* 1973). In addition to the associations of tracheal-muscular junctions, the morphology of regions of basal lamina in close proximity to muscle fibers has a distinctly porous or "spongy" texture similar to the morphology of the matrix surrounding the cardia (Romoser *et al.,* 2004). Since RVFV has been observed to bud directly into the cardial matrix (basal lamina) (Lerdthusnee *et al.,* 1995, Romoser *et al.,* 2004) it is logical to assume that virions may be able to infect midgut associated muscle cells via transport through a porous basal lamina. Indeed, circular and longitudinal midgut muscle have been identified as important tissues for the amplification and dissemination of WNV in *Cx. pipiens quinquefasciatus* mosquitoes (Girard *et al.,* 2004).

Systematic analyses of closely related viruses with disparate mosquito infectivity phenotypes are scarce. When we compare the *in vivo* (mosquito) phenotypes of the wt YFV Asibi strain and its vaccine derivative 17D, multiple differences can be identified: 1) Asibi can infect and disseminate in *Ae. aegypti* whilst 17D is midgut restricted (McElroy *et al.,* 2006, McElroy *et al.,* 2006, Miller & Adkins 1988, Whitman 1939); 2) although 17D can infect midgut epithelial cells, significantly fewer mosquitoes become infected following oral ingestion of equivalent titers to Asibi (McElroy *et al.,* 2006, McElroy *et al.,* 2006); 3) generally lower titers of infectious virus are produced following replication of 17D in midgut epithelial cells (McElroy *et al.,* 2006, McElroy *et al.,* 2006); and 4) Asibi and 17D have differential tropisms for specific regions of the mosquito gut. Within the mosquito midgut Asibi appears to be able to spread, most likely cell-to-cell, through the posterior and anterior midgut into the cardial epithelium followed by amplification in fatbody and nervous tissues (McElroy *et al.,* 2008). YFV 17D, on the

other hand, appears to be restricted to the posterior and anterior midgut (McElroy *et al.,* 2008). When taken together these data suggest that, both a partial MIB and a complete MEB function synergistically to restrict 17D in *Ae. aegypti*. Although viral genes which control these phenotypes have been identified the mechanism(s) have yet to be defined. However, the identification of attenuating mutations in genes involved in virion binding, replication, assembly, release, and membrane rearrangement/replicative complex anchoring, strongly suggests a multi-factorial mechanism (McElroy *et al.,* 2008, McElroy *et al.,* 2006, McElroy *et al.,* 2006).

B. Salivary gland infection and escape barriers

Salivary gland infection and escape barriers to successful arbovirus transmission have also been described (Grimstad *et al.,* 1985, Kramer *et al.,* 1981, Paulson *et al.,* 1989, Reisen *et al.,* 1993). In these vectors virus is released from the midgut into the hemoceol but either fails to infect or to be released from the salivary glands. Typically, salivary gland infection and escape barriers are time, temperature, and/or dose dependent. However, the nature of these barriers remains poorly understood.

1.3 THE GENUS *ALPHAVIRUS*

The alphaviruses (genus *Alphavirus*, family Togaviridae) are a group +ssRNA viruses characterized by the expression of a single subgenomic RNA. The vast majority of the alphaviruses are transmitted between vertebrate reservoir/amplifying hosts by biting flies (primarily mosquitoes) although a few exceptions exist (Weaver *et al.,* 2000). The alphaviruses can be divided based on geographic distribution into New and Old World viruses and based on sero cross-reactivity into seven distinct antigenic groups (**Figure 1.9;** Calisher & Karabatsos 1988). Sequence analysis of the alphaviruses suggests that this group evolved from a single common ancestor and was subsequently

translocated between hemispheres. However, since members of the Sindbis virus (SINV) and Semliki Forest virus (SFV) serocomplexes exist in both the New and Old world it is unclear as to the direction of these multiple introductions (Levinson *et al.,* 1990, Weaver *et al.,* 1993). This geographic distribution appears to correlate with distinct differences in vertebrate pathogenecity. The New World viruses, such as EEEV, WEEV, and VEEV equine encephalitis viruses, are capable of causing severe encephalitis, whilst the Old World viruses, such as chikungunya (CHIKV), O'nyong nyong virus (ONNV), Ross River virus (RRV), SINV, and SFV, are associated with polyarthritis and rash.

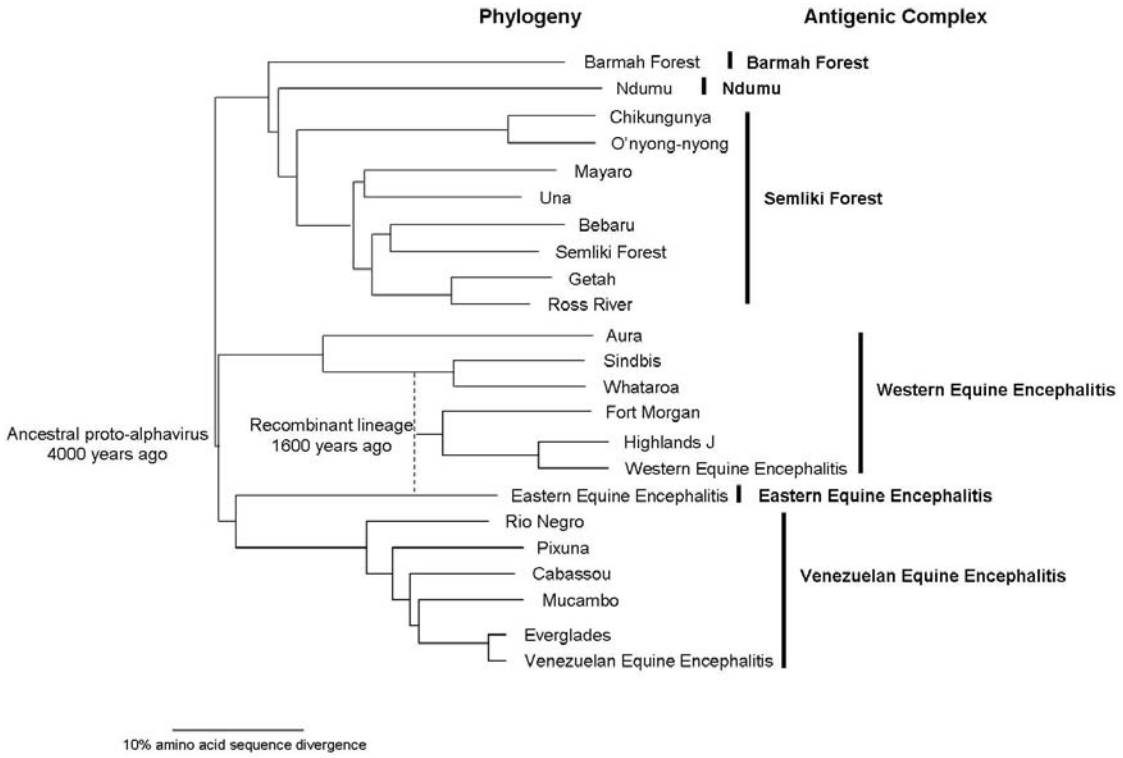


FIGURE 1.9 PHYLOGENETIC TREE OF REPRESENTATIVE ALPHAVIRUSES AND SEROCOMPLEXES.
Phylogenetic relationships based on comparison of amino acid sequences from the E1 glycoprotein (complied and adapted from Strauss and Strauss 1997; Griffin 2001).

A. Chikungunya virus

CHIKV is an *Aedes* mosquito-vectored alphavirus in the family Togaviridae and is the etiological agent of human chikungunya fever and the associated polyarthralgic disease. It has been postulated that the earliest recorded incidence of human disease caused by CHIKV occurred in Batavia-Jakarta, Cairo in 1779 with subsequent sporadic activity in Zanzibar, India, Hong Kong, and the West Indies through the early 1900s (Carey 1971). CHIKV was first isolated in Tanganyika from both acute human sera samples and *Ae. aegypti* mosquitoes and confirmed serologically by Ross in 1953 (Ross 1956). Mosquito-borne transmission of CHIKV by *Ae. aegypti* mosquitoes was demonstrated using an artificial bloodmeal and *in vitro* salivation technique followed by intra-cerebral inoculation of diluted mosquito expectorate in mice (Ross 1956). CHIKV remains epizootic throughout much of Africa and Asia (Jupp & McIntosh 1988, Powers & Logue 2007, Woodall 2001). Two distinct types of mosquito-borne transmission cycles have been described for CHIKV. Sylvatic transmission has been described in Africa where it is believed that epizootic cycles of transmission between *Aedes spp.* and non-human primates allow for CHIKV maintenance (Jupp & McIntosh 1988, Powers & Logue 2007). Epidemic transmission of CHIKV in both Africa and Asia results from transmission cycles involving humans and predominantly *Ae. ageypti* and *Ae. albopictus* mosquitoes (Jupp & McIntosh 1988, Powers & Logue 2007).

Recently, epidemic transmission of CHIKV (2004-2008) has resulted in millions of human infections in Africa, the Indian Ocean islands, and India (Tsetsarkin 2009). Furthermore, this epidemic activity has resulted in the importation of human cases of CHIKV into the United States, the Caribbean, and Europe (Townson & Nathan 2008). *Ae. albopictus* driven transmission of CHIKV in Romagna, Italy was responsible for a local outbreak resulting in 248 human cases following the introduction of a single acute

phase human infection (Sambri *et al.,* 2008). This potential for rapid expansion into previously non-endemic regions due to human/mosquito sustained transmission cycles, coupled with the debilitating nature of infection, has resulted in renewed interest in CHIKV research specifically with regard to vaccine development (Wang *et al.,* 2008).

B. Sindbis virus

SINV is the prototypical alphavirus (family Togaviridae) and as such much of what is known regarding basic alphavirus molecular biology has been defined using SINV model systems (Calisher *et al.,* 1980, Niklasson 1988, Strauss & Strauss 1994). The virus was first isolated from *Culex* mosquitoes in Sindbis, Egypt (Lvov *et al.,* 1984, Taylor *et al.,* 1955) with subsequent isolation and verification of disease etiology in humans (McIntosh *et al.,* 1964, Woodall *et al.,* 1964). SINV has a vast geographic distribution and remains endemic in Australia, Africa, Europe, and the Middle East (Griffin 2001, Griffin 2001). Enzootic transmission of SINV involves mosquitoes of the genera *Culex* and *Culiseta* and passerine birds, with incidental human infections resulting from transmission by *Aedes sp.* bridge vectors (Griffin 2001). In humans, SINV disease manifestations include fever, rash, and arthritis.

C. Overview of alphavirus genome, life cycle, and particle organization

The alphavirus genome is a single stranded RNA molecule of ~11-12Kb in length and is depicted schematically **Figure 1.10 A**. Viral RNA is protected at the 5' terminus by a covalently linked 7-methylguanosine cap and at the 3' end by a poly-adenosine tail similar to host mRNAs. The viral coding sequence is divided into two distinct ORFs, which are flanked by 5' and 3' untranslated sequences. The 5' two thirds of the alphavirus genome, first ORF, encodes the nonstructural proteins (nsP1, nsP2, nsP3, and nsP4). The viral structural (capsid (C) and envelope glycoproteins (E1 and E2)) and

accessory proteins (E3 and 6k) genes are expressed from a second ORF under the control of a subgenomic promoter (SGP). These two ORFs are separated by a short non-coding sequence (~60nt). Within the mature virion (~70nm in diameter) the genome is associated with multiple copies of C arranged to form the virion core or icosahedral nucleocapsid (T=4). This nucleocapsid is enveloped in a host cell plasma membrane derived lipid bilayer into which the viral envelope proteins are embedded. The envelope proteins are also arranged in an similar icosahedral organization consisting of 80 heterodimers of non-covalently associated E1-E2 trimers (compiled from Strauss & Strauss 1994).

A general overview of important steps in the alphavirus lifecycle is depicted in **Figure 1.10 B**. The first interaction between the alphavirus virion and host cell is receptor binding. Entry into the host cell via receptor mediated endocytosis is facilitated by interactions between E2 and host membrane protein(s) (Schlesinger & Schlesinger 2001), which may include high affinity laminin receptors, C-type lectins, and heparin sulfate (Byrnes & Griffin 1998, Klimstra *et al.,* 2003, Klimstra *et al.,* 1998, Strauss *et al.,* 1994, Wang *et al.,* 1992). Particles are then endocytosed and these vesicles trafficked into the host cell endosomal pathway (Schlesinger & Schlesinger 2001). Upon acidification, the E1-E2 heterodimers disassociate resulting in exposure of the E1 protein followed by a pH induced conformational change in E1 that facilitates fusion of the viral envelope and endosomal membrane (Kielian 2006). This process results in the release of the viral nucleocapsid into the host cell cytoplasm. Following nucleocapsid release interactions between host cell ribosomes and the RNA-associated C monomers result in nucleocapsid disassociation to allow for transcription and protein expression (Wengler *et al.,* 1992).

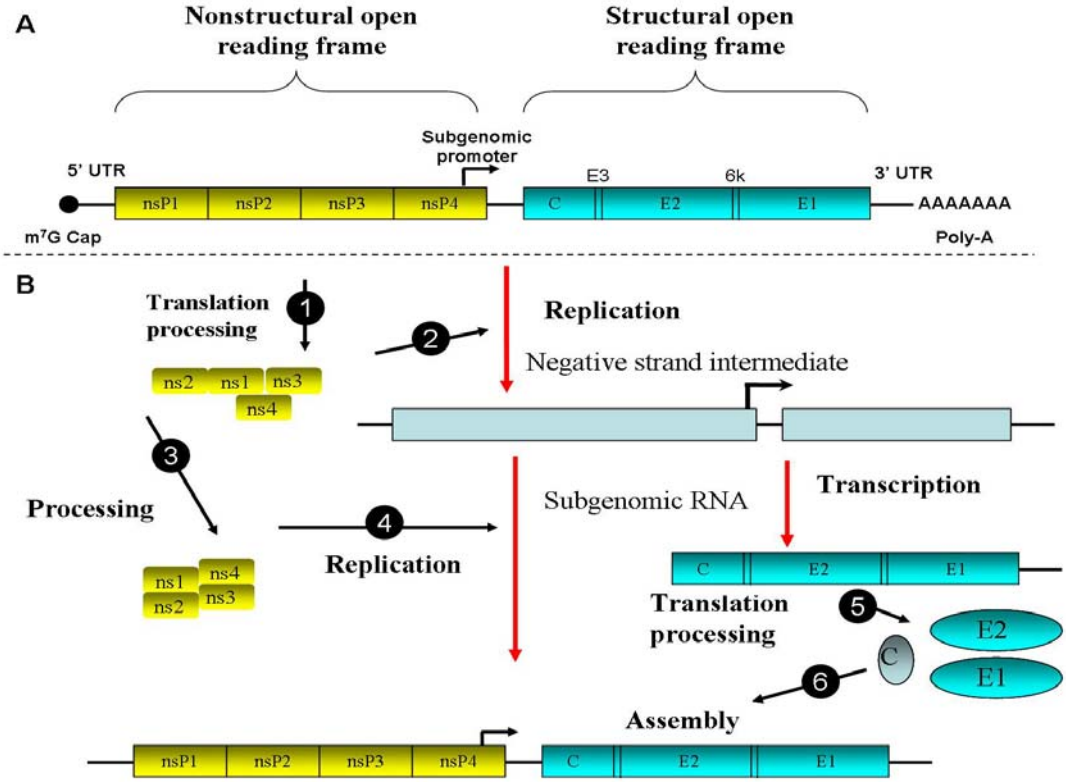


FIGURE 1.10 SCHEMATIC REPRESENTATION OF ALPHAVIRUS GENOME ORGANIZATION AND LIFE-CYCLE.
A, Stylized alphavirus genome containing: nonstructural and structural open reading frames, 5' and 3' untranslated regions, m^7G Cap and poly-adenosine tail, and subgenomic promoter. B, Alphavirus life-cycle: 1) Ribosome dependent translation of nonstructural polyprotein and co/post-translational processing; 2) Synthesis of genomic length negative sense viral RNA; 3) Further processing of the nonstructural replicate complex; 4) Synthesis of positive sense genomic and subgenomic RNAs; 5) Ribosome dependent translation of the structural polyprotein and co/post-translational processing/maturation; 6) Genome encapsidation, assembly, and release of mature virions via budding from the plasma membrane.

The alphaviral nonstructural ORF contains the sequences necessary for expression of the replicative machinery (nsP1, nsP2, nsP3, and nsP4) as well as the viral SGP from which the structural subgenomic RNA is expressed. The nonstructural ORF, which comprises the 5' two thirds of the viral coding sequence, is translated by host ribosomes immediately upon nucleocapsid disassociation. The alphavirus nonstructural ORF is

translated as either one (nsP1234) or two (nsP23 and nsP1234) distinct polyproteins depending on the absence or presence of a virus specific "leaky" stop codon between nsP3 and nsP4 (Strauss & Strauss 1994). These polyproteins contain the individual non-structural proteins that provide all the functions necessary for synthesis of positive and negative sense genomic length RNA and subgenomic RNA. nsP1 is required for initiation of minus strand synthesis and provides the methyltransferase capping function. nsP2 contains a helicase and a protease domain to facilitate unwinding of template RNAs and cleavage of nonstructural polyprotein precursors. nsP3 is required for plus and minus strand synthesis via unknown function(s). nsP4 is the viral RdRp (Schlesinger & Schlesinger 2001, Strauss & Strauss 1994). Upon transcription, the nsp1234 precursor is rapidly auto cleaved by the nsP2 protease (de Groot *et al.,* 1990, Hardy & Strauss 1989) to form nsP123 and nsP4. The replicative complex is then formed by association of nsP123, nsP4, and various host factors (Strauss & Strauss 1994) in association with modified host cell membranes (Froshauer *et al.,* 1988). This complex then initiates *de novo* replication of the positive sense viral RNA to generate full length negative sense intermediates (Strauss & Strauss 1994, van Dijk *et al.,* 2004). Following 3-4h of protein expression and negative strand synthesis, a critical concentration of nsP123 is reached which results in further proteolytic processing of the nonstructural polyproteins. Upon proteolytic cleavage, the affinity of the viral replicase switches from negative to positive strand templates (Strauss & Strauss 1994). This results in the synthesis of both full length +ss viral RNA and subgenomic RNAs which are expressed from the viral SGP.

The alphaviral structural proteins are also expressed as a single polyprotein exclusively from subgenomic transcripts. The C protein is comprised of two functional domains, a serine protease (Aliperti & Schlesinger 1978) and an RNA binding domain (Weiss *et al.,* 1989). The serine protease domain facilitates auto-cleavage of C protein

which then rapidly associates with positive sense genomic templates to form the viral nucleocapsid via interactions between the RNA binding domain and RNA packaging signal sequences (Frolova *et al.,* 1997, Volkova *et al.,* 2006). The nascent structural polypeptide is then translocated to the endoplasmic reticulum where the immature viral envelope proteins precursor (p)E2 and E1 are embedded (Strauss & Strauss 1994). Following synthesis, the envelope glycoproteins are folded and posttranslationally modified as they are trafficked through the ER and golgi towards the plasma membrane. Critical to this maturation is the proteolytic cleavage of pE2 which results in the generation of mature E2 (Schlesinger & Schlesinger 2001). Interactions between the cytoplasmic domains of the mature E1-E2 heterodimers trigger the nucleocapsid to contract/mature and bud through the plasma membrane resulting in the release of mature alphavirus virions (Coombs *et al.,* 1984, Garoff & Simons 1974).

D. Alphavirus reverse genetics

As previously mentioned, +ssRNA virus genomes have the unique property of being fully infectious to the host cell even in the absence of their structural proteins. It is this property that makes these viruses so amenable to molecular manipulation for both the study of basic viral mechanisms and development of vaccines. Infectious clones (i.c.), also known as reverse genetics systems, of +ssRNA viruses can be defined as complementary (c) DNA copies of the viral RNA genome that have been cloned into a double stranded circular autonomously replicating DNA molecule immediately downstream of an exogenous promoter sequence (**Figure 1.11**). Several full length alphavirus i.c.s have now been developed including: SINV (Rice *et al.,* 1987), VEEV wt and vaccine strains (Davis *et al.,* 1989, Kinney *et al.,* 1993), RRV (Kuhn *et al.,* 1991), SFV (Liljestrom *et al.,* 1991), CHIKV (Tsetsarkin *et al.,* 2006, Vanlandingham *et al.,* 2005), and ONNV (Vanlandingham *et al.,* 2006). Additionally, a number of full length

chimeric alphavirus constructs have been generated; SINV/RRV (Kim *et al.,* 2000, Kuhn *et al.,* 1990, Strauss & Strauss 1997), SINV/SFV (Smyth *et al.,* 1997), VEEV sub-type chimeras (Powers *et al.,* 2000), WEEV/EEEV (Schoepp *et al.,* 2002), ONNV/CHIKV (Vanlandingham *et al.,* 2006), and EEEV/SINV (Kenney *et al.,* 2010).

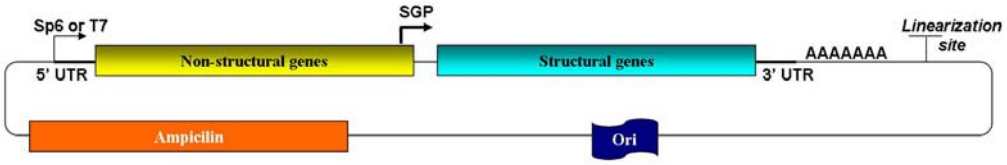


FIGURE 1.11 SCHEMATIC REPERESENTATION OF GENERALIZED ALPHAVIRUS INFECTIOUS CLONE OR REVERSE GENETICS SYSTEM.

One particularly useful characteristic of these i.c.s is the ability to express an exogenous gene of interest (GOI) from the viral ORF. Classically this has been achieved via duplication of the viral subgenomic promoter sequences followed by insertion of the exogenous gene (Hahn *et al.,* 1992) in either a 5' or 3' orientation relative to the viral structural expression cassette (**Figure 1.12**). Furthermore, it is possible to divide the alphavirus genome into two or more segments and generate a replicon/defective helper system (**Figure 1.12**). An alphavirus replicon is defined as a self-replicating genome containing regulatory and nonstructural gene coding sequences but deficient for the expression of the structural genes. Xiong and colleagues (1985) described the first SINV replicon genomes into which had been cloned a chloramphenicol acetyltransferase reporter gene (Xiong *et al.,* 1989). These genomes are competent for replication and high levels of exogenous gene expression; up to 25% of the total cellular protein content (Pushko *et al.,* 1997), but are incapable of packaging or infectious spread. However, if the structural genes are complemented by a defective helper genome, which is deficient for autogenous replication but contains the sequences necessary for *trans* replication and

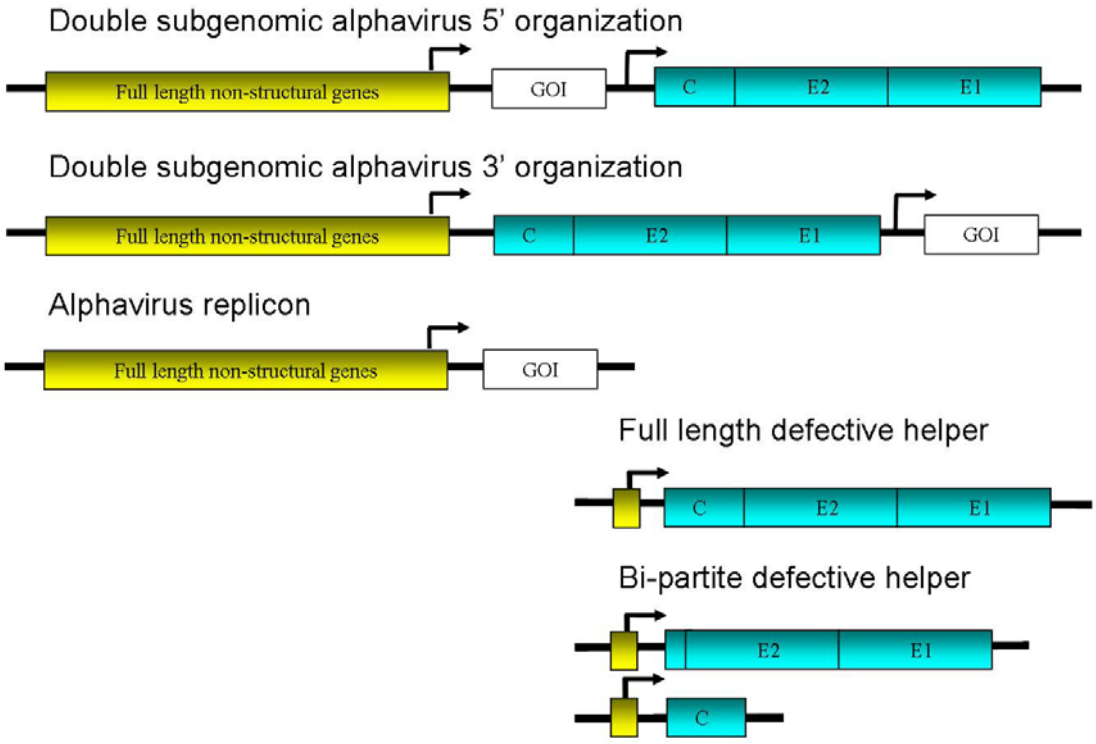


FIGURE 1.12 ALPHAVIRUS EXOGENOUS GENE OF INTEREST (GOI) EXPRESSION SYSTEMS.

structural gene expression (Geigenmuller-Gnirke *et al.,* 1991), replicon genome packaging can be restored. This technology has been used extensively in attempts to develop alphavirus vectored vaccines for heterologous infectious agents (Davis *et al.,* 1996, Hevey *et al.,* 1998, Hubby *et al.,* 2007, Kamrud *et al.,* 2008, Lee *et al.,* 2002, Lee *et al.,* 2003, Mok *et al.,* 2007, Pushko *et al.,* 2000, Pushko *et al.,* 2001, Pushko *et al.,* 1997, Rayner *et al.,* 2002, Reap *et al.,* 2007, Reap *et al.,* 2007). Early in the development of this technology it was noted that replication competent virus could be recovered from these co-transfections (Pushko *et al.,* 1997, Weiss & Schlesinger 1991). In an effort to eliminate the possible generation of full length virus, the so-called bi-partite or dual

helper system (**Figure 1.12**) was devised whereby the capsid and envelope glycoproteins are expressed from two distinct helper genomes.

E. Superinfection, co-infection, and persistence

Superinfection exclusion (also known as homologous interference) is a process by which primary cellular infection with one virus prevents the establishment of a productive secondary infection by a related virus. Initial *in vitro* reports of alphavirus superinfection resistance involved infection with a primary virus followed by secondary infections with either homotypic or heterotypic alphaviruses at increasing time intervals SINV/SINV (Johnston *et al.,* 1974) or VEEV/WEEV (Zebovitz & Brown 1968). In these experiments it was demonstrated that replication of the secondary or "challenge" virus could be suppressed if it was inoculated as little as 15 minutes after primary challenge with maximal suppression achieved by 60m (Johnston *et al.,* 1974). Furthermore, similar suppression could be observed when two viruses were inoculated simultaneously but at different moi (Zebovitz & Brown 1968). Interestingly, using a SFV system, Singh *et al.,* (1997) demonstrated that increasing the multiplicity of infection of the challenge virus could allow for escape from superinfection exclusion up to 30m post primary infection (Singh *et al.,* 1997).

While homotypic exclusion appears to be long lived, reports exist that suggest the ability of persistently infected cells to replicate heterotypic alphaviruses may recover (Eaton 1979, Stollar & Shenk 1973). Eaton (1979) observed that SINV superinfection resistance in CHIKV infected cells could be established by 1h, but noted that partial recovery could be achieved if CHIKV and SINV infections were separated by eight or more days. However, SINV titers observed in these CHIKV infected cells (>8 days) were still approximately ten-fold less than for non-CHIKV infected controls (Eaton 1979). Interestingly, only a small subpopulation of CHIKV infected cells (8-10%)

appeared to have recovered the ability to become infected with SINV. In a contradictory report, arthropod cell lines persistently infected with SINV (>1 year) were observed to retain their ability to resist replication of Aura virus, SFV, and RRV (>10^3 decrease in peak titers) (Karpf *et al.,* 1997).

Alphavirus superinfection resistance has also been demonstrated in various mosquito species. Lam and Marshall (1968) demonstrated that cytopathic replication of SFV in *Ae. aegypti* salivary glands blocked the ability of these mosquitoes to subsequently transmit superinfecting WNV and SINV (Lam & Marshall 1968). However, if the mosquitoes were inoculated in the reverse order, WNV or SINV primary infection followed by superinfection with SFV, no such resistance was observed (Lam & Marshall 1968). As such, this resistance was likely due to disruptions in salivary gland physiology via the cytopathic replication of SFV and not the establishment of an antiviral state. Homotypic superinfection resistance to SINV and SFV has been observed in *Ae. aegypti* and *Cx. annulirostris* mosquitoes respectively, provided primary and secondary infections were separated by >24h (Davey *et al.,* 1979, Peleg 1975). However, it was noted that *Cx. tarsalis* mosquitoes harboring a primary infection with EEEV remained completely susceptible to a secondary infection with WEEV when oral infections were separated by seven days (Chamberlain & Sudia 1957). Mosquitoes dually infected in this way were observed to replicate both viruses to comparable titers as individually infected control mosquitoes. Additionally, concurrent transmission of both viruses by individual mosquitoes was also observed.

Although the phenomenon of alphavirus superinfection resistance has been well documented both *in vitro* and *in vivo*, the mechanism of this suppression remains poorly defined. It has been proposed that alphavirus superinfection resistance results from some block to secondary virus negative strand synthesis imposed by the primary infection.

Potential impediments to negative strand synthesis include: degradation of the nsP123 polyprotein precursor of the secondary virus by the nsP2 protease of the primary virus and/or over-competition for necessary host factors by the primary virus (Strauss & Strauss 1994). Indeed, early studies suggested that superinfection resistance was the result of some block to RNA replication (Eaton 1979, Johnston *et al.,* 1974, Stollar & Shenk 1973, Zebovitz & Brown 1968). This hypothesis was strongly supported by the observation that resistance was the same regardless of whether or not the challenge virus was delivered as infectious virions or transfected RNA, suggesting that the blockage was not at the levels of attachment, entry, fusion, or uncoating (Zebovitz & Brown 1968). However, a contradictory report provided evidence that homotypic SFV resistance could result from deficiencies in receptor binding, endosomal release, nucleocapsid disassociation, and negative strand synthesis depending on the temporal stage of the primary infection (Singh *et al.,* 1997). Regardless of the mechanism(s), it is clear that superinfection resistance represents a major impediment to the establishment of a productive sequential co-infection. As such, it therefore follows that superinfection resistance may potentially influence recombination by preventing the establishment of a productively co-infected cell containing two distinct viral genomes.

Reports of naturally occurring alphavirus co-infection are scarce, despite the fact that alphavirus infection in the vector is considered to be persistent. This is perhaps not surprising if we consider that only a small population (~100) of mosquito midgut cells may be susceptible to primary alphavirus infection (Smith *et al.,* 2008). If this is true and we consider that following a primary infection only perhaps 10% of midgut cells may recover from superinfection resistance this would indicate that $\leq$10 cells would be permissive to establish a productive intra-vector co-infection upon subsequent challenge (Eaton 1979). However, presumably dual alphavirus infection must have occurred at

least once to result in the generation of the recombinant WEEV lineage (see Chapter 2: Introduction). Highly localized co-circulation of EEEV and Highlands J virus (HJV; WEEV sero-complex) has been demonstrated in geographically associated populations of *Cx. pipiens* and *Cs. melanura* mosquitoes (Andreadis *et al.,* 1998). In fact one pool of *Cs. melanura* mosquitoes (n=20), collected in Stonington, Connecticut was positive for both EEEV and HJV (T.G. Andreadis personal communication). Given that a pool represents multiple individuals, and the relatively high field infection rates observed for these two viruses in the trapping area ($\geq$1:200), it is likely that two mosquitoes, each with an individual alphavirus infection, contributed to this dual positive pool. Nevertheless, the isolation of two distinct viruses from a pool of only 20 mosquitoes from a single site collected in a single trapping night demonstrates the potential for co-circulation and the potential for an individual to become co-infected. Vertebrate infection of alphaviruses is generally considered to be acute followed by immunity, however, the potential for RRV and CHIKV to establish persistent infections in vertebrate macrophages has been demonstrated and postulated to be a mechanism for the establishment of persisting/relapsing arthralgia in humans (Labadie *et al.,* 2010, Way *et al.,* 2002). How the establishment of persistent macrophage infection by these viruses will affect the potential for alphavirus co-infection and the results of this persistence in terms of viral evolution in nature remains unknown.

1.4 THE GENUS *FLAVIVIRUS*

The family Flaviviridae contains three genera of viruses of specific human and veterinary importance: *Hepacivirus* (Human Hepatitis C virus), *Pestivirus* (bovine viral diarrhea virus and classical swine fever virus), and *Flavivirus*. The flaviviruses, genus *Flavivirus*, of which there are approximately 70 members, can be further subdivided into those viruses which are transmitted by tick (tick-borne flaviviruses), mosquito (mosquito-

borne flaviviruses), and those with no known vector (Strauss & Strauss 2002). The mosquito-borne flaviviruses, such as YFV, WNV, JEV, and the four serotypes of DENV 1-4, are responsible for millions of infections and thousands of deaths annually (Gubler 2004, Gubler 2006, Mackenzie *et al.,* 2004, Petersen & Marfin 2005).

The changing global pattern of human activity, including increased travel and encroachment on sylvatic habitat, coupled with recent explosions in competent vector populations have dramatically increased the global distributions of many of these viruses (**Figure 1.13**). WNV and SLEV are now both widely distributed across North America (**Figure 1.13**) and share a transmission cycle involving *Culex* mosquitoes and passerine birds. Furthermore, the rapid global expansion of the DENV 1-4 (Mackenzie *et al.,* 2004) has lead to *Aedes* mosquito driven co-circulation of all four serotypes coincident with YFV transmission in large areas of South America and Africa (**Figure 1.13**). As a result, a number of flaviviruses with similar ecological niches now have overlapping geographic regions of endemic/epidemic transmission dramatically increasing the potential for arthropod vectors and/or vertebrate hosts to become co-infected with multiple viruses. The use of live attenuated flavivirus vaccines, to control transmission in regions of epidemic/endemic transmission has been postulated to further increase the potential risk for co-infection and subsequent recombination (Holmes *et al.,* 1999, Seligman & Gould 2004, Worobey *et al.,* 1999).

A. Yellow fever virus

YFV has long been known to be a significant cause of human morbidity and mortality. It is believed that the first recorded outbreak of human disease caused by YFV occurred in 1648 in present day Yucatan, Mexico (Barrett & Higgs 2007, Carter 1931). Between the years of 1668-1893 there were approximately 135 outbreaks of YFV in the Americas with case fatality rates as high as 30% (Barrett & Higgs 2007, Monath 1988)

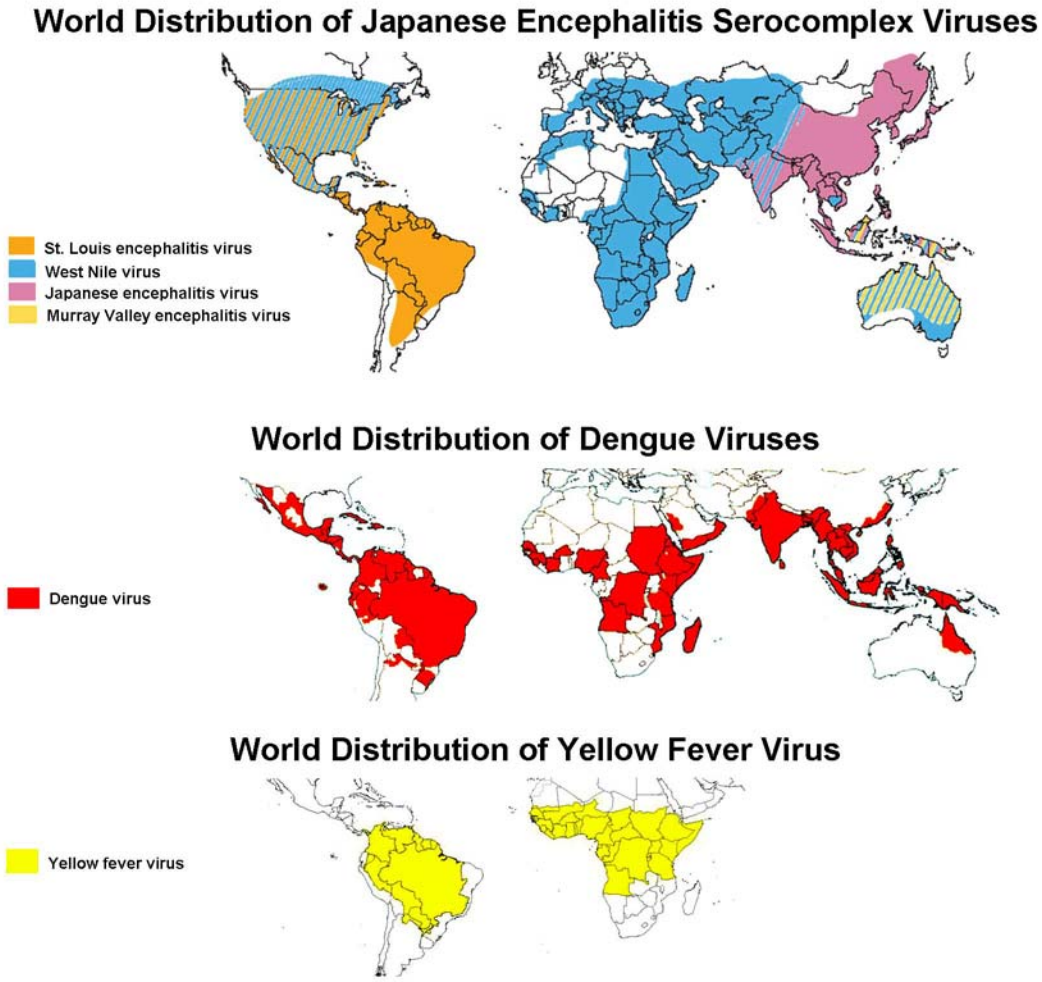


FIGURE 1.13 WORLDWIDE DISTRIBUTIONS OF MOSQUITO-BORNE FLAVIVIRUSES OF SIGNIFICANT PUBLIC HEALTH IMPORTANCE.
(compiled and adapted from World Health Organization http://www.who.int/ith/en/; and Mackenzie *et al.,* 2004)

(S. Cope personal communication; http://www.entomology.montana.edu/historybug/). YFV is the prototypical member of the genus, *Flavivirus* and it was indeed the first virus to be demonstrated to be transmitted between infected humans by a mosquito vector; *Ae. aegypti* (*Culex fasciatus;* Reed *et al.,* 1900). Phylogenetic analyses of distinct genotypes suggest that YFV likely emerged in Africa and was subsequently introduced into the

Americas (Barrett & Higgs 2007, Lepiniec *et al.,* 1994, Mutebi *et al.,* 2001) presumably in association with human activity. Presently distinct genotypes appear to remain geographically restricted largely due to vector control and vaccination (Twiddy & Holmes 2003). YFV is maintained in three distinct mosquito-borne transmission cycles (Monath 1988): an urban or epidemic cycle involving *Ae. aegypti* mosquitoes and humans (Beeuwkes & Hayne 1931, Reed & Carroll 1900, Reed & Carroll 1901, Reed *et al.,* 1900), a sylvatic/jungle cycle has been described between forest dwelling *Aedes*, *Hemogogous*, and *Sabathes spp*. mosquitoes and non-human primates (Monath 1988, Soper 1935, Soper 1937), and a rural/savannah cycle supported by humans coming into contact with sylvatic vectors (Bugher *et al.,* 1944, Soper 1935, Soper 1937).

The prototype wt strain of YFV known as "Asibi," (**Figure 1.14**) named for the febrile African male, from whom it was collected, was isolated in 1927 in the Gold Coast, Africa by Bauer and Mahaffy (Lloyd *et al.,* 1937, Stokes *et al.,* 1928, Stokes *et al.,* 2001). The 17D live attenuated vaccine (LAV) viruses were developed by serial passage of the wt Asibi strain through various tissues/cell types (Theiler 1951); **Figure 1.14**). This passage protocol resulted in the accumulation of 67nt substitutions associated with 33aa changes scattered throughout the genome, with the exception of the 5'UTR and capsid sequences which remain identical. 17D is characterized by two important phenotypic changes with respect to the wt parental (Asibi) strain; 1) avirulent whilst highly immunogenic in humans/non-human primates (Theiler & Smith 1937), and 2) a loss of transmissibility by *Ae. aegypti* mosquitoes (Jennings *et al.,* 1994, McElroy *et al.,* 2006, McElroy *et al.,* 2006, Miller & Adkins 1988, Whitman 1939). There are currently two distinct substrains of the 17D vaccine that are used to immunize people at risk for YFV infection; the 17D-204 strain which is produced/administered in the United States, United Kingdom, France, Colombia, Russia, Senegal, and Switzerland at passages 235-238, and

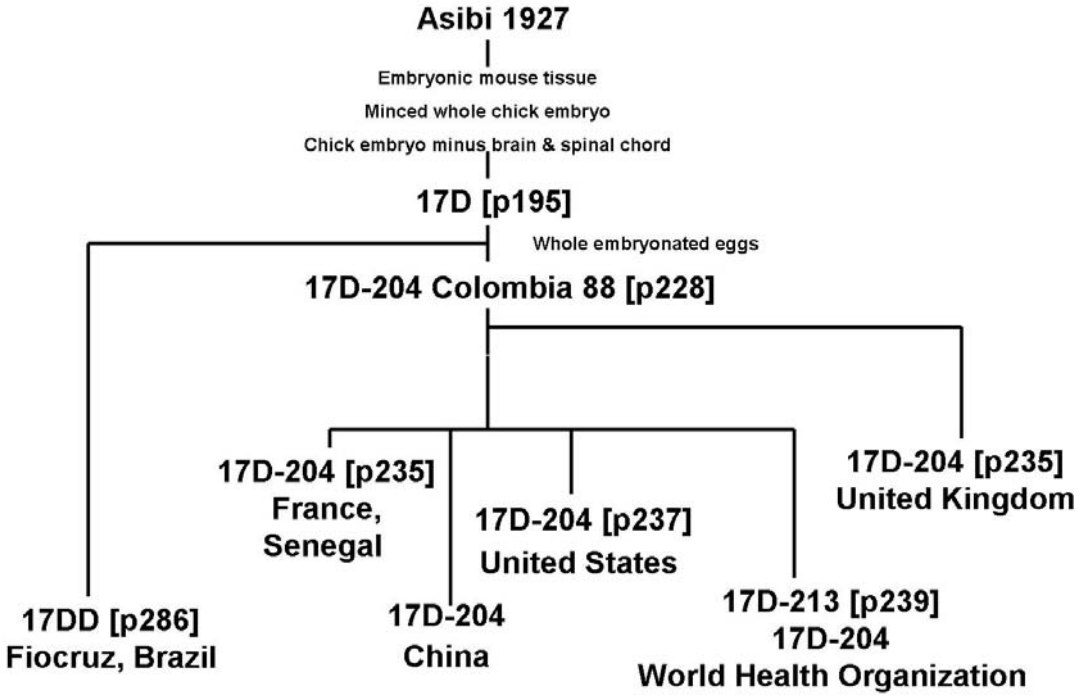


FIGURE 1.14 PASSAGE HISTORY OF THE EMPERIACAL DERIVATION OF THE 17D VACCINE SUBSTRAINS FROM THE ASIBI WILD-TYPE VIRUS.
(adapted from Monath 2005)

the 17DD strain which is exclusively used in Brazil at passage 284-285 (Monath 2005). As a result of vaccination with the 17D viruses and vector control strategies, epidemic YFV has largely been controlled in the Americas with little evidence of urban/epidemic transmission since 1942 (Barrett & Higgs 2007). Despite the availability of this highly efficacious vaccine, YFV remains endemic throughout tropical regions of South America and sub-Saharan Africa largely due to sylvatic transmission cycles between non-human primates and forest dwelling mosquitoes (Barrett & Higgs 2007, Barrett & Monath 2003). As a result, epidemic activity of YFV continues to be observed in Africa where an estimated 600 million people remain at risk (Barrett & Higgs 2007).

In humans, YFV infection is viscerotropic and generally presents as a three phase disease process. Primary infection is characterized by a non-specific febrile presentation including fever, malaise, headache, nausea, and vomiting and is concurrent with high titer viremia. Generally this initial phase remits and in up to 50% of patients no further complications are observed. However, infection in the remaining ≥50% of patients will progress to the more severe stage of intoxication. In these patients, severe hemorrhagic fever develops characterized by jaundice, cardiovascular distress, renal failure and hematemesis. Neurological complications can also develop including; delirium, seizures, and coma. Patients progressing to this late-stage hemorrhagic presentation generally exhibit a case fatality rate of up to 50% (compiled from Monath 1987, Tsai 2000).

Unfortunately, the study of YFV pathology has been extremely hampered by the lack of an adequate small animal model. Although non-human primates, such as cynomologous and rhesus monkeys, accurately recapitulate human YF disease (Hoskins 1935, McGee *et al.,* 2008, Monath 1987, Stokes *et al.,* 1928), few detailed comparative studies of YFV infection exist due to the economic and ethical concerns associated with their use. A mouse model of YFV has been used extensively to characterize virulence determinants of wt YF, however YFV infection in mice presents as a neurotropic infection and as such is not a good approximation of human YFV pathogenesis (Barrett & Gould 1986, Engel 2006, Ryman *et al.,* 1997). A Syrian hamster model of YFV has been developed via serial passage of the wt Asibi to generate a passage seven viscerotropic strain (Tesh *et al.,* 2001, Xiao *et al.,* 2001). In a recent study, Meier *et al.,* (2009) developed the first viscerotropic mouse model of YFV infection using various interferon knockout strains (Meier *et al.,* 2009).

B. Dengue

Human epidemics of "dengue-like" disease have been reported as early as the late 18th century (1779-1780) in Asia and North America (Carey 1971). However, as a result of similar clinical presentations during the early febrile stage of disease, it is possible that some of these early accounts may have been CHIKV infections which were mistakenly classified as "dengue." Carey (1971) suggested that the first record of dengue disease that could be attributed to DENV infection was a report of an epidemic in Philadelphia by Rush in 1780. Dengue is somewhat unique among the flaviviruses in that it is caused by four related but serotypically/antigenically distinct viruses (dengue viruses 1-4). The prototype strains of the DENV were isolated from Hawaii (1944) DENV 1 (Sabin 1952), New Guinea (1944) DENV 2 (Sabin 1952), and the Philippines (1956) DENV 3 and DENV 4 (Hammon *et al.,* 1960). Each of these serotypes can be further subdivided into multiple genotypes (Holmes & Twiddy 2003, Rico-Hesse 2003, Rico-Hesse 2007, Twiddy *et al.,* 2003, Twiddy *et al.,* 2002). Some of these distinct genotypes have been associated with increased virulence in humans (Leitmeyer *et al.,* 1999, Rico-Hesse 2003). Interestingly, phylogenetic analyses suggest that the virulent Asian genotype of DENV 2 may be displacing the more attenuated American genotype in South and Central America (Rico-Hesse *et al.,* 1997).

Phylogenetic analyses of DENV envelope sequences indicated that "all DENV arose in the Asian-Oceanic region and diverged into the four serotypes recognized today" (Wang *et al.,* 2000). The geographic distribution of the DENV has undergone rapid global expansion. Epidemic transmission concurrent with the circulation of all four serotypes has now been recognized in over 100 countries distributed throughout tropical and subtropical regions of Central and South America, Africa, the Middle East, Asia, India, Australia, and the Philippians (Mackenzie *et al.,* 2004). Similar to YFV, the

DENV are maintained in three distinct but overlapping transmission cycles: a sylvatic cycle involving non-human primates and forest dwelling *Aedes sp.* mosquitoes as well as rural and urban cycles where human/mosquito transmission is sustained by peridomestic vector species such as *Ae. aegypti* and *Ae. albopictus* (Barrett & Higgs 2007, Gubler 1988, Rudnick *et al.,* 1967, Rudnick *et al.,* 1965).

In humans, DENV infection can be asymptomatic or result in varying degrees of severity. Most patients that present with clinical manifestations exhibit a mild self-limited febrile illness known as dengue fever (DF) characterized by fever, chills, headache, rash, and generalized myalgia. However, an estimated 6% of cases (~2.1 million cases annually; http://www.pdvi.org/about_dengue/GBD.asp) progress to the more severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). This stage of the disease is characterized by subcutaneous hematoma, gastrointestinal hemorrhage, epistaxis, and increased capillary permeability (compiled from (Vaughn & Green 2000)). Unfortunatley, the lack of an animal model system that accurately mimics human disease has greatly impeded the study of the pathological mechanism(s) responsible for these disease presentations.

One interesting characteristic of DENV infection in humans is the propensity for individuals experiencing a secondary infection with a heterologous serotype to develop DHF/DSS. This phenomenon, known as antibody dependent enhancement (ADE), occurs when a secondary infection is established in the presence of a DENV heterotypic humoral response. Support for the potential exacerbation of DENV infection via an ADE dependent mechanism has been demonstrated by the observations of: 1) enhanced DENV viremia in non-human primates, 2) enhanced infection of mononuclear phagocytic cells *in vitro*, and 3) increased disease severity in human children (Halstead 2003, Halstead *et al.,* 1973, Halstead *et al.,* 1973, Marchette *et al.,* 1973). ADE is believed to result when

heterotypic antibodies bind to, but do not neutralize, a virus particle. Interactions between these antibodies and specific immune effector cells result in the enhanced recruitment/infection of these cells with non-neutralized particles (Halstead 1988). The infection of monocytes and macrophages with non-neutralized virus has been postulated to result in increased expression of proinflammatory cytokines resulting in increased vascular permeability and plasma leakage (Rothman 2003, Rothman & Ennis 1999). Therefore in order to prevent vaccine associated enhancement a successful DENV vaccine platform will have to simultaneously stimulate long lasting tetravalent immunity.

C. Overview of flavivirus genome, life cycle, and particle organization

The flavivirus genome is ~11-12 Kb, and is comprised of a +ssRNA molecule that contains a 5'methylguanosine cap but no poly-adenosine tail. This genome contains a single ORF, which encodes three structural (capsid (C), premembrane (prM), and envelope (E)) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins, which is flanked by 5' and 3' UTRs (**Figure 1.15 A**). The flavivirus virion is ~50nm in diameter and is comprised of an electron dense nucleocapsid core surrounded by an endoplasmic reticulum derived lipid bilayer (Hase *et al.,* 1987, Heinz & Allison 2003, Mackenzie & Westaway 2001). Within this lipid bilayer are embedded the mature membrane and E proteins. The E protein is comprised of three distinct domains; a receptor binding domain, a central linker domain, and a dimerization domain which contains the membrane fusion loop (Modis *et al.,* 2004, Nybakken *et al.,* 2006, Rey *et al.,* 1995). The flavivirus virion contains 180 E proteins associated as heterodimers that are oriented parallel to the lipid bilayer in an icosaheldral symmetry which give the particle a smooth "golf-ball-like" appearance (Kuhn *et al.,* 2002).

The flavivirus life-cycle is depicted schematically in **Figure 1.15 B**. The flavivirus virion gains entry into the cell via receptor mediated endocytosis. Although

flavivirus interactions with various unidentified cell surface proteins have been described, virus specific receptors remain largely unknown (Lindenbach & Rice 2003). The envelope protein then facilitates fusion of the viral and endosomal membranes, resulting from pH induced conformational changes via a mechanism similar to as described for alphaviruses (Kielian 2006). Following nucleocapsid disassociation, by an as of yet unidentified mechanism, the viral RNA is recognized by host ribosomes and translated 5'→3' as a single polyprotein in association with the host endoplasmic reticulum (**Figure 1.15 C**). The viral polyprotein is then co- and post-translationally cleaved by host proteases as well as the viral serine protease, comprised of NS2B/NS3, as indicated in **Figure 1.15 A** (Arias *et al.,* 1993, Chambers *et al.,* 1991, Chambers *et al.,* 1993, Lindenbach & Rice 2001, Strauss & Strauss 2002).

The highly basic RNA binding C protein is translated first and requires two cleavages to facilitate release from prM and a membrane anchor/translocation signal sequence (Lindenbach & Rice 2001, Nowak *et al.,* 1989). The second cleavage occurs late in infection to allow for generation of mature C protein. prM is then translated and rapidly folds and associates with the immature E protein monomers. Analysis of TBEV glycoprotein expression suggest these interactions are required to stabilize and facilitate E protein folding (Allison *et al.,* 1995, Heinz & Allison 2003, Lindenbach & Rice 2003, Lorenz *et al.,* 2002, Wengler & Wengler 1989). It has been suggested that the main function of prM may be to prevent pH induced conformational changes following synthesis in an acidic environment (Guirakhoo *et al.,* 1992, Guirakhoo *et al.,* 1991, Kaufmann *et al.,* 2010, Li *et al.,* 2008, Yu *et al.,* 2008, Zhang *et al.,* 2003). The flavivirus nonstructural proteins are then translated, processed, and function in various aspects of RNA replication (see below).

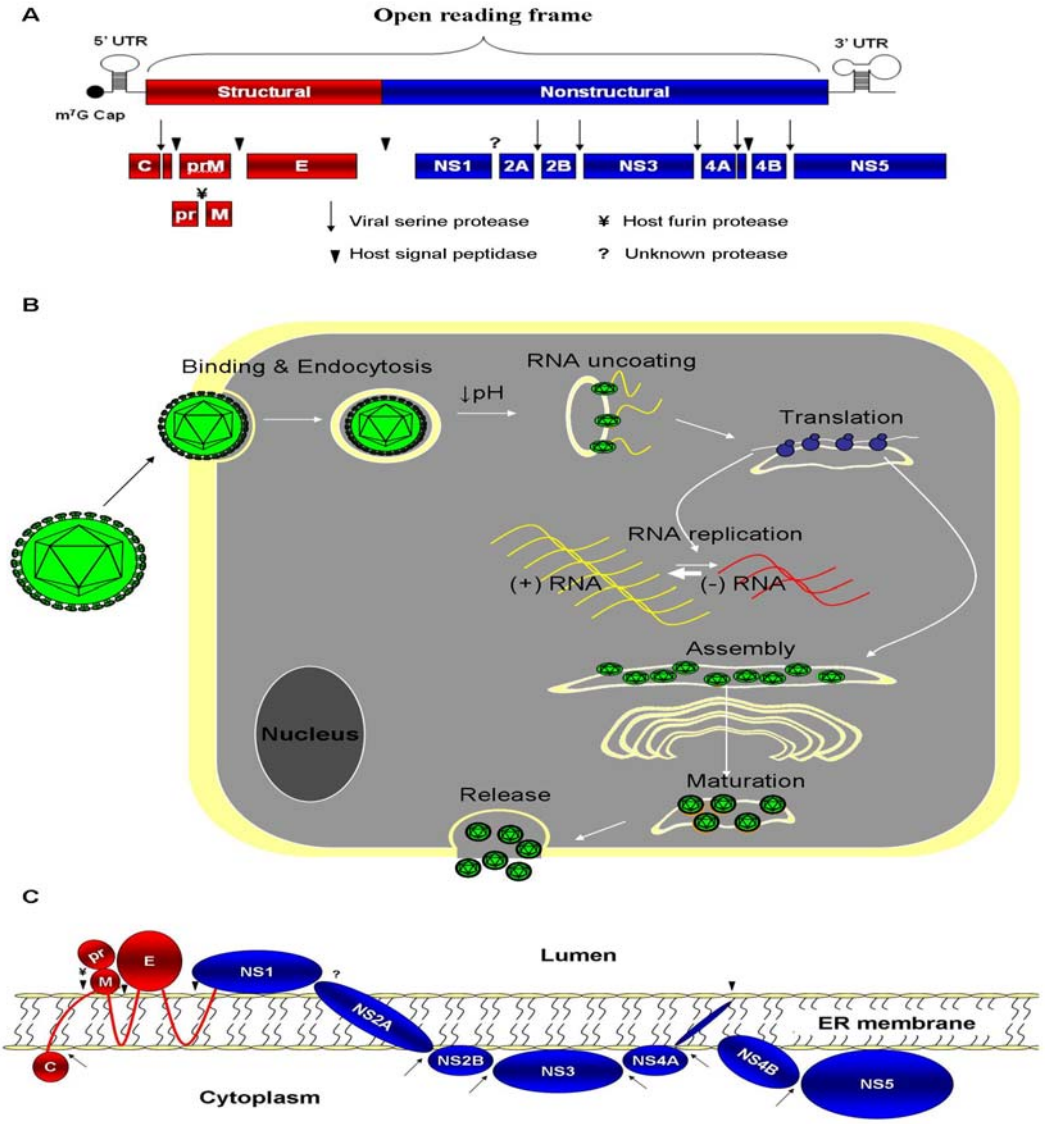


FIGURE 1.15 SCHEMATIC REPRESENTATIONS OF THE FLAVIVIRUS GENOME, LIFE-CYCLE, AND POLYPROTEN PRECURSOR.
A, Schematic representation of the flavivirus genome topology including the location of important polyprotein cleavage sites. B, The flavivirus life-cycle which is comprised of the following sequential steps: receptor binding, receptor mediated endocytosis, pH dependent fusion, RNA uncoating, translation, RNA replication, virion assembly, maturation, and release. C. Schematic representation of the topology of the flavivirus polyprotein topology relative to the membrane of the endoplasmic reticulum. (compiled and adapted from Lindenbach & Rice 2001, Lindenbach & Rice 2003).

Interestingly, flavivirus infection has been observed to result in the rapid formation of highly organized intra-cellular membrane complexes (Murphy *et al.,* 1968). The formation and coordinated rearrangements of these membrane structures, which are required for replication, may be regulated by the covalently attached precursor NS4A/NS4B and NS4A and NS4B cleavage products (Aligo *et al.,* 2009, Miller *et al.,* 2007, Roosendaal *et al.,* 2006). Replication occurs via asymmetric synthesis of negative and positive sense genomic RNA, by the viral replicative complex, in association with these complex membrane structures (Mackenzie *et al.,* 1996, Mackenzie *et al.,* 1998, Westaway *et al.,* 1999, Westaway *et al.,* 1997). Indeed, it has been proposed that the replicative intermediates of flavivirus genome synthesis may be sequestered in double membrane compartments (Uchil & Satchidanandam 2003). Structural and molecular analyses have also identified protein-protein interactions between NS2A and NS3 and between NS4A and NS1, which are required for replication potentially via anchoring/targeting the replicative complex to specific membrane compartments (Aligo *et al.,* 2009, Lindenbach & Rice 1999, Miller *et al.,* 2007). The flavivirus replicative complex is comprised of viral and potentially host proteins necessary for RNA synthesis including: NS1 which may function as a membrane anchor for the complex (Lindenbach & Rice 2003), NS3 which functions as the viral helicase (Gorbalenya *et al.,* 1989), and NS5 which functions as the viral RdRp (Koonin & Dolja 1993, Rice *et al.,* 1985) and methyl transferase (Egloff *et al.,* 2002, Koonin & Dolja 1993). Newly synthesized RNAs are encapsidated following cleavage of the C membrane anchor (Amberg *et al.,* 1994, Yamshchikov & Compans 1993), followed by budding into the endoplasmic reticulum (Mackenzie & Westaway 2001). These immature particles are trafficked through the golgi and exocytosed with maturation of the prM-E heterodimers likely facilitated by a host furin protease shortly before release (Lindenbach & Rice 2001).

D. Flavivirus reverse genetics

A number of infectious clones of flaviviruses (**Figure 1.16**) have been produced including: DENV (Kapoor *et al.,* 1995, Kinney *et al.,* 1997, Lai *et al.,* 1991), Kunjin virus (Khromykh & Westaway 1994), Muray valley encephalitis virus (Hurrelbrink *et al.,* 1999), JEV (Sumiyoshi *et al.,* 1995), tick-borne encephalitis virus (Campbell & Pletnev 2000, Mandl *et al.,* 1997), WNV (Rossi *et al.,* 2005, Shi 2002, Yamshchikov *et al.,* 2001), and YFVs Asibi (McElroy *et al.,* 2005, McElroy *et al.,* 2006, McElroy *et al.,* 2006) and 17D (Bredenbeek *et al.,* 2003, Rice *et al.,* 1989). Initial strategies required the bifurcation of the viral ORF into a dual plasmid system due to construct instability and toxicity in transformed bacteria cells. Generation of viral RNAs from clones produced in this way required an additional *in vitro* ligation step to restore the full complement of viral sequence to a single DNA molecule. However, the use of low copy number plasmids (<10 copies per bacterial cell) such as bacterial artificial chromosomes has greatly enhanced the ability to generate full length flavivirus clones.

The flavivirus genome organization confounds the insertion of synthetic/reporter genes due to the necessity to preserve the viral polyprotein ORF to facilitate successful translation of the viral nonstructural genes. GOI have been cloned into recombinant flavivirus genomes in a 3' orientation under the control of exogenous promoter sequences (**Figure 1.16**) (Khromykh & Westaway 1997, Pierson *et al.,* 2005, Rossi *et al.,* 2005, Scholle *et al.,* 2004). However, attempts to generate full length flavivirus-GOI constructs using similar strategies have resulted in construct instability and the rapid emergence of replication competent mutants with GOI sequence deletions (Pierson *et al.,* 2005) (Frolov, Zhao, Mason, and Higgs unpublished data).

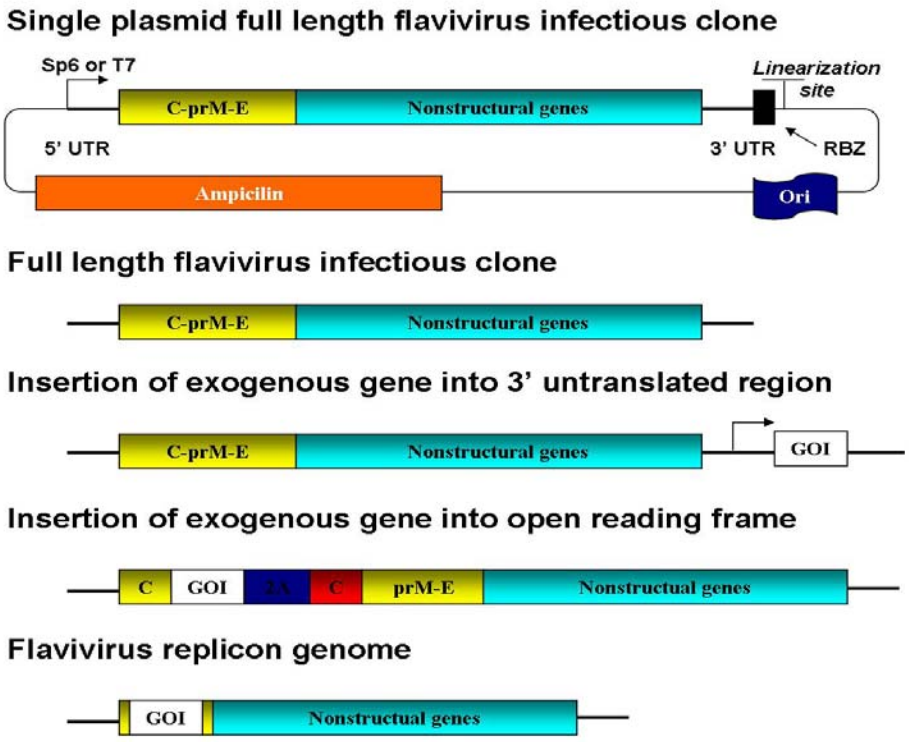


FIGURE 1.16 FLAVIVIRUS REVERSE GENETICS SYSTEMS
C-capsid, prM-premembrane, E-envelope, UTR-untranslated region, RBZ-Hepatitis Δ rybozyme, GOI-gene of interest, Ori-origin of plamid replication.

Khromykh and Westaway (1997) noted that the majority of the flavivirus structural genes were dispensable for replication and could be replaced with an exogenous gene, when fused in-frame, provided vital sequences involved in protein expression, replication, and preservation of membrane topolology (**Figure 1.15 C**) were maintained. These flavivirus replicons (**Figure 1.16**) have been used extensively to facilitate exogenous gene expression, study basic mechanisms of primary flavivirus infection, and analyze recombination. This technology has also been utilized for the construction of replicon based vaccine candidates for WNV, JEV, YFV, and DENV (Ishikawa *et al.,* 2008, Mason *et al.,* 2006, Shustov & Frolov 2010, Shustov *et al.,* 2007,

Widman *et al.,* 2008, Widman *et al.,* 2008). Using the replicon GOI expression strategy of Shustov *et al.,* (2008), insertion of additional genes between the native capsid and prM sequences, has allowed for the generation of full length flavivirus-reporter constructs with increased *in vitro/in vivo* stability (**Figure 1.16**) (McGee *et al.,* 2009) (Frolov unpublished data).

E. Superinfection, co-infection, and persistence

The seminal observation of flavivirus homologous interference (superinfection resistance) was made by Hoskins (1935) who observed that simultaneous or subsequent (within 48h) inoculation of *Macacus rhesus* with YFV viscerotropic (Asibi) and neurotropic strains could ameliorate the lethal phenotype of YFV Asibi (Hoskins 1935). However, since no detailed molecular techniques were available at this time it is difficult to determine if this resulted from superinfection resistance at the cellular level or via some immune/interferon mediated mechanism. *In vitro* analyses of DENV co- and superinfection indicate that the suppression of heterotypic replication can be induced very rapidly. Dittmar *et al.,* (1982) noted a 40% decrease in cells antigen positive for DENV 3 following simultaneous inoculation with DENV 1 and nearly complete resistance to DENV 3 if superinfection was separated by 20h (Dittmar *et al.,* 1982). A similar time dependent decrease in output titers was observed following DENV 2 and DENV 4 mixed/superinfections (Pepin *et al.,* 2008). This type of resistance can be quite long lived as DENV persistently infected C6/36 cells have been observed to produce significantly lower titers of a secondary challenge virus even when infections were separated by 50 days (Igarashi 1979). A recent study of WNV superinfection has begun to provide some insights into the mechanism of flavivirus superinfection resistance (Zou *et al.,* 2009). Zou *et al.,* (2009) demonstrated that replication of WNV was significantly decreased in cells harboring a persistently WNV replicon infection. Interestingly, this resistance could

be overcome by specific mutations in E and NS4A which appeared to increase particle-receptor binding interactions and the affinity for interactions with host factors required for replication. As such, it was suggested that flavivirus superinfection resistance may result from sequestering of critical host factors by the replicative complex of the primary virus, which exists in abundance relative to the secondary virus (Zou *et al.,* 2009).

Despite the fact that cells and potentially vertebrate host harboring a primary flavivirus infection appear to be somewhat refractory to challenge with a secondary virus several reports exist that indicate natural co-infections occur. Dual flavivirus infection in humans was first demonstrated by Gubler *et al.,* (1985) in Puerto Rico when two distinct isolates of DENV (serotypes 1 and 4) were isolated from a single febrile male (Gubler *et al.,* 1985). Concurrent infection of DENV 2 and DENV 3 in humans has also been demonstrated, in Taiwan, Sri Lanka and Brazil, using classical virus isolation and serological identification and/or sequence analyses (Araujo *et al.,* 2006, Wang *et al.,* 2003, Wenming *et al.,* 2005). Dual infections of DENV 1/DENV 3 and DENV1/DENV 2 have also been demonstrated in human serum samples collected in Brazil and New Caledonia respectively (dos Santos *et al.,* 2003, Laille *et al.,* 1991). Interestingly, the presence of dual infections of DENV comprised of recombinants and parental viruses have also been reported both in humans and mosquitoes (Aaskov *et al.,* 2007, Craig *et al.,* 2003).

Arthropod-borne flaviviruses have long been known to cause persistent life long infections in arthropod vectors thus allowing for multiple transmission events to occur. Although vertebrate infection with these viruses is generally considered to be acute, several studies have demonstrated the potential for flaviviruses (genus *Flavivirus*) to establish persistent infection in vertebrates. This should perhaps not be that surprising considering the natural life-cycle of the hepaciviruses and pestiviruses involves persistent

infections in humans and agricultural animals, respectively. Persistence has been documented for a number of flaviviruses including the un-vectored Modoc virus in hamster kidney cells (Johnson 1970), TBEV in non-human primates (Bochkova *et al.,* 1981, Fokina *et al.,* 1982, Malenko *et al.,* 1982, Pogodina *et al.,* 1981, Pogodina *et al.,* 1981, Pogodina *et al.,* 1981) and a number of Japanese encephalitis serocomplex viruses (Murray *et al.,* 2010, Pogodina *et al.,* 1983, Ravi *et al.,* 1993, Slavin 1943, Tesh *et al.,* 2005).

JEV and WNV have been shown to be capable of establishing persistent infection of the nervous system in humans and non-human primates with isolatable virus detected up to 5 ½ months after infection (Pogodina *et al.,* 1983, Ravi *et al.,* 1993). Interestingly, WNV appears to have some specific propensity to establish persistent infection in the kidneys resulting in virus being shed in the urine (Murray *et al.,* 2010, Pogodina *et al.,* 1983, Tesh *et al.,* 2005). In fact the detection of WNV RNA in human urine samples collected up to 6.7 years following primary infection suggests that persistent infection may result in the chronic symptomology experienced by some patients (Murray *et al.,* 2010). However, infectious virus has never been isolated from serum samples in any persistently infected animals/humans beyond the initial acute viremia (5-7 days post infection). Therefore the epidemiological implications in terms of the ability to facilitate natural arthropod-borne transmission remain unknown.

1.5 IMPETUS FOR THIS PROJECT

Live attenuated vaccine viruses have proven efficacy to protect humans and animals against viral disease via stimulating cellular and humoral immunity following the inoculation of a single dose. Empirically developed vaccines to poliovirus and YFV have significantly decreased epidemic transmission of these agents worldwide. The robust and protective responses elicited by these viruses are a direct result of replication and

subsequent presentation of immunizing epitopes in the vertebrate host. However, the *in vivo* replication of vaccines allows for evolution and potential reversion to a virulent phenotype. Analyses of post immunization samples, from patients with vaccine associated paralytic poliomyelitis, have identified heterotypic vaccine/vaccine and vaccine/non-vaccine recombinant viruses. As such, recombination has been implicated as a direct mechanism for virulent reversion of human vaccines, when administered as multivalent formulations and/or in wild-type endemic regions. Furthermore, the changing patterns of human activity coupled with recent explosions in the geographic distributions of competent mosquito vectors has resulted in increased potential for naturally occurring RNA arbovirus co-infections.

Recombination has now been identified in all three genera of the family Flaviviridae (*Flavivirus*, *Hepacivirus*, and *Pestivirus*) as well as in the family Togaviridae, genus *Alphavirus*. However, much regarding the potential for and consequence of RNA arbovirus recombination remains unknown. Therefore, the studies described in this dissertation were designed to evaluate the potential for and consequences of RNA arbovirus recombination. Given that when these studies were initiated, flavivirus recombination had never been quantified in the laboratory, experiments were designed using alphavirus reverse genetic systems to allow for detailed analysis of the conditions and sequences that would favor the generation and purification of recombinant viruses. It was thought that these systems would provide basic insights into the nature of RNA arbovirus recombination and allow for optimization of experimental detection methods towards the detection of recombinants in a similarly designed heterologous flavivirus system. Furthermore, experiments were designed to evaluate the *in vitro* and *in vivo* phenotypes of theoretical "worst-case" wild-type/vaccine recombinant viruses towards understanding the potential for a naturally occurring

flavivirus recombinant to establish virulent human-mosquito transmission cycles. A complete understanding of the potential for and consequences of RNA arbovirus recombination coupled with extensive characterization of the molecular constraints on the generation, fitness, and phenotype of viable recombinants will greatly enhance our ability to predict the consequences of naturally occurring wild-type/wild-type and wild-type/vaccine co-infections.

SPECIFIC AIM 1: To conduct a comprehensive comparative analysis of the sequences and conditions that favor RNA virus recombination using a chikungunya virus and Sindbis virus replicon/defective helper reverse genetics.

SPECIFIC AIM 2: To use the yellow fever virus live attenuated vaccine 17D reverse genetics and alphavirus optimized conditions to evaluate the potential for flavivirus homotypic recombination.

SPECIFIC AIM 3: To generate and evaluate the mosquito infectivity and vertebrate pathogenicity phenotype of flavivirus live attenuated vaccine/wild type experimental recombinant viruses using yellow fever virus 17D, ChimeriVax DEN4, and yellow fever virus Asibi.

CHAPTER 2: HOMOTYPIC AND HETEROTYPIC RECOMBINATION OF CHIKUNGUNYA AND SINDBIS VIRUSES

2.1 INTRODUCTION

Based on sequence analysis, functional similarities, and genome topology, it has been hypothesized that the alphaviruses may have arisen from a common ancestor approximately 4000 years ago (Koonin & Dolja 1993, Strauss & Strauss 1994, Strauss & Strauss 1997). Regression analysis of the mutation rates of members of the genus *Alphavirus* strongly support the emergence of the western equine encephalitis virus (WEEV) lineage as resulting from a dual recombination event occurring between ancestral "eastern-equine-encephalitis-like" and "Sindbis-like" viruses some 1600 years ago (Hahn *et al.,* 1988, Strauss & Strauss 1997, Weaver *et al.,* 1997). The generation of this recombinant lineage (that includes: WEEV, Fort Morgan virus, Buggy Creek virus, and Highlands J virus) was coincident with a double cross-over event (**Figure 2.1**) the first of which occurred within the E3 protein coding sequence and the second in the 3' untranslated region (UTR). The existence of a naturally occurring double recombinant is surprising given that such an event has never been empirically quantified under laboratory conditions. Nevertheless, this recombinant genome topology may have been paramount to the success of the WEEV recombinant lineage because analyses of rationally designed alphavirus chimeric genomes have identified major *in vitro*

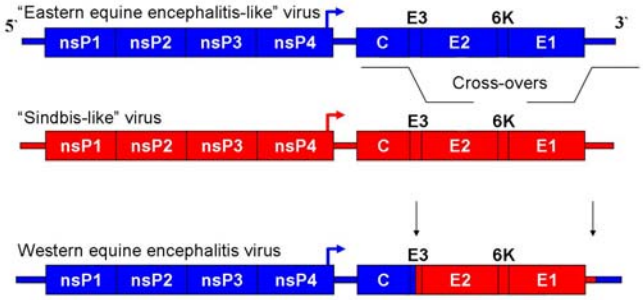


FIGURE 2.1 GENERATION OF WESTERN EQUINE ENCEPHALITIS VIRUS BY RECOMBINATION. (adapted from Hahn *et al.,* 1988).

attenuations associated with chimerization (Kuhn *et al.,* 1996, Lopez *et al.,* 1994, Smyth *et al.,* 1997, Strauss & Strauss 1997, Yao *et al.,* 1996). The effects of recombinant chimerization on replication competence must be evaluated on a case-by-case basis since reciprocal chimeras do not display equivalent phenotypes. Examples include: chimeric swaps of Ross River virus (RRV) and Sindbis virus (SINV) capsid coding sequences (Lopez *et al.,* 1994, Strauss & Strauss 1997), and viruses containing exchanges of the Semliki Forest virus (SFV) and SINV glycoprotein coding sequences (Smyth *et al.,* 1997). These analyses suggest that there may be need to preserve virus specific sequences in cis for functionality, which may explain the necessity of WEEV to preserve the "EEEV-like" 3' UTR sequence subsequent to either single double recombinant event or sequential single crossovers.

Initial laboratory studies of alphavirus recombination, modeled upon picornavirus recombinant systems, employed the use of high levels of co-infection of panels of complementary temperature sensitive (ts) SINV and SFV mutants (Atkins *et al.,* 1974, Burge & Pfefferkorn 1966, Pfefferkorn 1971). Recombinant viruses were undetected using these methodologies likely due either to lack of detection sensitivity (estimated limit of detection 10^{-4}), decreased recombinant fitness, and/or an inability to achieve sufficient levels of co-infection. Subsequent observations by Brawner and Sagik (1971) suggest the latter may be the reason for a lack of recombinant detection (Brawner & Sagik 1971). In these experiments, mixed parental inocula containing ultraviolet (UV) light inactivated wild-type (wt) and ts SINV particles, was pre-treated with $MgCl_2$ (as described by (Abel 1962, Kirvaitis & Simon 1965)) causing an aggregation of particles. This particle agglutination increased the probability of parental genome co-localization. Progeny virus could subsequently be isolated from cells infected by these aggregates that were competent for replication at the restrictive temperature, presumably resulting from

recombination events between the UV lesion inactivated and ts parental template genomes. However, as there were no detailed sequence analyses associated with these studies, it was difficult to determine if true recombination was observed.

Defective interfering (DI) SFV and SINV genomes have been observed following serial passage high multiplicity of infection (moi) (Lehtovaara *et al.,* 1981, Monroe & Schlesinger 1984). These DI genomes are characterized by major deletions of the protein coding sequences but retain the regulatory sequences required for replication and packaging and have been observed to incorporate cellular sequences to promote RNA synthesis (Pettersson 1981). DI genomes were the first definitive evidence to indicate that the alphaviral replicative complex was capable of generating recombinant genomes. However, these alphavirus recombinants are completely illegitimate and parasitic, in that they are only capable of replication in the presence of a full length virus from which they can exploit the replicative and packaging machinery. Since the studies described in this dissertation are concerned with the generation of fully functional recombinant viral progeny (containing a full complement of functional viral genes) the phenomenon of alphavirus DI RNA generation (as reviewed by (Strauss & Strauss 1997) will not be further discussed here.

Weiss and Schlesinger provided the seminal observations that alphavirus recombination was a mechanism capable of generating full length replication competent genomes (Schlesinger & Weiss 1994, Weiss & Schlesinger 1991). Analysis of cellular RNA following co-transfection of various SINV replicon/defective or mutated helper genomes resulted in the generation of recombinant "full-length-like" genomes. However, these recombinant genomes could not be enriched in cell culture and had to be biochemically extracted using non-denaturing electrophoresis conditions followed by re-transfection. Sequence analysis of purified putative recombinant genomes determined

that recombination was aberrantly homologous and characterized by insertions, deletions, and promoter duplications. Furthermore, early experiments in the development of packaging systems for alphavirus-vectored replicon vaccine platforms, using Venezuelan equine encephalitis virus (VEEV), identified a high incidence of recombination. Regeneration of replication competent infectious virus was readily detected via plaque purification ($<10^2$ – $2x10^5$ PFU/mL) (Pushko *et al.,* 1997, Vasilakis *et al.,* 2003). Sequence analysis of recombinant genomes suggested that although the amount of homologous sequence existing between two genomes plays a role in recombination efficiency, it may be that specific sequences in the packaging helper RNAs were responsible for recombination efficiency (Pushko *et al.,* 1997). As was previously observed for SINV recombinant genomes, VEEV recombinants contained aberrantly homologous cross-overs characterized by truncated exogenous gene sequence and subgenomic promoter (SGP) duplication.

Detailed analyses of SINV recombination using nonreplicative RNAs have identified several important characteristics of the mechanism of alphavirus recombination. Fusion of the recombinant template likely occurs during negative strand synthesis. Primary initiation of negative strand synthesis by the RNA dependent RNA polymerase (RdRp) occurs on the donor template (containing 3' regulator sequences necessary for negative strand synthesis initiation (Raju *et al.,* 1995)) followed by pausing upon encountering specific stem-loop structures in the donor sequence (Hajjou *et al.,* 1996). The RdRp/nascent RNA complex then disassociates from the donor template and re-initiates negative strand synthesis on the 5' acceptor template. This initiation was thought to be influenced by the length and sequence complexity/homology of the nascent RNA as well as by interactions between the RdRp and cryptic promoter sequences within the acceptor template (Hajjou *et al.,* 1996, Hill *et al.,* 1997). Additionally, the

introduction and removal of putative pausing and initiating sequences may influence the topology of recombinant genomes (Hajjou *et al.,* 1996, Hill *et al.,* 1997, Raju *et al.,* 1995). It was suggested that the viral RdRp may be competent to scan a variety of intracellular RNAs of both viral and cellular origin thus allowing for the generation of viral/viral and viral/cellular recombinant progeny (Hajjou *et al.,* 1996, Hill *et al.,* 1997, Raju *et al.,* 1995).

It is clear that recombination has played a significant role in alphavirus evolution and has facilitate the emergence of a new virus lineage, namely WEEV, which is capable of mosquito borne transmission and encephalitic disease presentation in humans. However, no systematic analysis of the conditions and sequences that favor alphavirus recombination, which would allow for the prediction of efficiency and success of such event in nature exists. An understanding of the complex interplay between co-infection, recombination, and competition between parental and recombinant genomes is vital to improve our ability to predict the potential emergence and phenotype of new alphaviruses and evaluate the effects of introducing live attenuated replication competent vaccines into alphavirus endemic regions.

2.2 AIMS AND HYPOTHESES

The **aim of these studies** was to conduct a comprehensive comparative analysis of the sequences and conditions that favor RNA virus recombination using chikungunya virus (CHIKV) and SINV replicon/defective helper reverse genetics. The hypotheses of these studies were as follows:

A. *Rational design of CHIKV replicon/defective helper systems should allow for selective packaging of only replicon and recombinant genomes and not defective helper genomes.*

B. *Engineering of fluorescent protein reporter gene sequences into defective helper genomes should allow for rapid phenotypic distinction between packaged helper genomes (if this occurs) and true recombinant genomes.*

C. *Recombination events occurring between replicon and defective helper genomes should result in the generation of "full-length-like" sized viral genomes containing a genetic sequence capable of expressing a complete viral protein complement.*

D. *The genome topology of CHIKV and SINV recombinants will likely be characterized by deletions, insertions, and/or promoter duplications indicating an aberrantly homologous mechanism.*

E. *Recombinant alphavirus genomes will be able to be readily purified using optimized cell culture passage protocols.*

F. *Co-electroporation of ten-fold serial dilutions of in vitro transcribed viral RNAs will allow for accurate quantification of the minimum levels of co-infection required to facilitate alphavirus recombination.*

G. *The efficiency of alphavirus recombination (minimum co-infection) will be related to specific functional constraints on the sequences participating in the recombinant event i.e. inter-genic recombination should be more efficient than intra-genic recombination.*

2.3 MATERIALS AND METHODS

A. Cells

Baby hamster kidney (BHK-21) cells (generously provided by Dr. Ilya Frolov) were maintained in alpha minimal essential medium (αMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1%

penicillin streptomycin (pen-strep), 1% L-glutamine (L-glu; Cellgro® Mediatech, Inc.) and vitamins and maintained at 37° C in the presence of 5% CO_2. African green monkey kidney (Vero) cells (generously provided by Dr. David Beasley) were grown in minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 2% bovine growth serum (BGS; Hyclone, Logan, UT), 1% pen-strep, 1% non-essential amino acids (NEAA; Sigma-Aldrich, St. Louis, MO), and 1% L-glu and maintained at 37° C in the presence of 5% CO_2. *Aedes albopictus* (C6/36) cells (generously provided by Dr. Robert Tesh) were grown in Liebovitz L-15 media supplemented with 10% FBS, 10% tryptose phosphate broth (TPB; Sigma-Aldrich, St. Louis, MO) 1% pen-strep, and 1% L-glu and maintained at 28ºC.

B. Plasmid constructs

All plasmid constructs including replicon, defective helper, and full length viral genomes (hereafter also referred to as infectious clones (i.c.(s)) or reverse genetic systems) were generated using standard polymerase chain reaction (PCR) based cloning methodologies (Sambrook 1989). All CHIKV constructs (nomenclature: each unique plasmid (p) name is associated with a unique p# that is indicated in parenthesis the first time the full plasmid name is used) were generated or derived from the parental full length constructs **pCHIK-LR i.c.** (p142), **pCHIK-LR-5'-GFP i.c.** (p145), and **pCHIK-LR-5'-Cherry** (p188) (whose construction was described by (Tsetsarkin *et al.,* 2006)) or parental replicons **pCHIK-LR-GFP-Rep** (p221) and **pCHIK-LR-Cherry-Rep** (p222) (**Figure 2.2**). Briefly, these complementary DNA (cDNA) clones were generated by RT (reverse transcription)-PCR amplification of RNA derived from the CHIKV LR2006 OPY1 strain provided by World Reference Center for Arboviruses at the University of Texas Medical Branch, Galveston, TX (Dr. Robert Tesh). Amplicons were cloned individually or sequentially into a modified pSinRep5 backbone (Invitrogen, Carlsbad,

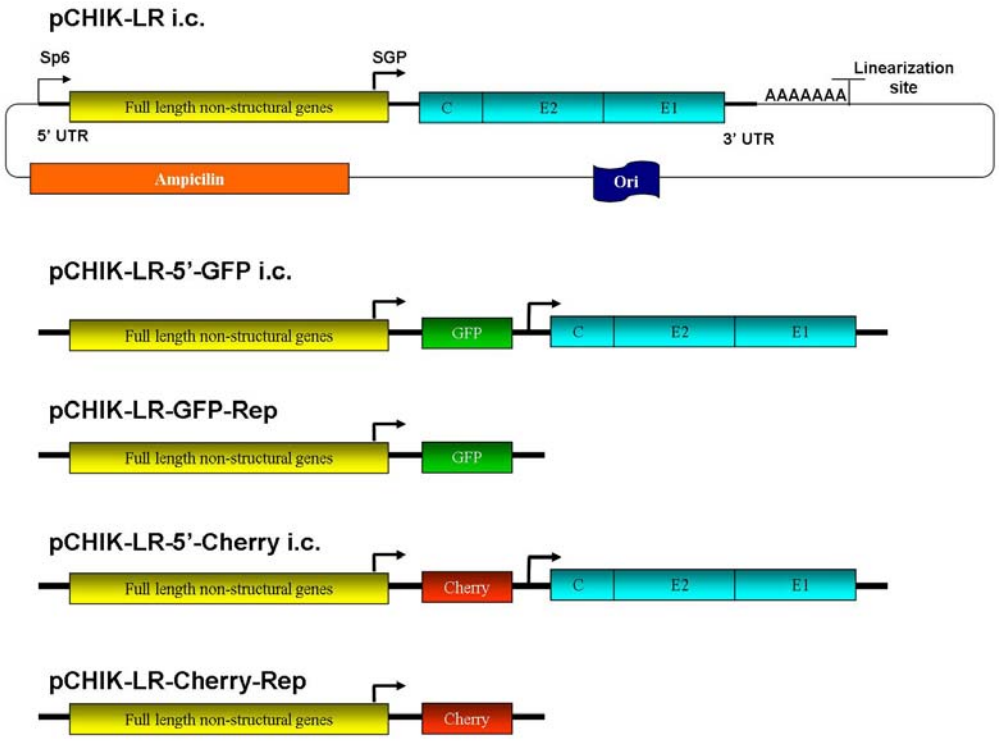


FIGURE 2.2 SCHEMATIC REPRESENTATION OF PARENTAL CHIKUNGUNYA VIRUS CONSTRUCTS.
All CHIKV constructs were cloned into a modified pSinRep5 backbone under the control of a Sp6 promoter sequence. C-capsid, E1/E2-envelope glycoproteins, i.c.-infectious clone, LR-LaReunion, Ori-origin of plasmid replication, Rep-replicon, SGP-subgenomic promoter, UTR-untranslated region.

CA), under the control of an exogenous Sp6 promoter (**Figure 2.2**). SINV replicon constructs were generated using introduction of exogenous genes into the pSinRep5 multiple cloning site. Brief descriptions of the construction (oligonucleotide primers (pr) and important restriction sites) or source of individual constructs are listed below:

pCHIK-LR-5'Δ-Rep (p300) was generated by single fragment ligation following digestion of p142 with BstB*I*.

pCHIK-LR-5'Δ-GFP-Rep (p301) was generated by single fragment ligation following digestion of p145 with BstB*I*.

pCHIK-LR-3'Δ-Rep (p302) was generated by three fragment cloning of V (vector) p142 digested with Sac*II*, Xho*I*, and calf intestinal phosphatase (CIP), I_1 (insert) p142 digested with Ase*I* and Sac*II*, and I_2 generated by PCR amplification of p142 (pr 41855 Bgl*I*F/41855 Xho*I*R) digested with Ase*I* and Xho*I*.

pCHIK-LR-3'Δ-Cherry-Rep (p303) was generated by two fragment cloning of V p188 digested with Bgl*I* and CIP and I_1 p302 digested with Bgl*I*.

pCHIK-LR-FL-ST-Help (p304) was generated by removal of 6,897nt of nonstructural gene coding sequence of p142 following digestion by Nhe*I* and religation of the V fragment.

pCHIK-LR-E2/E1-Help (p305) was generated by two fragment cloning of V p304 digested with Nhe*I*, Xho*I*, and CIP and I_1 fusion PCR amplicon of sequences from template p304 (pr CHIK LR Nhe*I*F/CHIK LR ΔC fusion R & CHIK LR ΔC fusion F/CHIK LR Xho*I*R) digested with Nhe*I* and Xho*I*.

pCHIK-LR-Cap-Help (p306) was generated by two fragment cloning of V p304 digested with Nhe*I*, Xho*I*, and CIP and I_1 fusion PCR amplicon of sequences from template p304 (pr CHIK LR Nhe*I*F/CHIK LR C-3'UTR fusion R & CHIK LR C-3'UTR fusion F/CHIK LR Not*I*R) digested with Nhe*I* and Xho*I*.

pCHIK-LR-Capsid-Rep (p308) was generated by two fragment ligation of V p142 digested with Bgl*I* and CIP and I_1 p306 digested with Bgl*I*.

pCHIK-LR-5'Δ-GFP-Help (p309) was generated by digestion of p301 with Nhe*I* and relegation of the V fragment.

pCHIK-LR-E2/E1-GFP-Help (p310) was generated by two fragment cloning of V p304 digested with Nhe*I*, Xho*I*, and CIP and I_1 fusion PCR amplicon of sequences from templates p145 (pr CHIK LR NheIF/ CHIK LR ΔC fusion R) and p304 (pr CHIK LR ΔC fusion F/CHIK LR Xho*I*R) digested with Nhe*I* and Xho*I*.

pCHIK-LR-Cap-Cherry-Rep (p311) was generated by three fragment cloning of V pCHIK-LR-Cap-Rep (p308) digested with Age*I*, Bgl*I*, and CIP, I_1 p188 digested with Age*I* and Bgl*I*, and I_2 digested with Bgl*I* (two sites).

pSinRep5-Cherry (p322) was generated by two fragment ligation of V pSinRep5 digested with Mlu*I*, Pme*I* and CIP and I_1 p222 digested with Asc*I* and Pml*I*.

pCHIK-LR-GFP-2A-FL (p324) was generated by two fragment cloning of V p145 digested with Asc*I*, Sfi*I*, and CIP and I_1 fusion PCR amplicon of sequences from templates pYFV-17D-GFP-RBZ i.c. (p1612; pr GFP Asc*I*F/2A Cap Fusion R) and p145 (pr 2A Cap Fusion F/41855 Mlu*I*R) digested with Asc*I* and Sfi*I*.

pCHIK-LR-5'-GFP-2A-Help (p325) was generated by two fragment cloning of V p310 digested with Asc*I*, Sfi*I*, and CIP and I_1 fusion PCR amplicon of sequences from templates pYFV-17D-GFP-RBZ i.c. (p1612; pr GFP Asc*I*F/2A Cap Fusion R) and p145 (pr 2A Cap Fusion F/41855 Mlu*I*R) digested with Asc*I* and Sfi*I*.

pCHIK-LR-Cherry-Rep2 (p326) was generated by site directed mutagenesis of p222 to introduce a unique Hind*III* restriction site 7457-7462nt using pr 41855 NS F1/NS4 Hind*III* mut R & NS4 Hind*III* mut F/41855 Xmn R and restriction sites Age*I* and Pme*I*.

pCHIK-LR-GFP-EcoR*V*-Help (p328) was generated by two fragment ligation of V p310 digested with Pme*I*, Sfi*I*, and CIP and I_1 fusion PCR amplicon of templates p145 (pr GFP 3'end F/Cap EcoR*V* R & Cap EcoR*V* F/41855 Mlu*I* R) digested with Pme*I* and Sfi*I*.

pCHIK-LR-Hind*III*-Rep (p337) was generated by one fragment ligation following digestion of p326 with Asc*I* and Pme*I* to remove the Cherry fluorescent protein coding sequence and treatment with T4 polymerase to generate a blunt end at the Asc*I* site.

pCHIK-LR-EcoR*V*-Help (p338) was generated by site directed mutagenesis of p304 using pr CHIK SP6 F2/Cap EcoR*V* mut R & Cap EcoR*V* mut F/41855 Mlu*I* R and restriction sites Nhe*I* and Sfi*I*.

pCHIK-LR-5'Δ-Help (p341) was generated by removal of 6,897nt of non-structural gene coding sequence of p300 following digestion by Nhe*I* and religation of the V fragment.

pSinRep5 was obtained from Invitrogen

pSinRep5-Cherry-CHIK-3'UTR (p345) was generated by two fragment ligation of V pSinRep5 digested with Mlu*I*, Not*I*, and CIP and I_1 p222 digested with Asc*I* and Not*I*.

pCHIK-LR-GFP-SIN-3'UTR (p346) was generated by two fragment ligation of V p221 digested with Pme*I*, Not*I*, and CIP and I_1 pSinRep5 digested with Pml*I* and Not*I*.

pCHIK-5'UTR-CHIK-SGP-GFP-SIN-SGP-Cherry-CHIK-3'UTR (p351) was generated by two fragment ligation of V p345 digested with BamH*I* (with T4 polymerase treatment), Sac*I*, and CIP and I_1 p328 (CHIK-LR-GFP-Cap-EcoR*V*-Help) digested with Sac*I* and Pme*I*.

pSIN-5'UTR-CHIK-SGP-GFP-SIN-SGP-Cherry-CHIK-3'UTR (p352) was generated by two fragment ligation of V p351 digested with Sac*I*, BamH*I*, and CIP and I_1 p1411 digested with Sac*I* and BamH*I*.

pSIN-5'UTR/SGP-CHIK-Help (p355) was generated by three step cloning:

1) Insertion of the CHIKV structural genes into a SINV replicon, to generate **pCHIK-ST-SIN-UTRs/SGP-Rep** (p347) by four fragment ligation of V p1411 digested with BamH*I*, Xho*I*, and CIP, I_1 pSinRep5 digested with BamH*I* and Xba*I*, I_2 PCR amplicon of p142 (pr CHIK-ST-

Begin Xba*I*F/CHIK-ST-End Pml*I*R) digested with Xba*I* and T4 polynucleotide kinase, and I_3 pSinRep5 digested with Pml*I* and Xho*I*.

2) Removal of the majority of the SINV nonstructural genes of p347, to generate **pCHIK-ST-SIN-UTRs/SGP-Help** (p348) by two fragment ligation of V p347 digested with Sac*I*, BamH*I*, and CIP and I_1 p1411 digested with Sac*I* and BamH*I*.

3) Re-insertion of the CHIKV 3'UTR, to generated p355 by two fragment ligation of V p304 digested with Sac*I*, Sfi*I*, and CIP and I_1 p348 digested with Sac*I* and Sfi*I*.

pSIN-FL-Help (pToto1411.01-BspM*II*-BamH*I* (1411) was generously provided by Dr. Ilya Frolov.

C. Basic molecular cloning methodologies

Extraction of viral RNA from infectious supernatant

Viral RNA was extracted from cell culture supernatant samples, following virus inoculation or RNA electroporation, using the QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA) in accordance with manufacturer protocols. Briefly, 140µL of sample was added to 560µL buffer AVL (supplemented with carrier RNA) and mixed thoroughly by pulse vortex ~15s. Following a 10m incubation at room temperature (rt), 560µL ethanol (100%) was added followed by a second round of pulse vortexing ~15s. RNA was then bound to the membrane of the QIAamp Mini spin column via centrifugation at 8000 rpm. The membrane bound RNA was then subjected to two sequential washes with buffers

AW1 and AW2 followed by a 60µL elution (AVE buffer) into sterile nuclease-free eppendorf tubes. RNA samples extracted in this way were stored at -80ºC.

Complementary DNA synthesis

Viral RNA was reverse transcribed to generate cDNA using the SuperScript® III First-Strand Synthesis Supermix Kit (Invitrogen, Carlsbad, CA). Viral RNA (3.0µL; extracted using the QIAamp protocol) was combined, in a 200µL PCR tube, on ice with 1.0µL each annealing buffer and random hexa-primer (50ng/µL) and 5.0µL nuclease-free water (nfw). The reaction mixture was incubated at 65ºC for 5m and then immediately placed on ice for at least 1m. In a second tube, on ice, enzyme master mix solution was prepared, which contained SuperScript III/RNaseOUT Enzyme mix and reaction buffer. Enzyme master mix was then added (12.0µL per reaction) to the primer annealed RNA reaction mixture. Reverse transcription was performed using the following thermo-cycling conditions: 25ºC for 10m; 50ºC for 50m; 85ºC for 5m; followed by incubation at 4ºC for up to 24h, and cDNA was stored at -20ºC.

Polymerase chain reaction amplification of cloning fragments

Cloning fragments that required amplification or modifications were generated using standard PCR methodologies. Priming oligonucleotides (**Table 2.1; Table 2.2**) were designed using the GeneRunner 3.05 software suite and supplied by Sigma-Genosys (Houston, TX). Amplification was performed as follows: individual PCR reactions were comprised of 2.5 Units (U) (1.0µL) *Pfu* DNA dependent DNA polymerase (DdDp; Stratagene, La Jolla, CA), 3.0µL cDNA reaction or ~25ng cloned DNA, 180ng each of forward and reverse primer, 1.5mM (6.0µL) deoxyribonucleotides mix (dNTPs; dATP, dTTP, dCTP, dGTP), and nfw (to a total volume of 60.0µL). PCR amplification was performed using the following conditions: 94ºC for 3m, followed by 25-40 cycles of

TABLE 2.1 SPECIFIC PRIMERS INVOLVED IN AMPLIFICATION, CLONING, AND SEQUENCING OF CHIKUNGUNYA AND SINDBIS VIRUS CONSTRUCTS.

Name	Sequence	Strain	Position
Rep5-R2	5'-CACGGAAATGTTGAATACTC	N/A	N/A
SinRep5-Sac*V*-F	5'-TATAGTCCTGTCGGGTTTC	N/A	N/A
Chik-Sp6-F2	5'-CTAGCATCGATTTAGGTGACACTATAGA TGGCTGCGTGAGACACACGT	N/A	N/A
CHIK-ns-F5	5'-ATTGGGCAGATGAGCAGGTACT	CHIK-Ross	669-690
CHIK-ns-R5	5'-GTAAAGCGTTGACCCGACTGAGA	CHIK-37997	898-920
41855-Ngo-F	5'- GTCATTCTCGGTGTGCACAT	CHIK-Ag41855	1055-1074
41855-Ngo-R	5'- GGCGTGACTTCTGTAGCA	CHIK-Ag41855	1115-1134
CHIK-ns-F6	5'-TCCCAGCCGAATTTGACAGC	CHIK-Ag41855	1408-1427
CHIK-nsF3	5'-TCAACTATAATCACAACATCTGCA	CHIK-Ag41855	2563-2586
CHIK-nsR3	5'-TGGCTTGTTGTACTCATTTG	CHIK-37997	2679-2698
41855-Bgl*II*-F	5'-GGAAGTGGAACATCAACA	CHIK-Ag41855	3411-3428
CHIK-nsR4	5'-GCGTTGACTACACACTCTTC	CHIK-37997	4121-4140
41855-nsF1	5'-TGCCGTTACGCCATGACTC	CHIK-Ag41855	4865-4883
CHIK-nsR2	5'-TTGACTTTCTGCACTCCTTC	CHIK-37997	4967-4986
41855-Eag-R	5'-TCTCCCTCGCCTTCTTCTG	CHIK-Ag41855	5281-5299
41855-Hind-R2	5'-TAACTCGTCGTCCGTGTCT	CHIK-Ag41855	5626-5644
41855-nsF2	5'-AGTCAGTACGCCAGTCAGTG	CHIK-Ag41855	5709-5728
41855-nsF5	5'- ATATCTAGACATGGTGGAC	CHIK-Ag41855	6106-6124
41855-nsF3	5'- ATACTGGGAAGAATTTGCTG	CHIK-Ag41855	6373-6392
41855-nsR1	5'-TATCAAAGGAGGCTATGTC	CHIK-Ag41855	6776-6794
41855-nsR2	5'-CCTAAATCCTCTAACAGCA	CHIK-Ag41855	6831-6849
CHIK-LR Nhe*I*F	5'-GAACAGCTTGCAGAGTGGC	CHIK-LR2006-OPY1	7215-7233
CHIK-LR Hind *III* F	5'-CTAGCGGCAGGTGACGAACA	CHIK-LR2006-OPY1	7271-7290
CHIK-LR nsP4F1	5'-ACGAACAGGGCTAATTGATG	CHIK-LR2006-OPY1	7339-7358
CHIK-LR Sgp R	5'-TATTTAGGACCGCCGTAC	CHIK-LR2006-OPY1	7483-7500
41855-Asc*I*R	5'-AGATGGCGCGCCTGATTAGTGTTTAGGTAC	CHIK-LR2006-OPY1	7543-7561
142 Cap 5' end F	5'-ATGGAGTTCATCCCAACCC	CHIK-LR2006-OPY1	7567-7585
CHIK-LR Cap F1	5'-CCGCGCCCTACTATCCAAG	CHIK-LR2006-OPY1	7627-7645
CHIK-LR Cap F2	5'-GCAGGAATCGGAAGAATAAG	CHIK-LR2006-OPY1	7751-7770
CHIK-LR EcoR*V* R	5'-TACCTTCGTGCTTGACTTCG	CHIK-LR2006-OPY1	7911-7930
41855-Bgl*I*F	5'-GTAATGAAACCAGCACACGT	CHIK-Ag41855	7966-7985
41855-Mlu*I*R	5'-AGCTCCTCCTAAGACTATG	CHIK-Ag41855	8244-8269
CHIK-LR-E2 R1	5'-GGTGGGGAGAACATGTTAAG	CHIK-LR2006-OPY1	8511-8530
41855-stF2	5'-GTAGCACTAGAACGCATCAG	CHIK-Ag41855	8635-8654
CHIK-LR-Xho*I*F	5'- TCCTGACCACCCAACACTCCTG	CHIK-LR2006-OPY1	9405-9426
41855-Xho*I*R	5'-ATACTTATACGGCTCGTTG	CHIK-Ag41855	9537-9555
41855-stF1	5'-ACTGCTTCTGCGACGCTGA-3`	CHIK-Ag41855	10271-10289
41855-stR1	5'-TCAACTTCTATCTCAGCTTC-3`	CHIK-Ag41855	11014-11033
41855-Pac-F	5'-CTGTTTAATTAACTTGACAACTAAGTATGA	CHIK-LR2006-OPY1	11314-11331
41855-Xmn-R	5'-ACCTACATCTCTTCCGTTTG-3`	CHIK-Ag41855	11672-11691
CHIK-LR Not*I*R	5'- ATTGTCTCATGAGCGGATAC	CHIK-LR2006-OPY1	11971-11990
SIN ns4 F1	5'-AGACTGAAAGGCCCTAAGG	SinRep5	N/A
SIN ns4 F2	5'-GACGACGAGCAAGACGAA	SinRep5	N/A
SIN ST F2	5'-CACCCTGCAGTATGTATCCG	p1411	N/A
SIN Cap R1	5'-ACCACTCTTCTGTCCCTTC	p1411	N/A
SIN Cap R2	5'-CAATGACTAGGGCACTGACG	p1411	N/A
SIN 3'UTR R	5'-CGGGGATCTAATGTACCAGC	SinRep5	N/A

Chikungunya sequence positions are relative to the LR2006-OPY1 genome.
N/A-not applicable.

94ºC for 20s; 54-56ºC for 20s; 70ºC for 1m/amplicon Kb, followed by a final elongation at 70ºC for 5-10m, and stored at either 4ºC or -20ºC.

Fusion PCR

Fusion of specific DNA sequences was achieved using fusion PCR (**Figure 2.3**). Briefly, this process was facilitated using rational primer design (**Table 2.2**) that results in the incorporation of terminal nucleotide duplications during first round PCR amplification to allow for annealing complementarities during second round PCR amplification. The reverse primers of the 5' templates were designed to contain a 20nt (nucleotides) overhang, the sequence of which is comprised of the 5' 20nt of the 3' template. The 3' template forward primers were designed to be complementary to their respective 5' reverse primers. Amplification of the 5' and 3' templates using primers designed in this way produced amplicons that contain 40nt of complementarity. These amplicons were then mixed in equimolar ratios and re-amplified using an amplicon A specific forward primer and an amplicon B specific reverse primer which resulted in the direct fusion of the two sequences of interest.

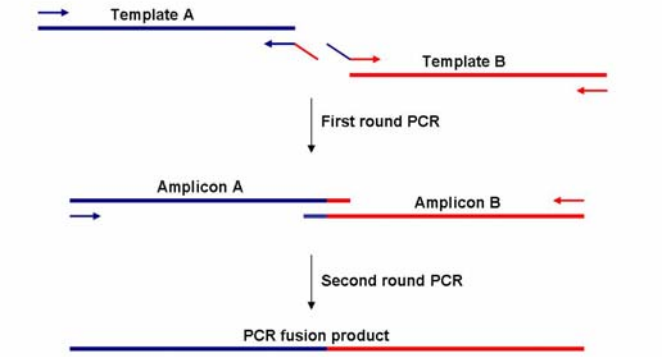


FIGURE 2.3 BASIC EXPERIMENTAL PROTOCOL: PCR MEDIATED FUSION OF DNA SEQUENCES.

Perpetration of cloning fragments for ligation

Cloning fragments were classified as either vector (V), those fragments which contain sequences necessary for replication and selection in bacteria cells, or inserts, (I) all other cloning fragments. DNA fragments were further processed in various ways to

TABLE 2.2 SPECIFIC PRIMERS INVOLVED IN AMPLIFICATION OF NON-VIRAL SEQUENCES, INSERTION OF RESTRICTION SITES, FUSION PCR, SITE DIRECTED MUTAGENESIS, AND RECOMBINANT AMPLICON SEQUENCING.

Name	Sequence
GFP 5'end R	5'-TCCTCGCCCTTGCTCACCAT
GFP 3'end F	5'-GCATGGACGAGCTGTACAAG
GFP-Asc F	5'-CCTTCGGCGCGCCATGGTGAGCAAGGGCGAG
GFP-Pme R	5'-CCTTCGTTTAAACTACTTGTACAGCTCGTCCA
Cherry Pst F	5'-GTGACCGTGACCCAGGACTC
Cherry Pst R	5'-GCGCAGCTTCACCTTGTAG
FMDV2A 3' end R	5'-AGGGCCAGGGTTGCTCTCCA
2A Cap fusion F	5'-TGGAGAGCAACCCTGGCCCTATGGAGTTCATCCCAACC
2A Cap fusion R	5'-GGGTTGGGATGAACTCCATAGGGCCAGGGTTGCTCTCCA
Cap EcoR*V* mut F	5'-GGCAACTTGCCCAGCTGATATCAGCAGTTAATAAACTGA
Cap EcoR*V* mut R	5'-TCAGTTTATTAACTGCTGATATCAGCTGGGCAAGTTGCC
NS4 Hind*III* mut F	5'-AGATCCAACTTCGAGAAGCTTAGAGGACCCGTCATAACTTT
NS4 Hind*III* mut R	5'-CAAAGTTATGACGGGTCCTCTAAGCTTCTCGAAGTTGGATCT
CHIK-LR Cap-3'UTR Fusion F	5'-CGAGGGGGCCGAAGAGTGGTAGCTTGACAATTAAGTATGAAGG
CHIK-LR Cap-3'UTR Fusion R	5'-CCTTCATACTTAATTGTCAAGCTACCACTCTTCGGCCCCCTCG
CHIK-LR ΔC Fusion F	5'-GAGTTCATCCCAACCCAAAACACAAATCAAAAGAAGCAG
CHIK-LR ΔC Fusion R	5'-CTGCTTCTTTTGATTTGTGTTTTGGGTTGGGATGAACTC
CHIK-LR-Pme F	5'- CCTTCGTTTAAACCATGGCCACCTTTGCAAG
CHIK-LR-Asc R	5'-AGATGGCGCGCCTGATTAGTGTTTAGATAC
SIN Cap Asc*I*F	5'-TAGCTGGCGCGCCATGAATAGAGGATTCTTTAAC
SIN ST-end Pme*I*R	5'-TAGCTGTTTAAACTCATCTTCGTGTGCTAGTCAG
SIN Cherry Xba*I*F	5'-CACTCTCTAGATGGTGAGCAAGGGCGAGGA
SIN Cherry Mlu*I*R	5'-CACTCACGCGTTACTTGTACAGCTCGTCCATGC
T_7	5'-AATACGACTCACTATAGGGC
T_3	5'- ATTAACCCTCACTAAAGGGA

generate desirable ends for ligation. Enzymatic modifications (New England Biolabs, Ipswich, MA) included digestion using various restriction endonucleases, generation of blunt ends via treatment with T4 DNA polymerase, and 5' phosphorlyation with T4 polynucleotide kinase (PNK).

Ligation

Following enzymatic processing, individual cloning fragments were isolated by agarose gel electrophoresis (Tris base-acetic acid-EDTA (TAE) running buffer) followed by extraction using either the QIAquick® Gel Extraction Kit (Qiagen) or the Zymoclean™ Gel KNA Recovery Kit (Zymo Research Corp., Orange, CA). Briefly,

following size fractionation, individual DNA fragments were excised from 1% agarose TAE gels using sterile scalpels and melted into approximately three volumes of the appropriate extraction buffer at 56ºC for 5-10m. QIAquick extractions were supplemented with 10µL 3M sodium acetate solution (NaAc) and 150µL 99% isopropanol. DNA was then bound to the appropriate column, washed with PE buffer (Qiagen) and eluted into 10-30µL nfw. Immediately following elution, 1.0µL of each extraction was re-fractionated by 1% agarose electrophoresis to assess yield, purity, and visually determine the volume to be used in the subsequent ligation reaction. Ligation of cloning fragments was performed using the T4 DNA ligase (Stratagene) and reactions were incubated at 12ºC for 15m-24h depending on the complexity of the reaction. Ligation reactions contained 1.0-9.0µL DNA fragments, 2.0µL ligation buffer (comprised of 9:1 10X ligase buffer:10mM ATP), 0.7µL T4 DNA ligase, and nfw to a total volume of 20.0µL. Ligation reactions were then transformed into competent *Escherichia coli* MC1061 cells.

Preparation and transformation of competent E. coli MC1061 cells

E. coli (hereafter referred to as MC1061 cells) were grown in 1.5xYT (13.0g tryptone, 6.5g yeast extract, 5.0g NaCl/liter water) broth/agar (supplemented with bacteriological grade agar powder). MC1061 glycerol stocks were generated by combining 1:1 cells from a fresh ~3.0mL overnight (o/n) culture, grown on an orbital rocker (300 rpm) at 37ºC, with 1.5xYT media supplemented with 15% glycerol. These stocks were stored at -80ºC.

Plasmid constructions and ligation reactions were always transformed into freshly made competent MC1061 cells as follows: A seed culture (2-3mL) was inoculated with 50-75µL of MC1061 glycerol stock and incubated at 37ºC with agitation (300 rpm) for 5-6h. Upon reaching a sufficient level of turbidity, 1.0mL of the seed culture was directly

inoculated into 50mL sterile 1.5xYT broth. This transformation culture was incubated at 37ºC with agitation (300 rpm) for 45m-1 ½ h until an optical density 0.6-0.8 OD_{600} was achieved. The transformation culture was then transferred to ice and chilled for at least 10m. Cells were pelleted at 4000 rpm in a refrigerated centrifuge pre-chilled to 4ºC, resuspended in 10mL B1 (0.01M MOPS, 0.01M RbCl, pH=7.0), recentrifuged as above, resuspended in 10mL B2 (0.1M MOPS, 0.05M $CaCl_2$, 0.01 RbCl, pH=6.5) and incubated on ice 10m-2h, recentrifuged as above, and finally resuspended in 200µL B2 per reaction (supplemented with 3.0µL DMSO per reaction; a maximum of 8 reactions could be generated per 50mL culture).

Two-hundred microliter aliquots of competent cells were placed in pre-chilled 15mL conical tubes and stored on ice (up to 8h) until being used for transformation. Individual plasmids (~10ng) or ligation reactions (15.0µL) were directly added to competent MC10610 cells, pulse vortexed, and incubated on ice for 10-30m. Cells were then heat shocked at 43.5ºC for 45s and placed back on ice for ~2m. Following heat shock, 3.0mL sterile 1.5xYT media was added to each transformation reaction followed by incubation at 37ºC for 30-60m. Cells were then pelleted as described above, media aspirated, resuspended in ~50µL and plated on 1.5xYT agarose plates supplemented with 1.0µL/1.0mL ampicillin (50mg/mL stock). Transformation plates were then incubated o/n at 32-37ºC. Individual colonies were screened for the presence of the desired plasmid construct by PCR analysis using *Taq* DNA polymerase as described above.

Confirmation of plasmid topology and sequence

Selected colonies identified as putatively positive for the desired plasmid constructs were subjected to further confirmatory analysis. Plasmid-bearing MC1061 cells underwent primary amplification as small cultures (~7.0mL) under ampicillin selection and plasmids extracted using the QIAprep Miniprep Kit (Qiagen) in accordance

with manufacturer's protocols. Briefly, pelleted cells were resuspended in 500µL P1 buffer (supplemented with RNase A), lysed by addition of 500µL P2 buffer, and neutralized with 700µL N3 buffer. Cellular debris were removed by centrifugation at 13,200 rpm for 20m. Plasmid DNA was bound to the column membrane, washed, and eluted in 50µL nfw. The presence of the desired cloning fragments and integrity of ligation sites was then verified using digestion analysis of the restriction sites involved in construct assembly. For those clones whose construction involved fragment amplification via PCR, the integrity of the region of manipulation was verified by sequence analysis.

SEQUENCING

Sequence analysis of plasmid constructs was performed as follows. PCR amplicons of regions of manipulation were directly sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence reactions were comprised of 1.0µL (30ng) appropriate primer, 4.0µL agarose-gel purified DNA, 1.0µL BigDye Terminator v3.1, 1.5µL 5X reaction buffer, and 2.5µL nfw, and thermo-cycled using the following conditions 96ºC for 1m; 25 cycles of 96ºC for 20s; 50ºC for 5s; 60ºC for 4m; and stored at 4ºC for up to 24h. Sequence reactions were purified using Performa® DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD) in accordance with manufacture's protocols, briefly, gel-cartridges were prepared by centrifugation at 4,200 rpm for 3m, 10µL sequencing reaction was then applied to the column, and eluted at 4,200 rpm for 3m. Sequencing was performed using an Applied Biosystems 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed using the GeneRunner 3.05 software suite.

LARGE SCALE CESIUM CHLORIDE PREPARATIONS OF PLASMID STOCKS

Stock preparations (preps) of all plasmid constructs (replicons, defective helpers, and full length viral i.c.s) were prepared using cesium chloride (CsCl) purification. Large cultures (250mL 1.5xYT broth) were inoculated with MC1061 cells bearing a clonal population of the confirmed (see above) desired plasmid, and allowed to grow, under antibiotic selection, o/n with agitation (300 rpm) at 37ºC. Following 15-18h of growth, cells were pelleted at 6,500rpm for 10m at 4ºC. Medium was discarded and all subsequent manipulations performed on ice; bacteria were resuspended in 8.0mL BF1 (0.025M Tris-HCL, 0.1M NaCl, 0.01M pH7.5 EDTA), lysed for 2-3m with 16.0mL BF2 (0.2M NaOH, 1.0% SDS), and neutralized for 2-3m with 12.0mL BF3 (150g KAc, 100mL glacial acetic acid, molecular grade water to 500mL). Preps were then transferred to 50mL conical tubes and cellular debris were removed via centrifugation in a Eppendorf centrifuge (model 5810R) at 4,000 rpm for 20m at 4ºC.

Following careful decanting (to ensure minimal debris carryover) into sterile 50mL conical tubes, nucleic acids were precipitated from supernatants using (70%) isopropanol and stored at -20ºC for at least 20m. Nucleic acids were pelleted at 4,000 rpm for 10m at 4ºC, briefly air dried, dissolved in 2.0mL TE (0.01M Tris-HCl, 0.001M EDTA pH 7.5-7.6), and contaminating RNAs removed via LiCl precipitation (2.0mL of a 5M solution) on ice for at least 10m. RNA was then pelleted (4,000 rpm for 10m at 4ºC), clarified supernatants transferred to sterile 15mL conical tubes, and DNA ethanol precipitated at -20ºC for at least 15m. DNA was then pelleted (4,000 rpm for 10m at 4ºC) and dissolved in 1.0mL TE.

DNA solutions were gently mixed with CsCl gradients (4.8g CsCl, 3.3mL TE, 40µL ethidium bromide (10mg/mL)) and heat sealed in ultra-centrifugation grade tubes. Samples were then centrifuged at 65,000rpm at 20ºC for 16-20h. Plasmid preps purified

in this way gave rise to two distinct bands; an upper band consisting of linearized or nicked DNA and a lower band comprised of supercoiled plasmid DNA. Supercoiled bands were aspirated using a 1.0mL syringe and 26 gauge needle, diluted with 1.0mL TE, and re-ethanol precipitated at -20ºC for 30-60m. DNA was then pelleted (4,000 rpm for 10m at 4ºC) redissolved in 400µL TE, transferred to sterile nuclease free 1.5mL eppendorf tubes, phenol-chloroform (pH 7.9) extracted, and re-ethanol (100µL 5M NaCl and 1.0mL 100% ethanol) precipitated. Plasmid preps were stored indefinitely at -20ºC as ethanol precipitations until needed for future experiments. Plasmid DNA was then pelleted (13,200rpm ≥10m), washed once with 75% ethanol, diluted in RNasin Plus RNase Inhibitor (Promega, Madison, WI) treated nfw, yield quantified using a BioRad SmartSpec Plus (BioRad, Hercules, CA) and the concentrations adjusted.

D. Transcription and transfection of plasmid derived viral RNAs

In vitro transcription

All cDNA viral genomes were cloned directly downstream of an optimized Sp6 exogenous promoter sequence from which viral RNAs could be transcribed. Prior to *in vitro* transcription alphaviral i.c.s were linearized by restriction digestion at unique sites engineered immediately 3' relative to the viral poly-adenosine (poly-A) tail. Digestion reactions were carried out using the appropriate restriction enzyme (Not*I* for CHIKV i.c.s, and Xho*I* for SINV i.c.s) supplemented with the optimal NEB restriction buffer and bovine serum albumin, at 30ºC for ~1h. Linearized plasmids were then purified by phenol-chloroform extraction. Briefly, the reaction volume was adjusted to 100µL with nfw, 100µL phenol-chloroform was added, the sample vortexed for ~15s, and phases separated by centrifugation at 13,200rpm for 3-5m. The aqueous phase was then removed and 100µL chloroform-isoamyl alcohol (24:1) added, vortexed ~15s, and re-

separated. Ethanol precipitation of the aqueous phase was then performed by adding 10µL 3M sodium acetate (NaAc) pH 5.2 and 200µL 100% ethanol, followed by inverting several times and storage at -80ºC.

Linearized plasmid DNA was then pelleted, 13,200rpm for ≥10m, washed once with 75% ethanol, and resuspended in 10µL RNasin treated nfw. *In vitro* transcription was performed using the mMESSAGE mMACHINE Sp6 Kit (Ambion, Applied Biosystems, Foster City, CA) in accordance with manufacturer protocols. *In vitro* transcriptions reactions were comprised of reagents in the following proportions: ~0.5µg linearized plasmid DNA, 1.0µL 10X Sp6 reaction buffer, 1.0µL 20mM GTP, 5.0µL NTP/Cap buffer (10mM each ATP, CTP, UTP, GTP; 8mM Cap analogue), and nfw to 10µL (total reaction volume varied based on desired yield). Reactions were incubated at 37ºC for 15-30m at which time a 1.0µL aliquot was analyzed by 1% agarose gel electrophoresis to determine RNA integrity and yield. After a total of 45-60m at 37ºC RNA was purified by addition of 1.5 reaction volumes each nfw and LiCl precipitation solution (7.5M LiCl, 50mM EDTA) and stored at -20ºC for ≥30m but for no longer than 24h. RNA was then pelleted (13,200 rpm for 15m at 4ºC), rinsed once with 75% ethanol, resuspended in RNasin treated nfw, yield quantified, and adjusted to a final concentration of 1.0µg/µL. RNAs synthesized and purified in this way were stored at -80ºC.

RNA transfections

All eukaryotic cell transfections were achieved via electroporation using cell specific parameters. Preparation of cells for electroporation was as follows regardless of cell type. Cells were grown to 70-80% confluence in 175 cm^2 tissue culture flasks under the appropriate conditions (see above). Medium was aspirated and monolayers washed once with ~10mL ice cold 1X Dulbecco's phosphate buffered saline (DPBS; without Ca^{2+} and Mg^{2+}; Cellgro), and trypsinized with ~3.0mL Trypsin-EDTA, 0.25%

(Invitrogen) for 3-5m at the appropriate maintenance temperature. Trypsin was subsequently diluted with ~7.0mL of maintenance medium per flask and cell suspensions ~10.0mL transferred to 50mL conical tubes. Cells were pelleted (1,200 rpm for 5m at 4ºC), rinsed three times with 1X DPBS (with centrifugation following every rinse), transferred to a sterile 15.0mL conical tube and resuspended in 400µL (~$1x10^7$ cells) per electroporation. Cells were then mixed with specific amounts of RNA based on the proportion of cells desired to become infected and electroporated. BHK-21 cells were placed in a 2mm gap cuvette and subjected to a single pulse (140 Volts, 25 mili-seconds) and immediately placed back on ice. Six hundred microliters of corresponding medium was then added and cells and medium removed diluted and plated in an appropriate growth vessel based on individual experimental conditions and stored at the appropriate growth conditions.

Specific infectivity (infectious centers assay)

Analysis of the electroporation efficiency (expressed as the number of cells successfully transformed via electroporation per µg RNA; defined here as specific infectivity) was quantified as previously described (McGee *et al.,* 2009, Tsetsarkin *et al.,* 2006). Vero cells were seeded into sterile six-well plates ($1x10^6$ cells/well) and incubated at 30ºC for ~4h to allow for attachment. Ten-fold serial dilutions of freshly electroporated BHK-21 cells were then seeded onto these sub-confluent Vero monolayers (~70% confluence) and allowed to attach for ~2h at 37ºC. Medium was then removed and cells overlayed. The overlay used was modified to take into account the cytopathicity of the viral constructs used. For those viruses competent for the formation of plaques, 2.0mL of a solid agarose overlay was used. This overlay was comprised of equal parts 1.5% agarose in sterile water, MEM supplemented with 2% BGS, 1% pen-strep, and 1% NEAA, and 2X modified eagle medium (Invitrogen) equilibrated to 43.5ºC.

Plates overlayed in this way were fixed using a 3.8% formaldehyde solution in 1X phosphate buffered saline (PBS) for 1 – 24h, agarose plugs removed, and counterstained with a 0.25% crystal violet solution in water. Whilst a methyl cellulose based, semi-solid, overlay (0.8% methyl cellulose in OptiMEM medium (Invitrogen) supplemented with 2% FBS, 1% pen-strep, and 1% L-glu) was used for those virus constructs which were not expected to cause cytopathic effect (CPE) and as such required immunological based detection methods. Plates overlayed in this manner were fixed either using 1:1 methanol:acetone solution (immunohistochemical staining) or 3:1 acetone:PBS solution (immunofluorescent staining).

E. Protocols involved in the analysis and conformation of alphavirus recombination

The basic experimental design for the generation and cell culture purification of full length alphavirus genomes was as depicted in **Figure 2.4**. Purified *in vitro* transcribed viral RNAs were electroporated, either individually or as mixtures of specific ratios, into BHK-21 cells as described above. For all alphavirus recombination experiments, immediately after electroporation cells were resuspended in 5.0mL BHK-21 maintenance media and 1/5 of the total electroporation suspension (~2x10^6 cells), with the addition of 1.0mL sterile BHK-21 maintenance medium, plated in a single well of a

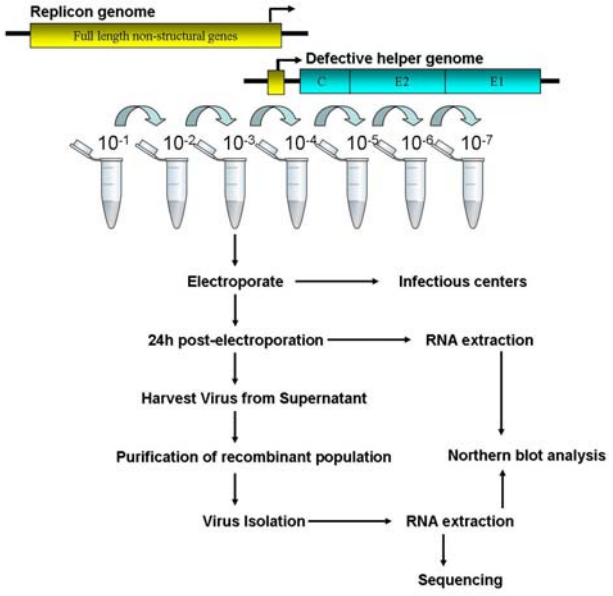


FIGURE 2.4 BASIC EXPERIMENTAL DESIGN: GENERATION, DETECTION, AND CHARACTERIZATION OF FULL LENGTH RECOMBINANT ALPHAVIRUS GENOMES.

six-well plate and incubated at 37ºC for 24h. At the time of electroporation, infectious centers assays were performed for those alphavirus constructs engineered to contain fluorescent protein expression cassettes. At 24h post-electroporation cell culture supernatant was removed and stored at -80ºC. Additionally, total cellular RNA was harvested.

Extraction of total cellular RNA

Total cellular RNA from cell culture mono-layers, grown in six-well plates (surface area ~9.6 cm^2/well) was extracted using TRIzol® Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol with the following modifications. Cell mono-layers ($\geq$90% confluent) were lysed by the addition of 800µL of TRIzol followed by incubation at rt for ~5m. TRIzol/lysates were then placed in 1.5mL eppendorf tubes with the addition of 200µL (24:1) chloroform: isoamyl alcohol solution followed by vigorous shaking for ~15s. Subsequent phase separation was achieved by centrifugation at 13,200 rpm for 15m at 4ºC. The aqueous phase was then removed and RNA precipitated by the addition of 500µL of 99% isopropanol. Samples extracted in this way were stored indefinitely as isopropanol-precipitations at -20ºC until needed for further analysis. RNA was then pelleted via centrifugation at 12,000 rpm for 10m at 4ºC, rinsed once with nuclease-free 75% ethanol solution, and dissolved in RNase-free molecular grade water supplemented with RNasin. RNA yield was then quantified using a SmartSpec Plus spectrophotometer.

Isolation of clonal populations of putative recombinant alphavirus genomes

Clonal populations of recombinant genomes were isolated via infection of $1x10^7$ Vero cells in suspension with 500µL of electroporation supernatant, with agitation for 1h at rt. Following this incubation cells were seeded into 96-well plates (~$3.5x10^4$ cells per

well; 150µL total volume per well) and allowed to incubate for 72h at 37ºC at which time all wells were examined visually. Total medium from those wells identified as positive for CPE was harvested and stored at -80ºC for additional analysis. Experimental conditions (dilutions), in which no wells were identified to be CPE positive at 72h, were amplified for a single passage in C6/36 cells. Five hundred microliters of electroporation supernatant was inoculated directly onto a >80% confluent monolayer of C6/36 cells in a single well of a six-well plate allowed to incubate at rt for 1h, inoculum was removed and 2.0mL C6/36 maintenance medium added, and stored at 28ºC for 48h. Supernatant samples were harvested from these infections and stored at -80ºC. C6/36 amplification supernatants were then inoculated onto Vero cells, plated in 96-well format, and examined as described above. Those samples in which no CPE in Vero cells was observed following inoculation with either electroporation of C6/36 amplification supernatants were ruled to contain no detectable levels of recombinant alphaviruses.

Up to a maximum of six isolates per experimental condition were selected for further analysis. Fifty microliters of CPE positive 72h Vero cell supernatant was inoculated onto a single well of C6/36 cells (six-well plate format) with the addition of 450µL sterile C6/36 maintenance medium. Following a 1h incubation at rt with agitation, the inoculum was removed and replaced with 2.0mL fresh C6/36 medium, plates were then incubated at 28ºC. Supernatant samples were harvested, following a 48h incubation, designated recombinant stocks and stored at -80ºC until analyzed for the presence of infectious virus by plaque assay. Additionally, RNA was extracted from supernatant samples (QIAamp Viral RNA Mini Kit) and C6/36 (TRIzol Reagent) cells and subjected to sequence and Northern blot analysis respectively.

Plaque assay

Amounts of infectious virus in all recombinant stocks were titered by plaque assay. Briefly, Vero cells ($1x10^6$ per well), seeded in six-well plates and allowed to attach and grow for 4-6h, were directly inoculated with 250µL of ten-fold serial dilutions of viral samples. Plates were incubated at 37ºC with agitation every 10-15m. Inoculum was then removed and cells overlayed with either a solid agarose or a semi-solid methyl cellulose overlay as described above (see Specific infectivity: infectious centers assay). Alphavirus plaque assay plates were then incubated at 37ºC for 48-72h (depending on the specific virus being assayed), fixed with 3.8% formaldehyde solution, stained with crystal violet, and scored.

Northern blot

The presence of full length recombinant viral genomes in TRIzol extracted cellular RNA was assessed by Northern blot using the NorthernMax®-Gly glyoxal-based system for Northern blots (Ambion). Resuspended RNA samples (see Extraction of total cellular RNA) were mixed 1:1 and RNA size markers 3:1 with Glyoxal loading dye and denatured at 56ºC for 30m. Denatured RNA were then pulse centrifuged and stored at -20ºC, for up to seven days. Horizontal 1% agarose glyoxal gels were prepared and cast to an approximate thickness of 0.6cm, and primed for sample analysis by subjecting them to a 5m pre-run in 1X glyoxal gel prep/running buffer at 55 Volts. Denatured virus specific RNA size markers, 3.0µL stock (hereafter referred to as ladders; see Construction of virus specific RNA ladders), and cellular RNA samples 10.0µg/lane were fractionated at 55V for 1 ½ h. RNA was then transferred to a pre-cut BrightStar®-Plus membrane (Ambion) via downward capillary transfer (**Figure 2.5**) for a minimum of 20m/mm gel thickness and no longer than 3 ½ h total. RNA bound membranes were then briefly rinsed in glyoxal running buffer and RNA bound to the membrane using a

Stratagene UV Stratalinker 1800 (auto-crosslink function). On occasion RNA transfer controls were included and visualized by reversible staining of ribosomal RNA with methylene blue solution (0.02% in 0.3M NaAc) (Sambrook 1989). Membranes were then pre-hybridized at 42ºC in equilibrated ULTRAhyb for at least 30m. Virus specific biotinylated 40nt oligonucleotide probes (**Table 2.3**) were then added directly to the pre-hybridization buffer to a final concentration of 1.5pM and hybridized o/n at 42ºC in a rotary hybridization oven. Membranes were then washed twice in low stringency wash buffer (10m at rt, 2m at 42ºC) with agitation and immediately developed.

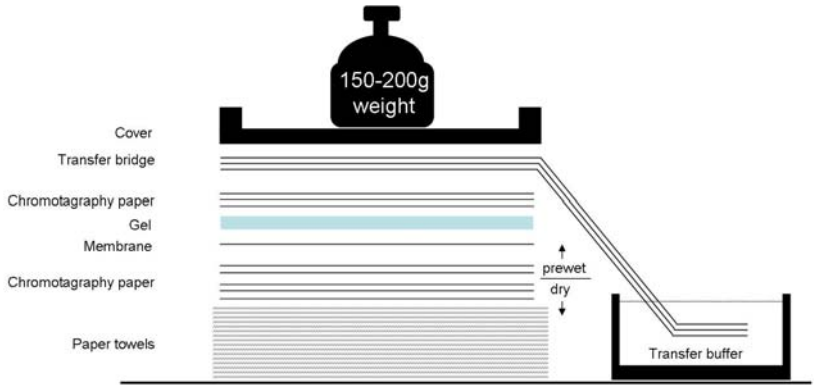


FIGURE 2.5 NORTHERN BLOT DOWNWARD CAPILLARY TRANSFER ASSEMBLY.
(adapted from NorthernMax®-Gly protocol manual)

TABLE 2.3 SEQUENCES OF ALPHAVIRUS SPECIFIC BIOTINYLATED OLIGONUCLEOTIDE PROBES.

Virus	Name	Sequence
Chikungunya	CHIKV-5'UTR BioProbe	5'- CACGTACACAGGATCCATGATGGGTTATTAATCTCTTGCT
Sindbis	SINV-5'UTR BioProbe	5'- AGTGCAATTGGTCGGCTGTTTGATTCAATAGTGTGTACTA
Sindbis	SINV-3'UTR BioProbe	5'- TTAATATAGTGGTTATGTGGCAACACTGCGCAGCATTATG

Nucleotides covalently linked to biotin molecules are highlighted in yellow.

Construction of virus specific RNA ladders

The use of mixtures of synthetic viral RNAs as size markers and virus specific biotinylated oligonucleotide probes allowed for efficient detection of both ladders and experimental samples using a single developing protocol. Virus specific RNA ladders were generated by mixing equimolar amounts of *in vitro* transcribed purified viral RNAs

derived from plasmids of varying lengths; i.e. full length ~12.0Kb, replicon ~8.0Kb, defective helpers ~2.0-5.0Kb. RNAs used in ladder generation were purified using low melting point (LMP) agarose (0.8% in TAE) cast at 4ºC and run at 60V for 45-60m. The desired bands were then excised using a sterile nuclease free scalpel, 20µL NEB restriction buffer #2 added, and melted at 65ºC for 5-10m with periodic pulse vortexing. RNA was then sequentially extracted, 3-5m centrifugations at 13,200 rpm, in the aqueous phase using one gel volume each of phenol (water-saturated pH 6.6), phenol-chloroform (pH 6.6), and chloroform-isoamyl alcohol (24:1). Following extraction, RNA was ethanol precipitated, (2.0µL 5M NaCl and 250µL 100% ethanol per 100µL sample volume, at -80ºC for ≥30m). RNA was pelleted and resuspended as described above. The concentration of each ladder that was used for Northern blot analysis was determined empirically.

Detection of membrane bound alphaviral RNAs

All Northern blot membranes were developed using the BrightStar® BioDetect™ nonisotopic detection kit (Ambion) immediately following low stringency washing. Membranes were washed, blocked, bound with streptavidin-alkaline phosphatase (Strep-AP; 1:10,000), re-washed, treated with assay buffer, and incubated in the CDP-Star substrate. Detection of sequence specific nucleic acids using this method was achieved as follows; all virus specific oligonucleotide probes contained three covalently attached biotin groups which form strong non-covalent bonds with the streptavidin group of Strep-AP, the alkaline phosphatase group then dephosphorylates dioxetane (within CDP-Star) generating an anion which decomposes predictably emitting light at a wavelength of 466nm. This chemiluminescent reaction was detected by exposing the treated membrane to Kodak Biomax XAR film (Sigma-Aldrich, St. Louis, MO) which was then developed.

Sequencing of cross-over sequence of putative alphavirus recombinants

Two clonal isolates, per recombination experiment, from the lowest dilution (minimum level of co-infection) were selected for sequence analysis of the putative junction/cross-over region. RNA was isolated from alphavirus recombinant stocks (C6/36 48h passage #1) using the QIAamp viral RNA extraction and cDNA generated using the SuperScript III First-Strand Synthesis kit. cDNA was then PCR amplified using *Taq* DdDp and the appropriate sequence specific primers and cycling conditions. Amplicons were phenol-chloroform extracted, ethanol precipitated (with the addition of 1.0µg Linear acrylaminde (Ambion)), enzymatically modified (T4 PNK, T4 polymerase) to generate ends appropriate for cloning, and LMP agarose extracted. Purified amplicons were then cloned into the multiple cloning site of pBluescript SK+ (Stratagene). The EcoR*V* site was chosen to facilitate efficient cloning of blunt end fragments between the T_7 and T_3 promoter/primer binding sites (**Figure 2.6**) to facilitate ease of insert detection

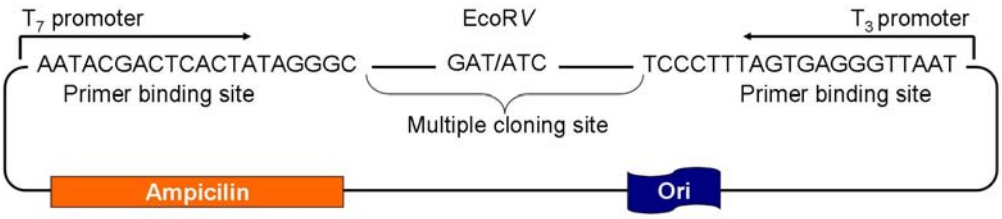


FIGURE 2.6 PBLUESCRIPT SK+ CLONING VECTOR FOR SEQUENCING OF ALPHAVIRUS RECOMBINANT AMPLICONS.

and sequencing. Ligations were then transformed into fresh competent MC1061 cells and incubated o/n at 32-34ºC. Insert positive colonies were identified by PCR using *Taq* DdDp and the T_7 and T_3 primers (**Table 2.2; Figure 2.6**), and five insert positive colonies per isolate amplified and QIAprep miniprep extracted. Plasmid DNA (1µg) was then

combined with specific primers in a 10µL reaction and submitted to the University of Texas Medical Branch (UTMB) Protein Chemistry Core. Sequence files were edited and analyzed using the GeneRunner 3.05 software suite.

F. Mosquito maintenance and infection

Mosquitoes

Aedes albopictus mosquitoes (Galveston, TX) generation F≥5, were chosen for use in all alphavirus mosquito infection experiments due to their proven susceptibility to CHIKV (Tsetsarkin *et al.,* 2006, Tsetsarkin *et al.,* 2009, Tsetsarkin *et al.,* 2007). Mosquitoes were maintained as previously described (Vanlandingham *et al.,* 2004) and all mosquito infections were conducted in a arthropod containment level 3 (ACL-3) insectary. Briefly, mosquitoes were maintained at 28ºC on a 14:10 hour light:dark cycle including a one hour period to simulate dusk/dawn conditions. Larvae were fed using a 1:1 mixture of TetraMin® fish flakes (Doctors Foster and Smith, Inc., Rhinelander WI) and ground Prolab® 2500 rodent diet (PMI Nutrition International LLC, Brentwood, MO) and adults were allowed to freely feed on a 10% sucrose solution. Additionally adult females were blood-fed once weekly on anesthetized hamsters, in accordance with NIH and UTMB humane laboratory animal use standards, to promote egg production.

Oral infection (per os)

Mosquitoes were orally infected by combining inoculum (infected cell culture supernatant) 1:1 with defibrinated sheep blood (Colorado Serum Company, Denver, Colorado). Infectious bloodmeal was warmed to 37ºC and transferred to a Hemotek arthropod feeder covered with a murine skin membrane (Discovery Workshops, Accrington, Lancashire, United Kingdom) housed in a sealed glove box. Blood feeding chambers were placed on the tops of mosquito cartons and females permitted to feed *ad*

libitum for 30-60m. Subsequent to the feeding period mosquitoes were chilled and engorged females, >4+ (Pilitt & Jones 1972), transferred to new cartons, placed in a sealed humidified container, provided with 10% sucrose solution and housed in a Precision model 818 environmental chamber (Precision, Winchester, Virginia) at 28ºC, with a 14h:10h light:dark cycle. An aliquot of infectious meal was stored at -80ºC for virus titration.

Intra-thoracic (IT) inoculation

Mosquitoes were chilled on ice and inoculations performed on a chill table (~4ºC) and visualized through a dissecting microscope. Briefly, 0.5µL of virus stock was directly inoculated into the mosquito thorax. This procedure was performed using a manual pressure delivery injection system through which virus was inoculated via the use of a calibrated glass-syringe. Inoculated mosquitoes were maintained as described above.

Infection and dissemination

Mosquitoes were sampled at various time points post-infection to assess whole body titer. Additionally, mosquito bodies and corresponding heads were assayed independently for infectious virus to determine overall infection and dissemination rates, respectively. Whole individual mosquitoes and mosquito bodies were triturated in a 2.0mL round bottom safe-lock micro centrifuge tube (Eppendorf) containing 1.0mL (heads in 300µL) Vero maintenance medium and a single zinc coated steel grinding ball (Daisy® Rogers, Arkansas) using a Retsch Mixer Mill MM301 operating at a frequency of 26 vibrations/sec for 4m, followed by centrifugation at 13,000X g for 6m. Clarified homogenates were then titrated by plaque assay as described above.

Tissue dissection

On occasion individual mosquito tissues were examined microscopically to assess virus infection. Cold anesthetized mosquitoes were surface sterilized in a 70% ethanol solution and rinsed twice in PBS. Salivary glands and midguts were dissected into small drops of PBS using finely honed dissecting needles and forceps. Heads were removed and the salivary glands exposed by placing gentle pressure on the thorax followed by careful removal of surrounding tissues. Midguts were removed from the posterior end of the abdomen via gently pulling on the terminal abdominal tergum and gently separating the midgut from the associated tissues. Dissected tissues were placed on Teflon-coated spot slides in a small droplet of PBS and examined visually for fluorescent protein expression (enhanced green fluorescent protein (GFP) and Cherry fluorescent protein (Cherry)). Expression in infected tissues was visually assessed using a Olympus IX51 compound fluorescent microscope and the appropriate filter (Higgs *et al.*, 1996).

2.4 RESULTS

A. Development and analysis of first generation CHIKV replicon/replicon and replicon/defective helper recombination system

Preliminary analysis of alphavirus recombination involved the generation of two distinct systems: 1) Two "suicide" replicon CHIKV genomes (replicon/replicon cross) containing complementary frame shift deletions (ΔFS) within the structural open reading frame (ORF) that require recombination to restore protein expression. 2) One suicide replicon CHIKV, containing a ΔFS in the E2 protein (capable of expressing functional capsid (C)), and a complementary defective helper (deletion of ~7Kb, nonstructural genes) competent for the expression of the CHIKV envelope proteins (replicon/defective helper cross). These two systems along with expected recombinant genome topology and protein products expressed when co-electroporated (crossed) are depicted in **Figure 2.7**.

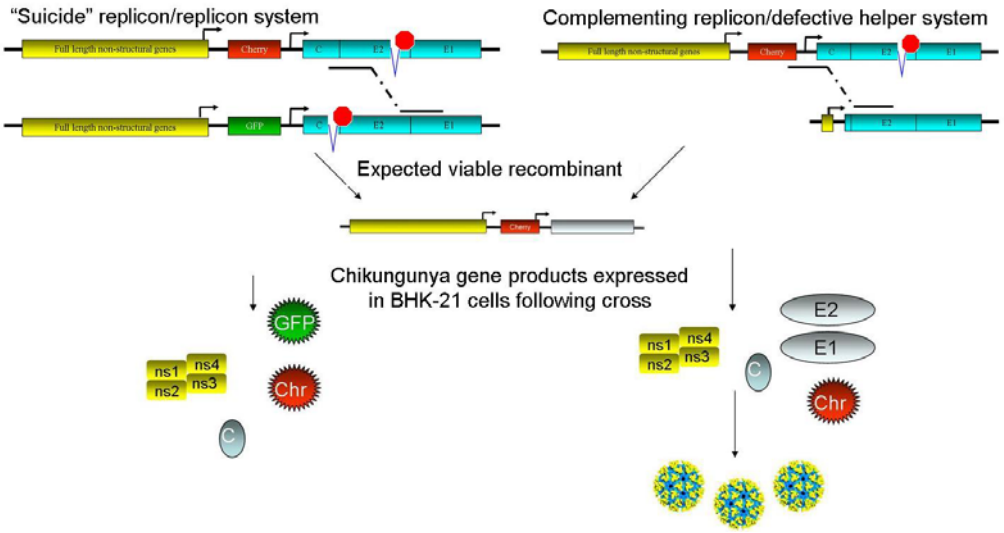


FIGURE 2.7 SCHEMATIC REPRESENTATIONS OF FIRST GENERATION REPLICON/REPLICON AND REPLICON/DEFECTIVE HELPER RECBOMINANT CROSSES. The suicide replicon/replicon system involved co-electroporation of CHIK-LR-5'Δ-GFP-Rep and CHIK-LR-3'Δ-Cherry-Rep RNAs. The complementing replicon/defective helper system involved co-electroporation of CHIK-LR-3'Δ-Cherry-Rep and CHIK-LR-E2/E1-Help RNAs.

The rationale behind the development of this dual strategy was as follows. Co-electroporation of the replicon/replicon cross would be completely deficient for envelope protein expression in the absence of recombination; therefore the use of these genomes was hypothesized to facilitate detection of putative recombinants by eliminating/minimizing the population of packaged competing replicon genomes. Because it was possible that recombinant genomes could in some way be attenuated with regard to structural protein expression the second replicon/defective helper cross was designed to allow for efficient expression of functional structural gene products for the packaging of putative recombinant genomes. However, the use of genomes competent for the expression of all protein products would produce a major population of packaged replicon genomes from which recombinants would have to be purified.

Frame shift deletions engineered into the CHIKV structural ORF were chosen based on ease of cloning. A 5' structural 182nt ΔFS was engineered into CHIKV i.c.s both with (pCHIK-LR-5'Δ-GFP-Rep) and without (pCHIK-LR-5'Δ-Rep) a GFP expression cassette. Complementary constructs containing a 3' ΔFS of 112nt of E2 sequence both deficient (pCHIK-LR-3'Δ-Rep) and competent (pCHIK-LR-3'Δ-Cherry-Rep) for the expression of Cherry were also generated. CHIKV defective helper genomes were constructed from the parental defective helper pCHIK-LR-FL-ST-Help. This full length CHIKV structural helper was used to generate a bi-parte helpers system, comprised of genomes competent for the expression of either the CHIKV envelope proteins E2/E1, pCHIK-LR-E2/E1-Help or capsid protein pCHIK-LR-Cap-Help.

Electroporation of suicide replicons individually and in concert with defective helper genomes confirmed that the engineered 5'/3' ΔFS had indeed compromised the ability of these genomes to spread in cell culture (**Figure 2.8**). Recombination co-electroporation experiments were performed by mixing ~10μg each of complementary ΔFS replicons; CHIK-LR-5'Δ-Rep & CHIK-LR-3'Δ-Rep as well as CHIK-LR-5'Δ-GFP-Rep & CHIK-LR-5'Δ-Cherry-Rep at an efficiency of ~$3.5x10^7$ total co-infections. Supernatant was harvested from these crosses at 24h, amplified a single time in C6/36 cells, and inoculated onto Vero monolayers (~70% confluent). However, no evidence to suggest the generation of a viable CHIKV full length recombinant such as virus induced CPE or fluorescent protein expression was detected.

Similar recombinant crosses were performed using CHIK-5'Δ-Cherry-Rep & CHIK-LR-E2/E1-Help ($1.0x10^7$ total co-infections) and passaged as above. Inoculation of C6/36 cells with electroporation supernatant from this experiment resulted in >95% of cells being Cherry positive at 24h (**Figure 2.9**). When either Vero or C6/36 cells were infected with supernatant samples harvested from C6/36 passage #1 (48-72h) small self-

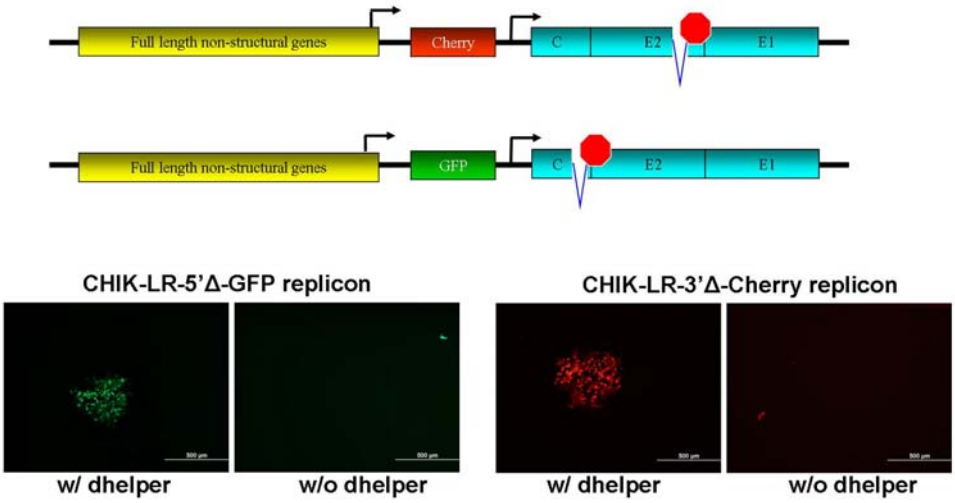


FIGURE 2.8 CONFIRMATION THAT 5' AND 3' STRUCTURAL FRAME SHIFT DELETIONS RESULT IN GENERATION OF CHIKUNGUNYA VIRUS "SUICIDE" GENOMES. Electroporation of CHIK-LR-5'Δ-GFP-Rep and CHIK-LR-3'Δ-Cherry-Rep RNA individually both with (w) and without (w/o) the presence of the appropriate defective helper (dhelper) RNAs CHIK-LR-E2/E1-Help and CHIK-LR-Cap-Help (not depicted schematically). Red octagons indicate polyprotein truncation resulting from stop codon introduction via frame shift.

restricted foci could be observed as early as two days post infection (dpi) (**Figure 2.9**). These foci continued to expand for approximately three to five days at which time they would appear to growth arrest. Two possible explanations could account for this plaque/foci phenotype. Either recombination was resulting in the generation of a quasi-stable genome topology that was rapidly degrading into complete dysfunction or defective helper genomes were being packaged at some efficiency resulting in foci generation as previously observed for SINV (Weiss & Schlesinger 1991).

B. Analysis of CHIKV defective helper packaging

Since the majority of the CHIKV nonstructural gene coding sequence was deleted from all defective helper genomes, proteins were only expressed from these genomes

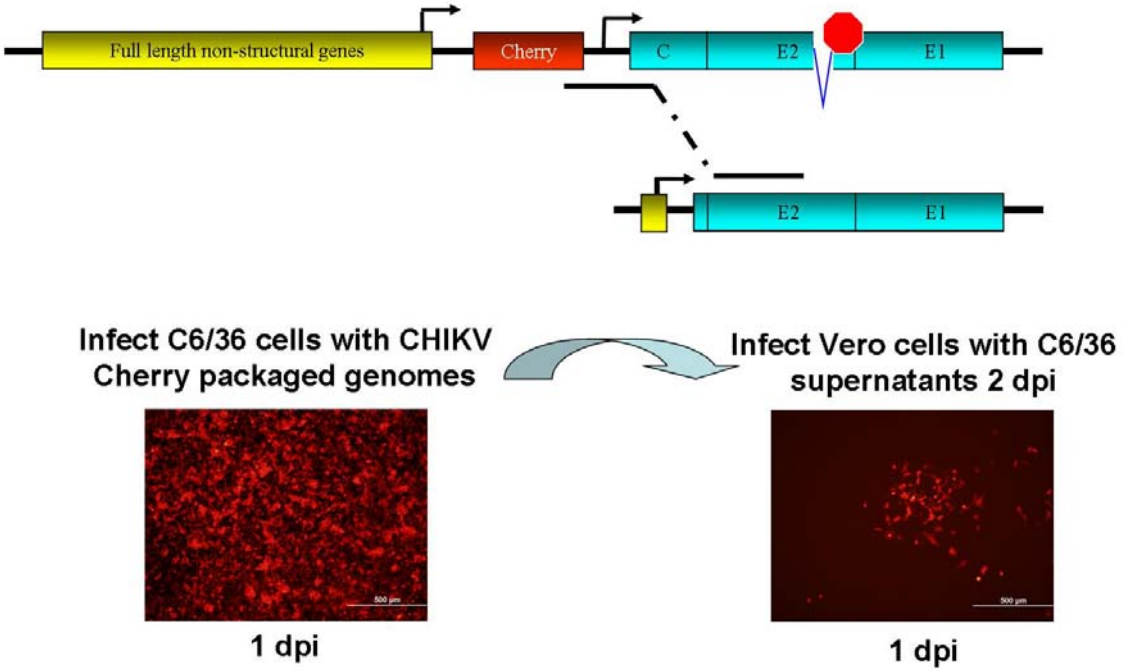


FIGURE 2.9 CHIKUNGUNYA FIRST GENERATION REPLICON/DEFECTIVE HELPER RECOMBINANT CROSS.
Co-electroporation of CHIK-LR-3'Δ-Cherry-Rep and CHIK-LR-E2/E1-Help RNA followed by passage of post-electroporation supernatant in cell culture as indicated.
CHIKV-chikungunya virus
dpi-days post infection

when there were spatially and temporally associated with a replicon genome. In such a co-infection circumstance, the replicative machinery was supplied in *trans* by the replicon genome. The replicase complex can recognize the regulatory sequences within the defective helper genome (such as the UTRs and subgenomic promoter (SGP)), and drives genome replication and protein expression (**Figure 2.10**). I therefore hypothesized that "tagging" defective helper genomes with fluorescent protein coding cassettes, under the control of the CHIKV SGP, should allow for convenient identification of co-electroporated cells and defective helper packaging during cell culture passage.

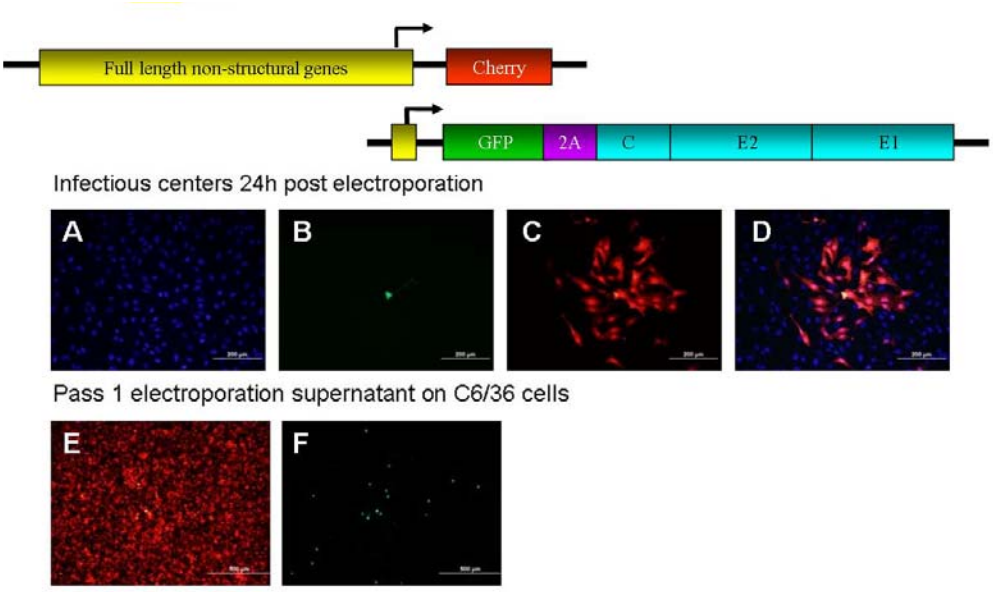


FIGURE 2.10 CONFIRMATION OF CHIKUNGUNYA VIRUS DEFECTIVE HELPER GENOME PACKAGING.
Schematic representation of replicon/defective helper systems employed to analyze defective helper genome packaging. CHIK-LR-Cherry-Rep and CHIK-LR-5'-GFP-2A-Help RNAs were co-electroporated followed by infectious centers assay (A-D) and passage in C6/36 cells (E-F). Images were captured at 20X magnification, scale bar is indicated.
A, DAPI nuclear counter stain. B, GFP filter. C, Cherry filter. D, Merge of images A/B/C. E, Cherry filter. F, GFP filter.

GFP was engineered into CHIKV defective helper genomes in two distinct orientations. pCHIK-LR-E2/E1-GFP-Help contained GFP using a classic 5' SGP duplication, whilst in pCHIK-LR-5'-GFP-2A-Help a mono-subgenomic topology was achieved via fusing GFP to the 5' end of the capsid gene. In the latter topology, cleavage of the GFP-capsid fusion product was achieved by introduction of a foot-and-mouth disease (FMDV) 2A protease. It was hypothesized that this topology would increase the efficiency to subgenomic protein production by eliminating the generation of two ~26S RNAs as was the case for more traditional double subgenomic orientation. To verify this topology would be functional and not compromise the replication kinetics of CHIKV, a

5'GFP-2A expression cassette was also engineered into a full length CHIKV i.c., pCHIK-LR-GFP-2A-FL. Virus derived from this construct was observed to have similar specific infectivity, foci/plaque size and morphology, and 24-48h post electroporation titers to

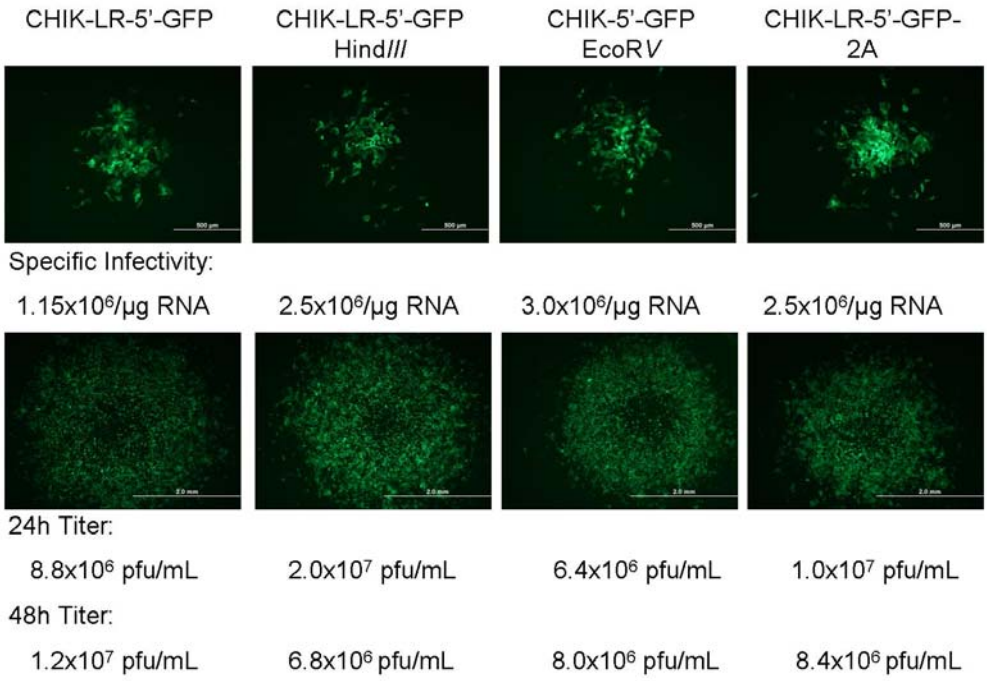


FIGURE 2.11 ANALYSIS OF THE EFFECTS OF MOLECULAR MANIPULATIONS (SITE DIRECTED MUTAGENESIS AND GFP-2A INSERTION) ON CHIKUNGUNYA REPLICATION.
pfu – plaque forming units

virus derived from both pCHIK-LR-5'-GFP i.c. (**Figure 2.11**) and pCHIK-LR i.c.

Replicon/defective helper pairs were then co-electroporated into BHK-21 cells and infectious centers assay performed. CHIK-LR-E2/E1-GFP-Help RNA was co-electroporated (10µg each) with RNA from pCHIK-LR-Cap-Cherry-Rep and CHIK-LR-5'-GFP-2A-Help with CHIK-LR-Cherry-Rep. Co-electroporation of these replicon/defective helper pairs resulted in the generation of foci where only the single "seed-cell" (BHK-21 cell at the foci center) was positive for both Cherry (replicon) and

GFP (defective helper) expression while all surrounding cells were positive for only Cherry (replicon genome only) expression (**Figure 2.10**). As such the electroporated cell from which the Cherry foci was generated was capable of expressing all CHIKV protein products; nonstructural genes from the replicon genome and structural genes from the defective helper. Replicon genomes were subsequently packaged, released, and infected Vero cells within the vicinity of the BHK-21 "seed-cell". When this foci was then visualized with a filter allowing for the detection of GFP only the cell at the center of the foci was positive (**Figure 2.10 B**). However, when examined using a Cherry filter the entire foci including the central cell (positive for both genomes) and all surrounding cells (replicon only) could be visualized (**Figure 2.10 C**). When these images were merged, the foci has a characteristic "bulls-eye" appearance where the central cell was positive for both fluorescent proteins with all surrounding cells being positive for only Cherry (**Figure 2.10 D)**. Unfortunately this foci morphology did not allow for differentiation between extremely low packaging efficiency or a complete loss of defective helper packaging. To determine if defective helper genomes were being packaged at any efficiency C6/36 cells were infected with electroporation supernatant samples (500µL) and visually examined at 24h. Since >95% of cells are infected with replicon genomes (Cherry positive; **Figure 2.10 E**) it can be assumed that any cell which becomes infected with a defective helper genome would be competent for GFP expression, and if any cells were observed to express GFP at 24h defective helper packaging would be confirmed. Indeed a population of GFP positive cells was observed when C6/36 cells were infected in this way (**Figure 2.10 F**) thus providing very strong evidence that defective helper CHIKV genomes were being packaged, by these systems, either individually or in combination with replicon genomes (as single particles with bipartite genomes).

C. Analysis of the threshold of detection of a full length virus titrated against a saturated background of replicon/defective helper genomes

It was clear that co-electroporation of replicon/defective helper recombinant crosses results in the release of a heterogeneous population of genomes housed within CHIKV particles with subpopulations of replicon only, defective helper only, and dually packaged genomes all potentially represented. Furthermore, if recombination occurred at any frequency within these crosses we would also expect subpopulation of "full-length-like" recombinant genomes to exist. However, since I expected recombination efficiency to be much less than defective helper packaging efficiency and recombinant genomes to have aberrant sequence duplications that would likely result in decreased replicative fitness, based on similar studies of SINV (Weiss & Schlesinger 1991). It would be safe to assume that any recombinants would likely be the minority population and be at a competitive disadvantage. Therefore experiments were designed to determine the minimum number of primary infections (limit of detection) of a full length virus (or simulated recombinant events) that allowed for cell culture based purification of that full length virus against a saturated background of replicon/defective helper genomes.

Briefly, ten-fold serial dilutions of CHIK-LR-5'-GFP RNA (range: 10.0-0.001ng) were titrated in triplicate against a saturated 10.0µg each of CHIK-LR-Cherry-Rep and CHIK-LR-FL-ST-Help (**Figure 2.12**) with infectious centers assay. One fifth of each electroporation was plated in a single well of a six-well plate ($2x10^6$ cells), supernatant harvested at 24h, blindly passaged three times in C6/36 cells (500µL infection, 72h incubation), and the recovery of CHIK-LR-5'-GFP evaluated by plaque assay of C6/36 passage #3 supernatant samples. GFP expressing full length CHIKV (CHIK-LR-5'-GFP) could be re-purified in this way to a minimum of 49 ± 21 primary infections. Additionally, plaque assay analysis of these samples identified the reproducible

generation of two other distinct phenotypic populations of foci/plaques namely foci that were Cherry positive and plaques that did not display fluorescent protein expression (**Table 2.4**). While it was possible that the colorless variant observed in these experiments could have represented the loss of CHIK-LR-5'-GFP expression cassette due to instability (as previously demonstrated (Tsetsarkin *et al.*, 2006)), this seemed unlikely since it was detected at a dilution approximately ten-fold below the limit of CHIK-LR-5'-GFP detection. Nevertheless, the Cherry population variant could only have arisen via a recombination event resulting in the insertion of the Cherry expression cassette into a full length replication competent background. Because defective helper packaging efficiency appears to be very low, I expected the population of particles containing dual packaged genomes to rapidly decrease during serial passage in cell culture. Therefore it was extremely unlikely that either the Cherry or colorless plaques resulted from co-packaged replicons and defective helpers. However, since these experiments involved the co-electroporation of three distinct genome topologies, full length, replicon, and defective helper, it was unclear which of these genomes were

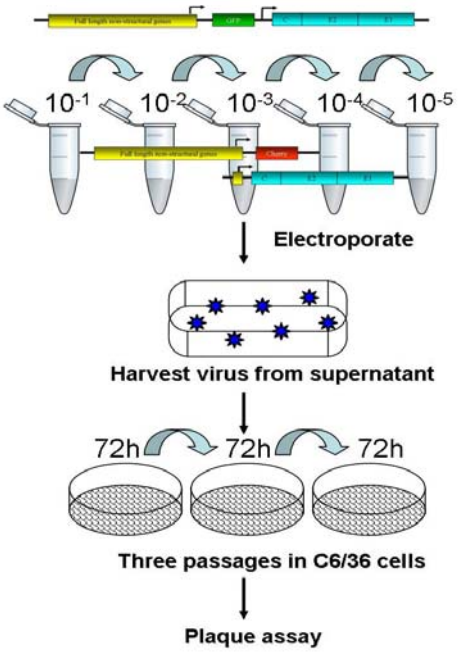


FIGURE 2.12 BASIC EXPERIMENTAL DESIGN: LIMIT OF DETECTION OF FULL LENGTH VIRUS TITRATED AGAINST A SATURATED LEVEL OF REPLICON/DEFECTIVE HELPER.
Empirical evaluation of limit of detection was performed by co-electroporating ten-fold serial dilutions of CHIK-LR-5'-GFP RNA against a constant 10µg per species CHIK-LR-Cherry-Rep and CHIK-LR-FL-ST-Help RNAs.

participating in the putative recombination events to give rise to the colorless and Cherry variants.

TABLE 2.4 LIMIT OF CHIK-LR-5'-GFP DETECTION PLAQUE ASSAY RESULTS.

	Replicate #1			
CHIK-LR-5'-GFP Dilution	**Green Foci FL infections**	**Green Plaque Titer**	**Cherry Plaque Titer**	**Colorless Plaque Titer**
-2 (10ng RNA)	660	5.0×10^6	2.0×10^4	n.d.
-3 (1ng RNA)	102	4.8×10^6	9.8×10^6	n.d.
-4 (.1ng RNA)	28	1.9×10^6	5.6×10^6	n.d.
-5 (.01ng RNA)	14	n.d.	4.0×10^5	n.d.

	Replicate #2			
CHIK-LR-5'-GFP Dilution	**Green Foci FL infections**	**Green Plaque Titer**	**Cherry Plaque Titer**	**Colorless Plaque Titer**
-3 (1ng RNA)	70	8.8×10^5	7.6×10^4	n.d.
-4 (.1ng RNA)	8	n.d.	n.d.	n.d.
-5 (.01ng RNA)	7	n.d.	n.d.	n.d.
-6 (.001ng RNA)	4	n.d.	n.d.	n.d.

	Replicate #3			
CHIK-LR-5'-GFP Dilution	**Green Foci FL infections**	**Green Plaque Titer**	**Cherry Plaque Titer**	**Colorless Plaque Titer**
-2 (10ng RNA)	50	4.0×10^4	n.d.	n.d.
-3 (1ng RNA)	28	n.d.	1.2×10^5	n.d.
-4 (.1ng RNA)	14	n.d.	n.d.	n.d.
-5 (.01ng RNA)	5	n.d.	4.0×10^3	4.4×10^6

plaque titers-plaque forming units (pfu/mL)
n.d.-not detected
plaque titers of putative recombinant virus are highlighted in yellow

Based on previous analyses of alphavirus recombination (Pushko *et al.*, 1997, Weiss & Schlesinger 1991) I anticipated putative recombinants to be characterized by aberrantly homologus cross-over events. Such an event should result in the entire helper structural cassette including SGP being incorporated into the replicon resulting in a duplication of the subgenomic promoter. Therefore I hypothesized that if GFP could be fused into the structural cassette of the defective helper genome (and remove the double subgenomic orientation) and co-electroporated with a replication competent Cherry

genome bi-colored recombinant genomes could theoretically generated (**Figure 2.13**). Infection with such a genome should result in a foci in which all cells would be positive for both fluorescent proteins and could be readily differentiated from the packaged helper "bull's-eye" phenotype.

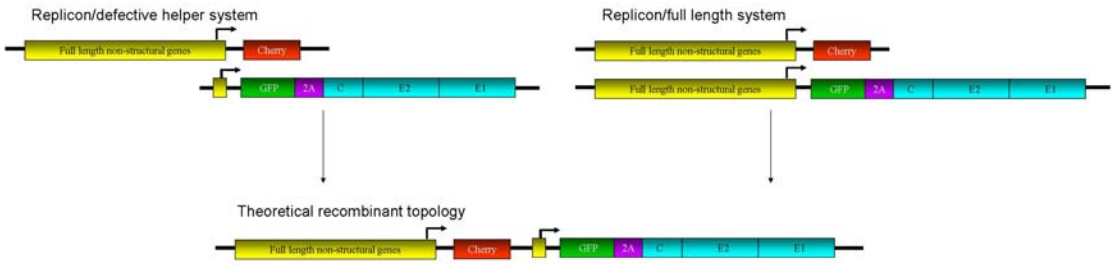


FIGURE 2.13 SCHEMATIC REPRESENTATION OF CHIKUNGUNYA VIRUS GENOMES USED TO GENERATE BI-COLORED RECOMBINANTS
The replicon/defective helper cross involved co-electroporation of CHIK-LR-Cherry-Rep2 and CHIK-LR-5'-GFP-2A-Help RNAs, while the replicon/full length system involved co-electroporation of CHIK-LR-Cherry-Rep2 and CHIK-LR-5'-GFP-2A-FL RNAs.

These experiments were performed by titrating ten-fold serial dilutions of either CHIK-LR-5'-GFP-2A-FL or CHIK-LR-5'-GFP-2A-Help (range 10-10^{-7}µg) RNA against a constant 10.0µg CHIK-LR-Cherry-Rep2 RNA. RNAs were co-electroporated and 1/5 of each electroporation plated in a single well of a six-well plate (~2.0x10^6 cells), supernatant harvested at 24h, blindly passaged three times in C6/36 cells (500µL infection, 72h incubation), and analyzed by plaque assay. Co-electroporation of CHIK-LR-5'-GFP-2A-FL and CHIK-LR-Cherry-Rep2 resulted in the recovery of high titer GFP positive plaques likely representing non-recombinant virus derived from CHIK-LR-5'-GFP-2A-FL. Low titer Cherry positive "plaques" were observed (co-infection levels 10^4-10^6, titers $\leq 10^3$), however these areas of Cherry fluorescence were always observed against a saturated background of GFP fluorescence (100% GFP positive cells).

Additionally, these areas of Cherry expansion could not be plaque purified. This observation suggested that the observed Cherry foci were a result of CHIK-LR-Cherry-Rep2 genomes that were being packaged by CHIKV structural genes supplemented in *trans* by CHIK-LR-5'-GFP-2A-FL and not a product of recombination.

However, it was possible that the GFP positive population could represent a mixed population of recombinant and non-recombinant genomes. Therefore, C6/36 passage #3 supernatant samples from all dilutions in which GFP plaques were recovered were analyzed by RT-PCR and restriction digestion analysis to determine if the nonstructural genes of any of these full length GFP genomes had been derived from CHIK-LR-Cherry-Rep2. This was possible because of the unique Hind*III* restriction site engineered into the nsP4 gene of CHIK-LR-Cherry Rep2 (**Figure 2.14**). Primers were chosen that would only allow for amplification of those genomes which contained the CHIKV nonstructural genes located 5' to the GFP-2A cassette (pr 41855 nsF3/FMDV2A 3' end R). The expected 1124nt amplicon was generated from all samples amplified in this way. None of these PCR amplicons were capable of being digested by Hind*III* indicating that the GFP positive plaques in all samples were a pure population of non-recombinant genomes. Therefore it was concluded that it was unlikely that the previously observed putative recombinants resulted from recombination between full length and replicon genomes.

Co-electroporation of CHIK-LR-Cherry-Rep2 (R326) and CHIK-LR-5'-GFP-2A-Help (H325) reproducibly resulted in the generation of viable recoverable putative recombinant genomes, limit of detection ~10^3 co-infections, that were characterized by the ability to efficiently grow and be selected for in cell culture. An unexpected result of these experiments was the presence of a mixed population representing four phenotypes: colorless plaques (CL; 5/10 dilutions, average titer $1.4x10^6$ pfu/mL), Cherry only plaques

(CO; 1/10 dilutions, average titer $8.0x10^2$ pfu/mL), Green only plaques (GO; 2/10 dilutions, average titer $1.8x10^5$ pfu/mL) and bi-color plaques (C/G; 2/10 dilutions $2.2x10^4$ pfu/mL) (**Table 2.5**). Although all four phenotypes were detected, only the colorless variant was reproducibly detected at all dilutions (range: 10^3-10^6 co-infections). Colored foci (CO/GO/CG) were detected sporadically and in general at a lower titer than colorless plaques suggesting that colored genomes may be less stable/fit and were evolving toward colorless genomes during cell culture passage. Indeed in only a single dilution, 1µg H325:10µg R326, were all four variants detected simultaneously, however in this sample colorless plaques were in 100-1000 fold abundance over colored foci (**Table 2.5**). Northern blot analysis (CHIKV-5'UTR BioProbe, **Table 2.3**) of plaque purified CL, GO, and CG putative recombinants confirmed the presence of CHIKV genomes of full-length -like size (**Figure 2.15 Lanes 5, 6, 8, 10 and 11**), CO foci were not analyzed in this way due to difficulties in plaque purifying this low titer variant. All further analyses were performed using only the sample from which all four phenotypic variants were simultaneously observed (1µg H325:10µg R326; **Table 2.5**). Experiments were designed to determine how early in the cell culture passage protocol putative recombinants could

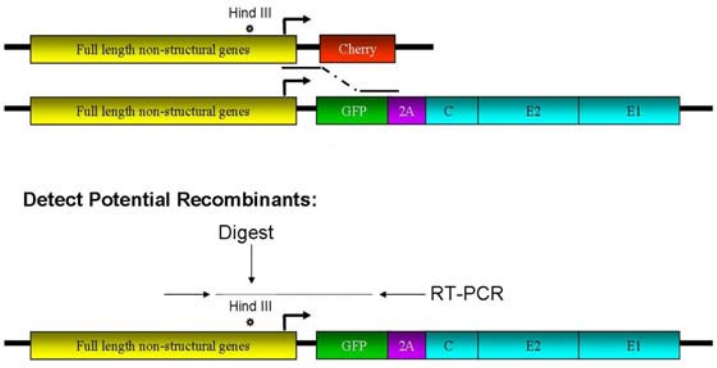


FIGURE 2.14 STRATEGY FOR RESTRICTION DIGESTION CONFIRMATION OF REPLICON/FULL LENGTH RECOMBINATION.
The presence of a unique Hind*III* restriction site in the replicon nsP4 sequence of CHIK-LR-Cherry-Rep2 allows for the differentiation of parental full length GFP virus (CHIK-LR-5'-GFP-2A-FL) and recombinant GFP virus by RT-PCR and restriction digestion analysis.

TABLE 2.5 CHIKUNGUNYA CHERRY REPLICON/GFP-2A DEFECTIVE HELPER PLAQUE ASSAY RESULTS.

	Plaque Assay Results				
H325 : R326 RNA input ratio	**Primary "co-infections"**	**GFP & Cherry (+) Plaques**	**GFP only (+) Plaques**	**Cherry only (+) Plaques**	**Non-Colored Plaques**
H325 10µg neg	--	n.d	n.d	n.d	n.d
R326 10µg neg	--	n.d	n.d	n.d	n.d
10µg : 10µg	$3.3x10^6$	n.d	$3.6x10^5$	n.d	$2.8x10^5$
10µg : 1µg	$2.0x10^5$	n.d	n.d	n.d	$5.2x10^6$
1µg : 10µg	$5.0x10^5$	$4.0x10^3$	$8.0x10^2$	$8.0x10^2$	$6.0x10^5$
10^{-1} µg : 10µg	$5.0x10^4$	$4.0x10^4$	n.d	n.d	$1.04x10^5$
10^{-2} µg : 10µg	$6.0x10^3$	n.d	n.d	n.d	$1.0x10^6$
10^{-3} µg : 10µg	$1.0x10^2$	n.d	n.d	n.d	n.d
10^{-4} µg : 10µg	86	n.d	n.d	n.d	n.d
10^{-5} µg : 10µg	9	n.d	n.d	n.d	n.d
10^{-6} µg : 10µg	n.d	n.d	n.d	n.d	n.d
10^{-7} µg : 10µg	n.d	n.d	n.d	n.d	n.d

H325 denotes defective helper CHIK-LR-5'-GFP-2A-Help RNA, R326 denotes replicon CHIK-LR-Cherry-Rep2 RNA. The amount of each RNA species in each electroporation was as indicated.
Titers are reported as plaque forming units per mL

be recovered and the nature of the early population heterogenecity. These analyses determined that clonal recombinant CHIKV genomes could not be isolated without a single amplification in C6/36 cells followed by infection of Vero cells ($1x10^7$) in suspension and analysis in 96-well plates (288 wells, ~$3.5x10^4$ cells per well). This was likely due to the fact that alphavirus genomes encapsidated with CHIKV protein coats had a 10-50 fold higher infectivity for C6/36 cells than for Vero cells. Primary clonal recombinants were all (20/20) characterized by the ability to express both GFP and Cherry fluorescent proteins suggesting that early recombination events likely resulted in the generation of a double subgenomic genome topology as expected. Six isolates were selected for further analysis, via passage three times in C6/36 cells with plaque assays following each passage, in attempt to recapitulate the previously observed phenotypic diversity. Rapid loss of reporter protein coding cassettes coincident with degradation into a four phenotype population was observed for all six isolates within a single passage in

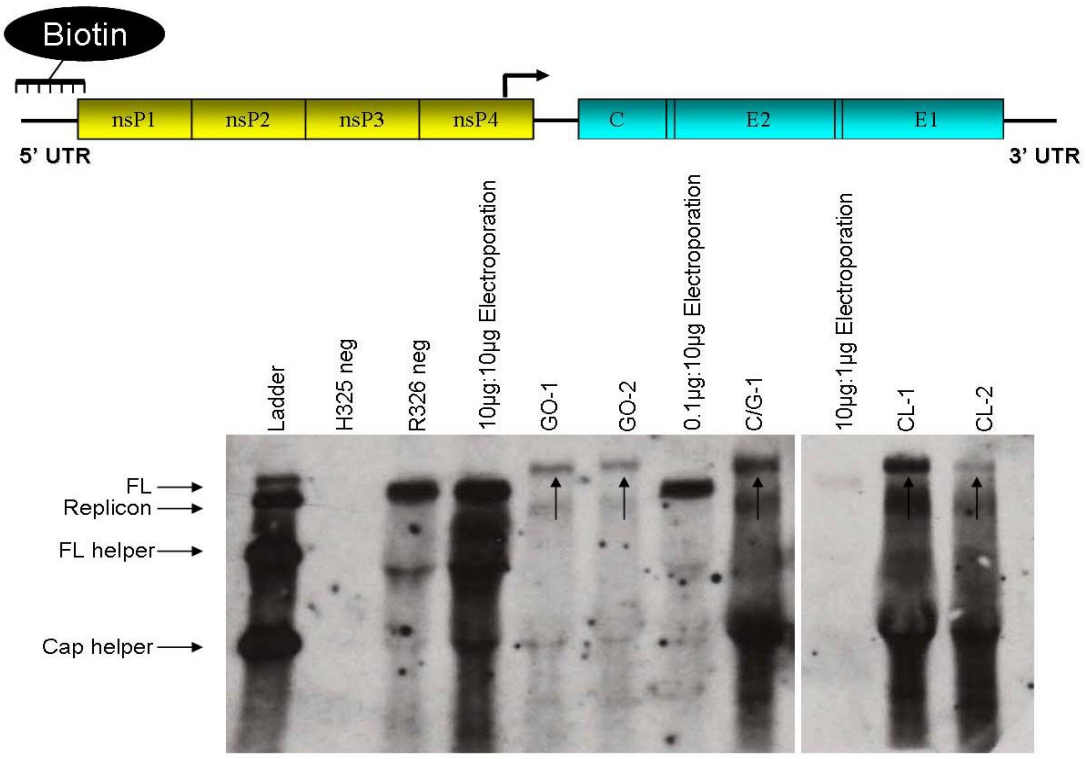


FIGURE 2.15 NORTHERN BLOT ANALYSIS OF PLAQUE PURIFIED CHIKUNGUNYA CHERRY/GFP RECOMBINANTS.
Northern blot analysis of plaque purified recombinant virus following co-electroporation of CHIK-LR-Cherry Rep2 (R326) and CHIK-LR-5'-GFP-2A-Help (H325) RNAs (not depicted schematically). Hybridized using CHIKV-5'-UTR BioProbe. Lane 1: CHIKV RNA ladder, Lane 2: defective helper only electroporation, Lane 3: replicon only electroporation, Lanes 4, 7, and 9; H325-R326 co-electroporations, Lanes 5 and 6: two GFP only (GO) isolates, Lane 8: Bi-colored isolate (C/G), and Lanes 10 and 11: two colorless (CL) isolates. Arrows indicated the presence of CHIKV specific bands of full-length-like size.

C6/36 cells (**Figure 2.16**). Furthermore, by the end of the passage protocol only the colorless variant was detected in all samples (6/6), likely a result of evolution to, and success of, the most fit genome topology (i.e. the genome containing the least amount of exogenous sequence). This rapid degradation precluded the ability to PCR amplify and sequence these recombinants. Nevertheless, the observation of the ability to generate bi-colored genomes of full-length-like size strongly supported the ability of these strategies

to generate viable and recoverable alphavirus genomes. Therefore due to the confounding nature of reporter stability all further analyses of alphavirus recombination were performed using replicon/defective helper systems that did not contain these cassettes. However, fluorescent protein constructs were used as electroporation efficiency controls within each experimental cross.

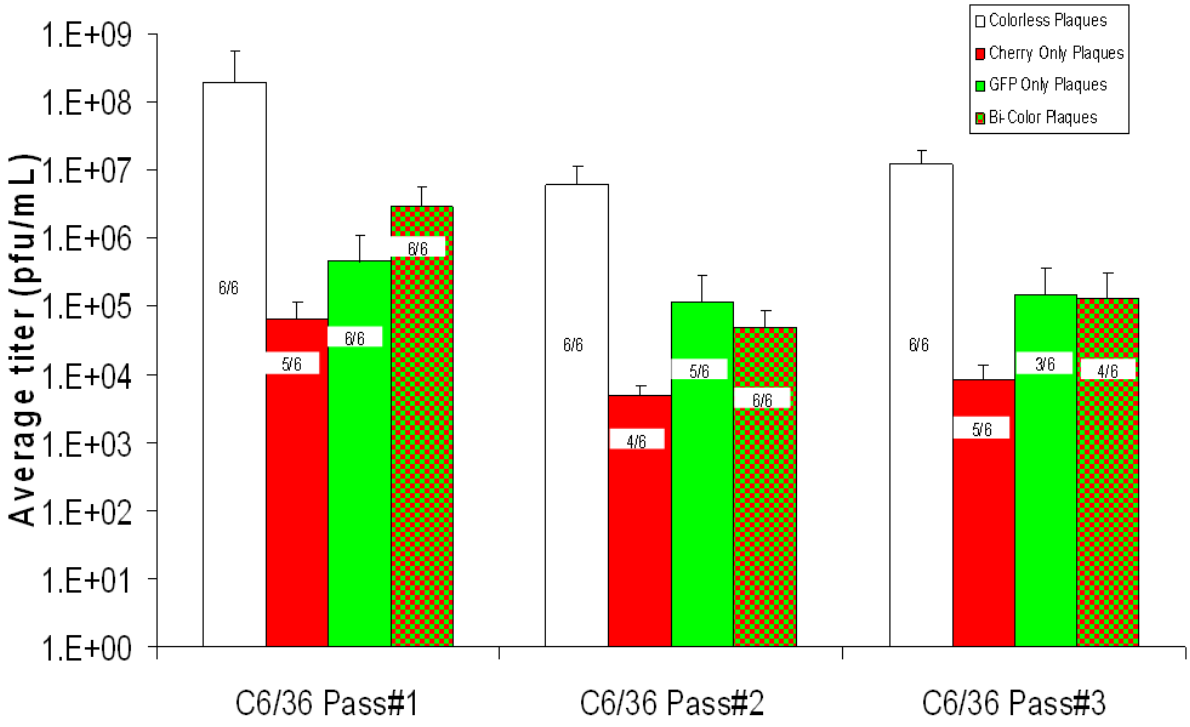


FIGURE 2.16 CHANGE IN TITER AND ABUNDANCE OF CHIKUNGUNYA RECOMBINANTS OVER THREE PASSAGES IN C6/36 CELLS.
Titers reported as plaque forming units (pfu)/mL

D. Mosquito infectivity of GFP/Cherry recombinants

CHIKV mixed phenotypic recombinants were assayed for their relative abilities to orally infect *Ae. albopictus* mosquitoes. Mosquitoes were sampled (n=12) at days 1, 3, 7, 14, and 21 post infection with a bloodmeal comprised of the four CHIKV recombinant

variants (C6/36 passage #1 of isolate #1); CL 1.6x10^7pfu/mL, GO 4.0 x10^4pfu/mL, CO 1.2 x10^5pfu/mL, and CG 1.3 x10^6pfu/mL. Whole mosquitoes from days 1, 3, and 7 and mosquito bodies and corresponding heads from days 14 and 21 were homogenized and the presence and relative titer of the four CHIKV variants was determined by plaque assay. Additionally, mosquito midguts were dissected at seven dpi and examined microscopically for GFP/Cherry fluorescence.

High titer colorless CHIKV was recovered from ≥90% of mosquitoes at all dpi. Viruses competent for the expression of fluorescent proteins were not detected beyond three dpi, day one; GO 2/12, CO 2/12, and CG 5/12 and day three; GO 1/12, CO 0/12, and CG 0/12, and were ≥10^4 fold less abundant as compared to CL variants (**Figure 2.17**). Interestingly the majority (>90%) of midguts were positive for both GFP and Cherry fluorescence at seven dpi indicating that CHIKV variants containing fluorescent protein coding cassettes were competent for infection, replication, and protein expression in *Ae. albopictus* mosquitoes. Analysis of mosquito heads identified a high proportion of mosquitoes had developed a disseminated infection of the colorless variant by days 14 (12/12; average titer 1.2x10^3pfu/mL) and 21 (11/12; average titer 2.2x10^3pfu/mL) suggesting these mosquitoes would likely have been able to transmit this CHIKV variant. Disseminated infections of GO, CO, and CG variants were not observed at 14 and 21 dpi.

E. Analysis of CHIKV inter-genic recombination

All further alphavirus recombination experiments involved the use of an optimized cell culture passage protocol (see Materials and Methods: isolation of clonal populations of putative recombinant alphavirus genomes; **Figure 2.3**). This protocol was empirically refined during the analysis of the bi-colored products of the CHIK-LR-Cherry-Rep2 and CHIK-LR-5'-GFP-2A-Help recombinant cross. Samples for which no

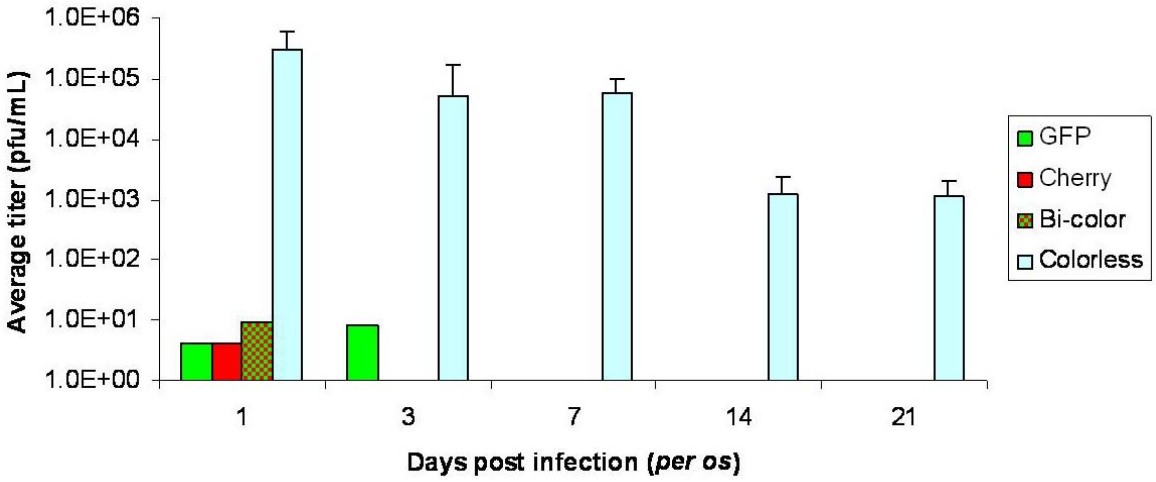


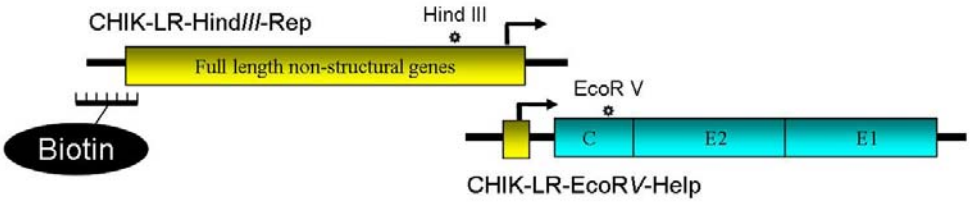
FIGURE 2.17 ANALYSIS OF THE RELATIVE MOSQUITO INFECTIVITY OF CHIKUNGUNYA CHERRY/GFP MIXED POPULATION RECOMBINANTS.
Average for whole mosquito homogenates are reported at days 1, 3, and 7 and mosquito body homogenates at days 14 and 21.
Titers are reported as plaque forming units (pfu)/mL.

Vero wells were CPE positive following inoculation with either electroporation or C6/36 passage #1 amplification supernatants were ruled to be recombination negative.

pCHIK-LR-Hind*III*-Rep has the following topology; 5'UTR, full length nonstructural genes with a engineered unique Hind*III* restriction site in nsP4, SGP, 68nt inter-genic sequence, and 3'UTR. Defective helper pCHIK-LR-EcoR*V*-Help contains the same nonstructural gene deletion as p304 followed by the full length CHIKV structural ORF and associated subgenomic promoter with the addition of a single silent mutation in the capsid sequence to allow for introduction of a unique EcoR*V* restriction site. It was expected that recombination using a replicon/defective helper system such as this would be aberrantly homologous and result in sequence duplications, deletions, and/or insertions allowing for easy identification of putative cross-over regions. However, should a completely homologous event have occurred, or result from post-recombination sequence

evolution (as observed in experiments with bi-colored recombinants), the resulting sequence would have been indistinguishable from the CHIKV (La Reunion) wt sequence. Because this strain was regularly used in the laboratory it was critical that putative recombinants have a sequence distinct from the wt virus. These unique Hind*III* and EcoR*V* restriction sites were specifically engineered into my replicon and defective helper genomes to facilitate the differentiation of putative recombinant genomes from potential laboratory contaminants should that situation arise. Additionally, both of these mutations were individually engineered into the CHIK-LR-5'-GFP i.c. and plaque size, specific infectivity, and post-electroporation titers (24 and 48h) evaluated to confirm that the inclusion of either the Hind*III* and EcoR*V* sites did not in any way compromise viral replication (**Figure 2.11**)

The efficiency of CHIKV inter-genic recombination (events facilitated by non-coding sequence) was determined by co-electroporating ten-fold serial dilutions of CHIK-LR-EcoR*V*-Help (H338) full length structural helper RNA against a saturated (10µg per electroporation) CHIK-LR-Hind*III*-Rep (R337) "empty" replicon. Additionally, CHIK-LR-Cherry-Rep2 and CHIK-LR-EcoR*V*-Help RNAs (10µg per species) were co-electroporated with infectious centers assay as efficiency controls ($5.0x10^6$/10µg each RNA) to ensure appropriate levels of co-infection ($\geq 1x10^6$ co-infections per electroporation) were achieved within the experimental set. CHIKV recombinants were purified (following a single 48h passage in C6/36 cells) to a minimum co-infection of $\sim 10^3$ (**Figure 2.18**). Up to six isolates per dilution were passaged once in C6/36 cells, followed by titer determination by plaque assay. Inter-genic recombinants from all dilutions were competent for replication in both vertebrate (Vero) and arthropod (C6/36) cells reaching average titers of $1.05\text{-}5.93x10^7$ pfu/mL by 48h post infection in C6/36 cells (**Figure 2.18**). Northern blot analysis (CHIKV-5'UTR BioProbe; **Table 2.3**)



H338 : R337 RNA input ratio	Primary "co-infections"*	Isolates Analyzed (#/total)	Average Titer C6/36 P1 (pfu/mL)
H338 10µg neg	--	n.d	--
R337 10µg neg	--	n.d	--
10µg : 10µg	~$1.0x10^6$	6/11	$1.05x10^7$
1µg : 10µg	~$1.0x10^5$	2/2	$4.2x10^7$
.1µg : 10µg	~$1.0x10^4$	2/2	$2.8x10^7$
.01µg : 10µg	~$1.0x10^3$	6/11	$5.93x10^7$
.001µg : 10µg	~$1.0x10^2$	n.d	--

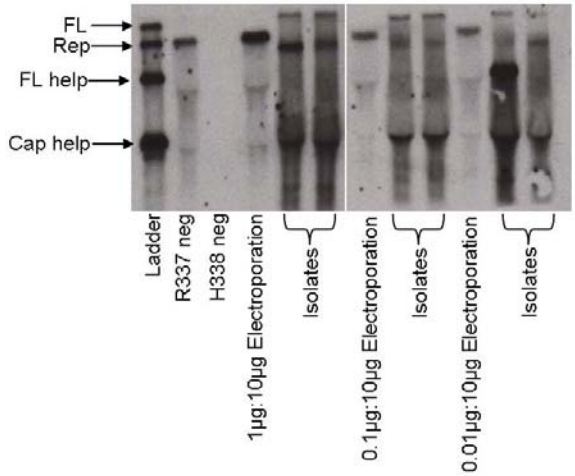


FIGURE 2.18 ANALYSIS OF INTER-GENIC RECOMBINATION OF CHIKUNGUNYA VIRUS. Average plaque assay titers for all dilutions (up to six isolates per dilution) in which putative recombinant CHIKV was detected following co-electroporation of CHIK-LR-HindIII-Rep (R337) and CHIK-LR-EcoRV-Help (R338) RNAs, including replicon and defective helper only negative controls, reported as plaque forming units (pfu)/mL. Minimum level of recombinant recovery highlighted in yellow. Northern blot analysis, hybridized using CHIKV-5'-UTR BioProbe, of selected CHIKV inter-genic recombinants. Lane 1: CHIKV RNA ladder, Lane 2: replicon only electroporation, Lane 3: defective helper only electroporation, Lanes 4, 7, and 10: H338-R337 co-electroporations, and Lanes 5, 6, 8, 9, 11, and 12: clonal recombinants.

of selected isolates, C6/36 48h passage #1 total cellular RNA, confirmed the presence of CHIKV RNA species of full-length-like size. These full-length-like viral RNAs were ~4.0Kb larger than replicon RNA (CHIK-LR-Hind*III*-Rep) and were only detected in cellular RNA samples infected with putative CHIKV clonal recombinant isolates (**Figure 2.18, Lanes 5, 6, 8, 9, 11, and 12**). As expected defective helper RNA from CHIK-LR-EcoR*V*-Help was not detected when electroporated alone, indicating that this genome was not competent for self replication (**Figure 2.18, Lane 3)**.

Sequence analysis of two clonal isolates from the lowest dilution from which recombinant genomes were detected (~10^3 co-infections) identified a single recombinant topology (**Figure 2.19**). Inter-genic recombinants were characterized by a double subgenomic organization whereby the entire CHIKV structural ORF and its native SGP were inserted into the replicon 3'UTR. Assuming that no post-recombinant evolution had occurred, to give rise to the observed topology, it appeared that the CHIKV RdRp synthesized nascent RNA from a replicon template and at 271nt into the 3'UTR this complex "jumped" into the truncated nsP4 defective helper sequence and resumed RNA synthesis to result in the aforementioned topology. This recombination event appears to have occurred despite a lack of secondary structure in the 3'UTR at the release site and a lack of sequence homology between the release and re-initiation sequence. Nevertheless, this aberrant double subgenomic organization was stable over five blind passages in Vero cells, with no signs to indicate evolution had occurred within the sequenced region ~600nt 5'/3' of the cross-over site.

F. Analysis of CHIKV intra-genic recombination

The relative efficiency of CHIKV intra-genic (occurring within coding sequence) recombination was determined using a CHIKV replicon/defective helper recombinant cross containing complementary 5' and 3' deletions in the structural ORF. RNA from

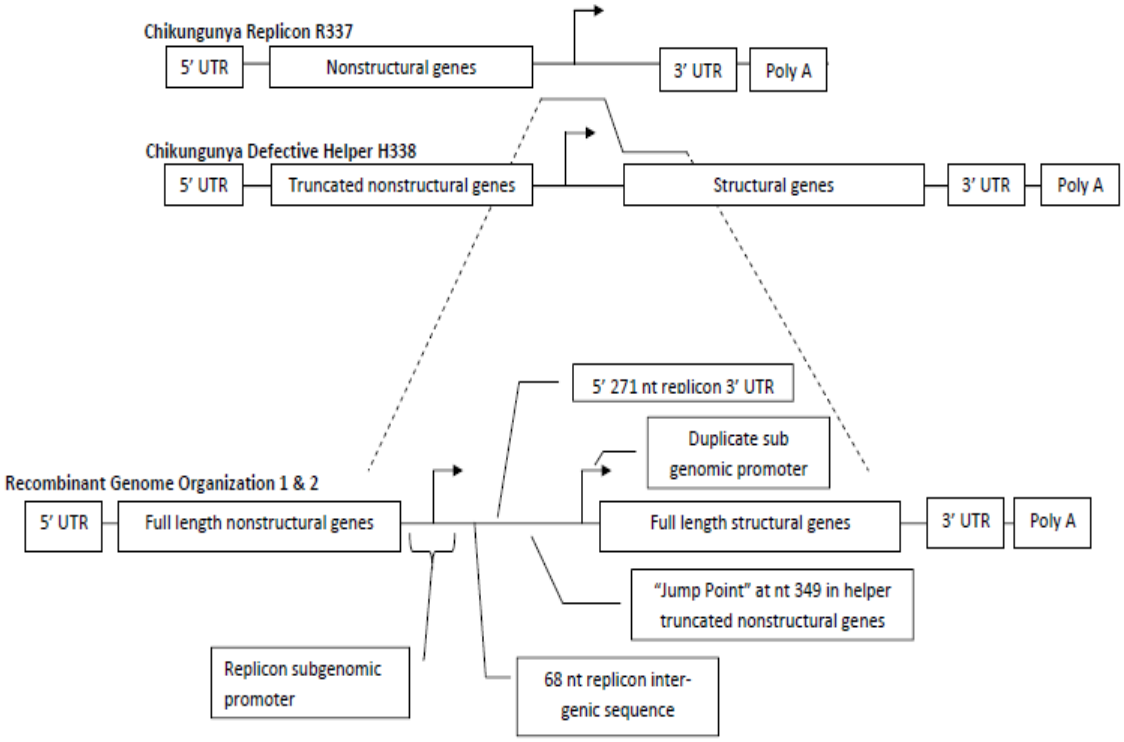
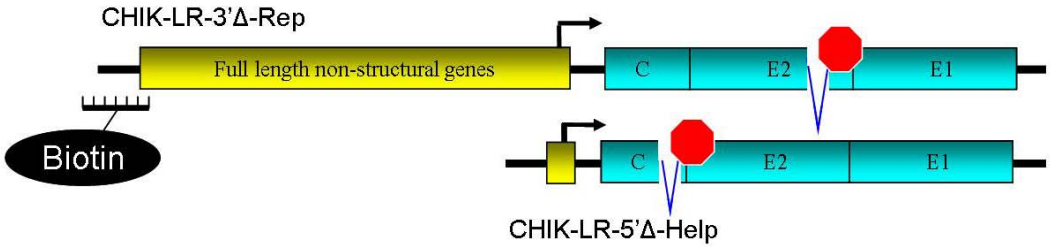


FIGURE 2.19 CHIKUNGUNYA INTER-GENIC RECOMBINANT GENOME ORGANIZATION. Schematic representation of parental replicon/defective helper recombinant cross (CHIK-LR-Hind*III*-Rep (R337) and CHIK-LR-EcoR*V*-Help (R338)) along with detailed sequence analysis of the cross-over region and resulting recombinant topology.

pCHIK-LR-3'Δ-Rep, containing the previously described 112nt FSΔ in E2, was co-electroporated against ten-fold serial dilutions of RNA from pCHIK-LR-5'Δ-Help, which contained a 182nt FSΔ in capsid. RNAs derived from pCHIK-LR-3'Δ-Cherry-Rep and pCHIK-LR-5'Δ-GFP-Help were also co-electroporated in parallel (10µg per RNA species) to verify co-infection efficiency within this experimental set, $1.2x10^7$/10µg each RNA.

Recombination was observed to occur within the 967nt of the structural ORF under investigation to a minimum of ~10^5 co-infections (**Figure 2.20**). Clonal isolates of intra-genic recombinant CHIKV could be directly purified from electroporation



H341 : R302 RNA input ratio	Primary "co-infections"*	Isolates Analyzed (#/total)	Average Titer C6/36 P1 (pfu/mL)
H341 10µg neg	--	n.d	--
R302 10µg neg	--	n.d	--
10µg : 10µg	~$1.0x10^6$	3/3	$3.07x10^7$
1µg : 10µg	~$1.0x10^5$	2/2	$2.8x10^7$
0.1µg : 10µg	~$1.0x10^4$	n.d.	--

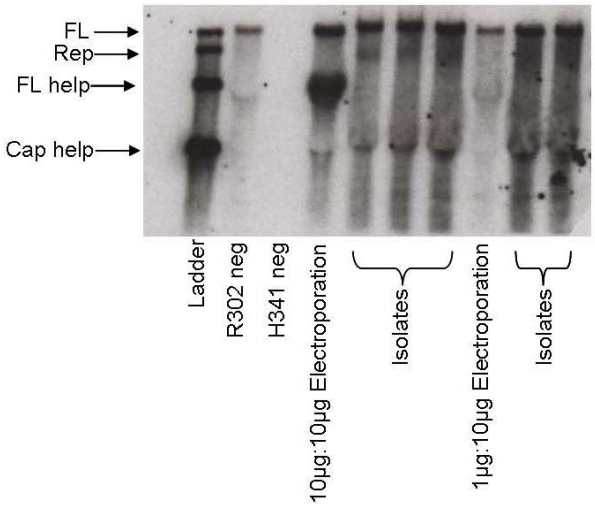


FIGURE 2.20 ANALYSIS OF INTRA-GENIC RECOMBINATION OF CHIKUNGUNYA VIRUS. Average plaque assay titers for all dilutions in which putative recombinant CHIKV was detected following co-electroporation of CHIK-LR-3'Δ-Rep (R302) and CHIK-LR-5Δ'-Help (H341) RNAs, including replicon and defective helper only negative controls, reported as plaque forming units (pfu)/mL. Minimum level of recombinant recovery highlighted in yellow. Northern blot analysis, hybridized using CHIKV-5'-UTR BioProbe, of selected CHIKV intra-genic recombinants. Lane 1: CHIKV RNA ladder, Lane 2: replicon only electroporation, Lane 3: defective helper only electroporation, Lanes 4 and 8: H341-R302 co-electroporations, and Lanes 5, 6, 7, 9, and 10: clonal recombinants. Red octagons indicated truncated open reading frames.

supernatant samples and did not require any additional amplification in C6/36 cells. Intra-genic recombinants were competent for replication in both Vero and C6/36 cells and reached average titers of ~$3.0x10^7$pfu/mL by 48h post-infection in C6/36 cells. It is important to note that CHIK-LR-5'Δ-Rep is only 112nt smaller in size than the CHIK-LR wt virus, therefore although Northern blot analysis can verify the presence of virus specific RNAs intra-genic recombinants size shift analysis was not possible in these experiments. Northern blot analysis (CHIKV-5'UTR BioProbe; **Table 2.3**) of all CHIKV intra-genic recombinants identified virus specific RNAs of full-length-like size in C6/36 total cellular RNA samples 48h post infection with putative intra-genic recombinants (**Figure 2.20, Lanes 5, 6, 7, 9, and 10**).

The putative cross-over sequence, entire region between the engineered 5'/3' ΔFS, of two isolates from co-infection dilution ~10^5 was sequenced to determine the resulting gene topology. Aberrantly homologous recombination appeared to have occurred between the two ΔFS mutations as expected and resulted in the generation of a stable in-frame duplication of structural gene coding sequence (**Figure 2.21**). This duplication was comprised of the 5' 34 amino acids (aa) (104nt) of the CHIK-LR-5'Δ-Rep E3 coding sequence followed by the 3' 20aa (61nt) of the CHIK-LR-3'Δ-Help capsid sequence and the generation of a new leucine codon (CTG) at the jump point. This topology likely resulted in retention of the capsid-envelope cleavage signal sequences allowing for efficient removal of the duplicated partial proteins during structural gene transcription and translation thus allowing for the efficient expression of full length functional C and E3. In this case the replicative complex appears to have released the primary or donor (replicon) template at a bulge within a predicted stem-loop in the E3 sequence (**Figure 2.22**) with re-initiation on the secondary or acceptor (defective helper) template likely being directed by sequence homologies in the structural

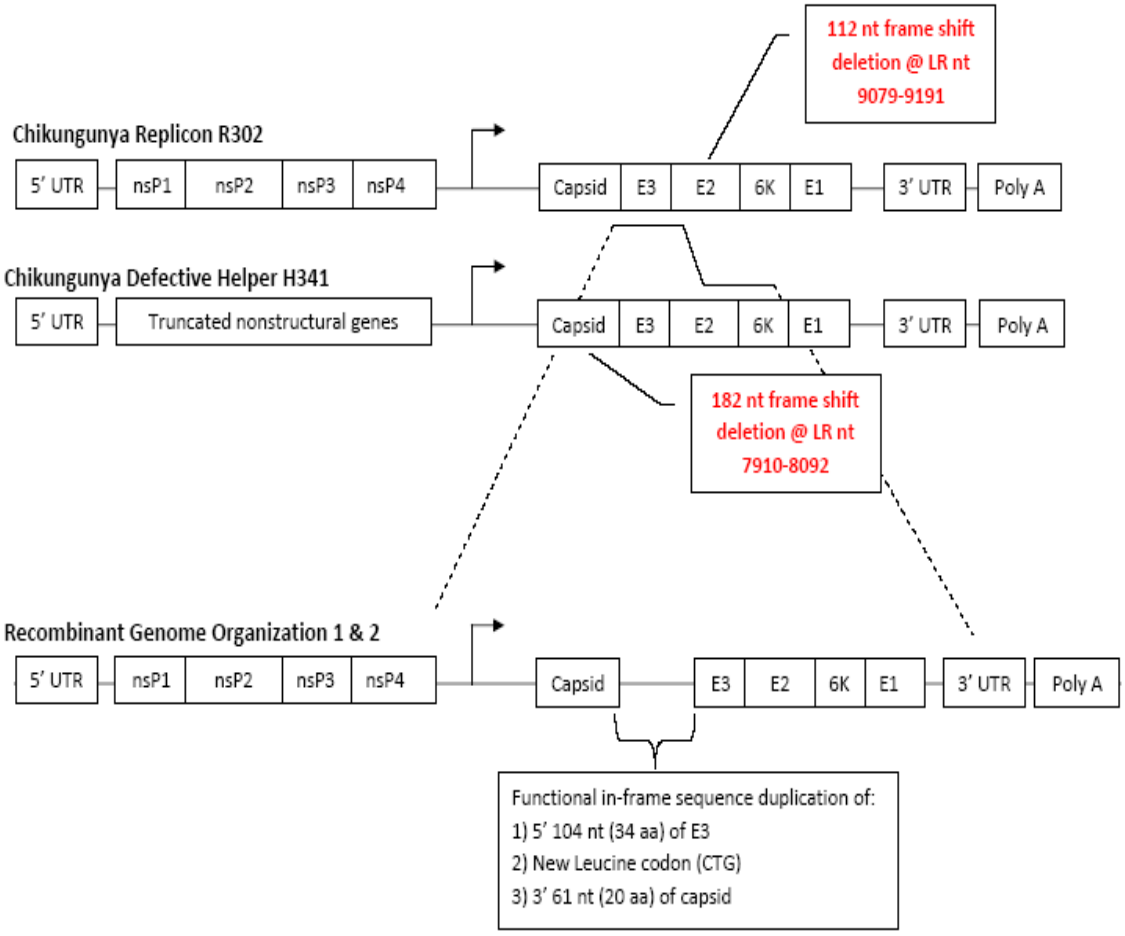


FIGURE 2.21 CHIKUNGUNYA INTRA-GENIC RECOMBINANT GENOME ORGANIZATION. Schematic representation of parental replicon/defective helper recombinant cross (CHIK-LR-3'Δ-Rep (R302) and CHIK-LR-5Δ'-Help (H341)) along with detailed sequence analysis of the cross-over region and resulting recombinant topology.

gene sequences. This topology remained stable over five blind passages in Vero cells with no evidence of sequence evolution or loss of duplicated structural gene sequence.

G. Analysis of CHIKV recombination between genomes containing in-frame deletions

Initial analyses of CHIKV intra-genic recombination using frame shift deletions (see previous section) identified recombination occurring within the structural gene coding sequence. Within these crosses very strong pressure for recombination was placed on the structural sequence overlap because only an event occurring within this sequence could give rise to expression of the CHIKV envelope proteins.

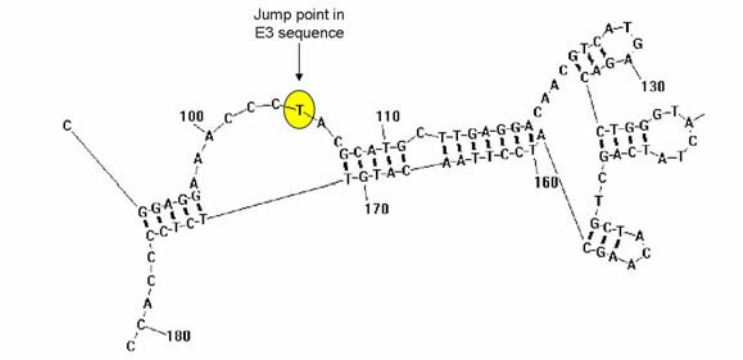
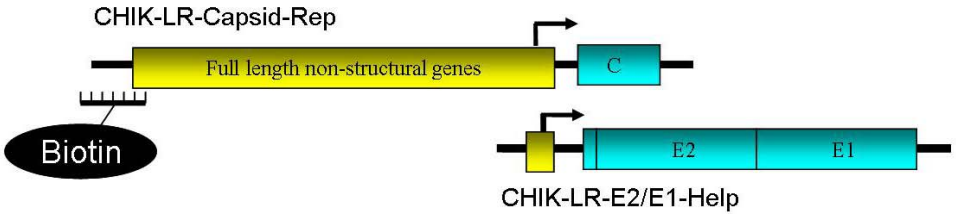


FIGURE 2.22 PREDICTED E3 STEM-LOOP STRUCTURE AT INTRA-GENIC RECOMBINATION REPLICON RELEASE POINT.
Stem-loop predictions generated using the RNAstructure 4.6 software package (Mathews *et al.*, 2004).

A CHIKV replicon/defective helper system containing in-frame structural gene sequence deletions, competent for the expression of all structural genes, was also constructed to determine if similar intra-genic recombination would be favored in a more natural circumstance, i.e. coding sequence intact. Ten-fold serial dilution of CHIK-LR-E2/E1-Help (H308) RNA were co-electroporated against a saturated 10µg RNA of a CHIKV replicon competent for the expression of only the capsid gene (pCHIK-LR-Capsid-Rep). CHIK-LR-Cap-Cherry-Rep and CHIK-LR-E2/E1-GFP-Help RNAs were also co-electroporated (10µg per species) with infectious centers and co-infection efficiency for this experimental set calculated as $8.5x10^6$/10µg each RNA.

Recombinant isolates of the CHIK-LR-E2/E1-Help and CHIK-LR-Capsid-Rep cross were only purified at a level of ~10^5 co-infections similar to intra-genic crosses using ΔFS constructs. Purification of these recombinants required a single round of amplification in C6/36 cells. In-frame recombinants replicated efficiently in both Vero and C6/36 cells however, average peak titers ($5.6x10^6$pfu/mL; **Figure 2.23**) were ~5-10



H305 : R308 RNA input ratio	Primary "co-infections"*	Isolates Analyzed (#/total)	Average Titer C6/36 P1 (pfu/mL)
H338 10µg neg	--	n.d	--
R337 10µg neg	--	n.d	--
10µg : 10µg	~$1.0x10^6$	n.d.	--
1µg : 10µg	~$1.0x10^5$	2/2	$5.2x10^6$
0.1µg : 10µg	~$1.0x10^4$	n.d.	--

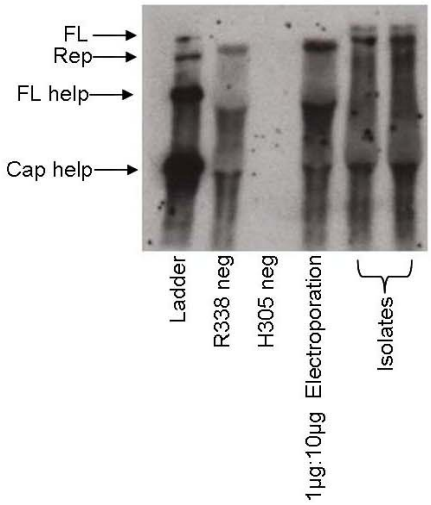


FIGURE 2.23 ANALYSIS OF RECOMBINATION OF CHIKUNGUNYA VIRUS BETWEEN GENOMES CONTAINING IN-FRAME DELETIONS.
Average plaque assay titers for all dilutions in which putative recombinant CHIKV was detected following co-electroporation of CHIK-LR-Capsid-Rep (R308) and CHIK-LR-E2/E1-Help (H305) RNAs, including replicon and defective helper only negative controls, reported as plaque forming units (pfu)/mL. Minimum level of recombinant recovery highlighted in yellow. Northern blot analysis, hybridized using CHIKV-5'-UTR BioProbe, of selected CHIKV in-frame recombinants. Lane 1: CHIKV RNA ladder, Lane 2: replicon only electroporation, Lane 3: defective helper only electroporation, Lane 4: H308-R305 co-electroporation, and Lanes 5 and 6: clonal recombinants.

fold lower than observed average titers of both intra and inter-genic recombinants (see above). Interestingly Northern blot analysis (CHIKV-5'UTR BioProbe; **Table 2.3**) of in-frame recombinants displayed a somewhat unique double banding pattern (**Figure 2.23, Lanes 6 and 5**) with both viral bands being larger than CHIK-LR-Capsid-Rep, potentially suggesting the presence of either a mixed population or recombinant genome instability. Sequence analysis of these in-frame isolates identified a classic double subgenomic recombinant organization with >800nt of sequence duplication. In this case, the entire defective helper (H305) structural cassette including its native SGP and ~150nt of nsP4 sequence were inserted into the replicon 3'UTR (**Figure 2.24**). Analysis of the cross-over point suggested that the replicative complex released the donor strand within the 3'UTR immediately 5' to a potential stem-loop forming sequence (**Figure 2.25**) with subsequent re-initiation on the donor strand without regard for sequence homologies. Interestingly, the blind passage (five) of in-frame isolates resulted in a complete reversion of this double subgenomic organization to the CHIKV-LR wt sequence again indicating that this particular organization was highly unstable.

H. Analysis of CHIKV recombination with a replicon-dual helper system

Previous analyses of alphavirus replicon/defective helper systems have determined that physical bifurcation of the packaging construct into two distinct genomes, namely capsid only and envelope helpers is an effective strategy to decrease full length virus generation to below detectable thresholds (Pushko *et al.,* 1997). To verify this was also the case for CHIKV, RNAs CHIK-LR-Hind*III*-Rep, CHIK-LR-E2/E1-Help, and CHIK-LR-Cap-Help were co-electroporated and assayed (as described for all other CHIKV crosses). No full length viable CHIKV recombinants were detected at any dilution following co-electroporation of this replicon dual-helper system,

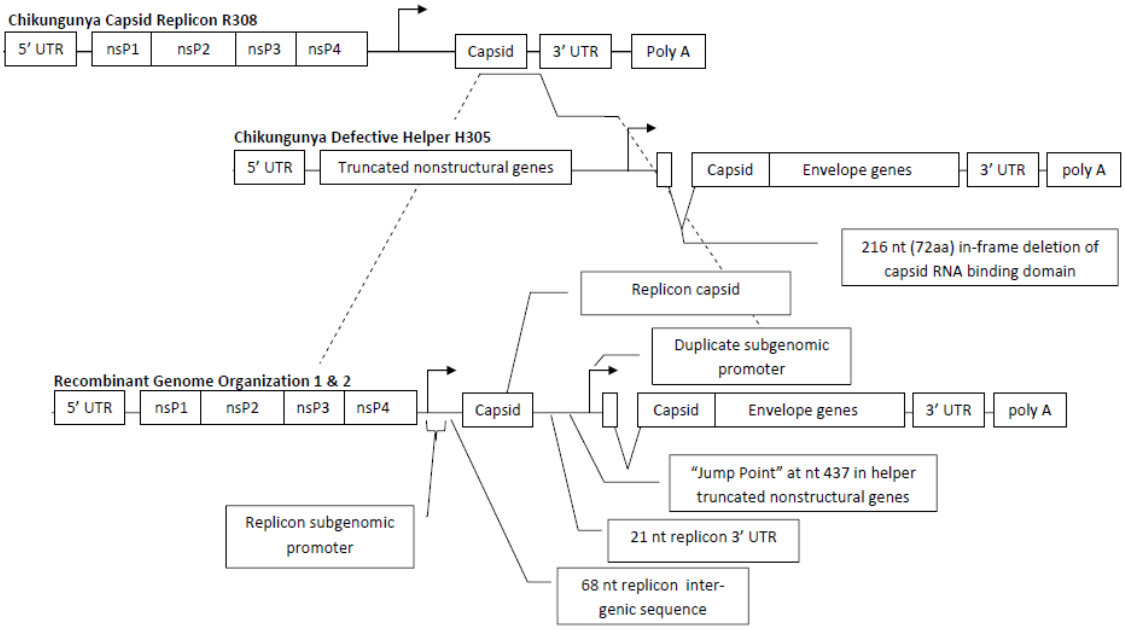


FIGURE 2.24 CHIKUNGUNYA IN-FRAME RECOMBINANT GENOME ORGANIZATION. Schematic representation of parental replicon/defective helper recombinant cross (CHIK-LR-Capsid-Rep (R308) and CHIK-LR-E2/E1-Help (H305)) along with detailed sequence analysis of the cross-over region and resulting recombinant topology.

maximum co-infection $1x10^7$/10µg of each RNA (efficiency control: (10µg each) CHIK-LR-Cherry-Rep2, CHIK-LR-E2/E1-Help, and CHIK-LR-Cap-Help).

I. Competition of CHIKV recombinant recovery and CHIK-LR-5'-GFP

Analysis of CHIKV inter-genic recombination, reported in this dissertation, has demonstrated that co-electroporation of CHIK-LR-Hind*III*-Rep and CHIK-LR-EcoR*V*-Help RNAs (10µg per species) reproducibly resulted in >95% of BHK-21 cells becoming infected with both genomes and generating recombinant progeny. These recombinant progeny were then readily cell culture purified from contaminating replicon/defective helper genomes for subsequent analysis. However, in a natural infection circumstance we would expect recombination to occur between two full length alphavirus genomes followed by competition of these recombinant and parental genomes with the most fit genome likely succeeding. As such, I designed experiments to quantify the minimum

amount of a full length CHIKV against which a recombinant CHIKV could successfully compete.

Ten-fold serial dilutions of CHIK-LR-5'-GFP (full length GFP virus; range: 10^{-1}-10^{-6}µg) were co-electroporated with 10µg per species CHIK-LR-Hind*III*-Rep and CHIK-LR-EcoR*V*-Help (recombinant cross). Electroporation supernatant samples were then blindly passaged three times in C6/36 cells (48h incubation per pass) and then analyzed by plaque assay. CHIK-LR-5'-GFP plaques were recovered at dilutions 10^{-1}-10^{-3} (corresponding to 10^{5}-10^{3} primary infections), while Cherry positive plaques (evidence of recombinant virus) were only recovered at an initial input of $\leq 10^{-3}$ CHIK-LR-5'-GFP RNA (**Figure 2.26**) which corresponded to ~10^{3} primary full length infections.

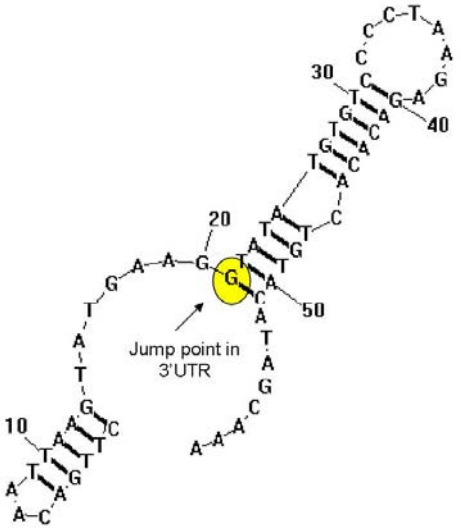


FIGURE 2.25 PREDICTED 3'UTR STEM-LOOP STRUCTURE AT IN-FRAME RECOMBINATION REPLICON RELEASE POINT. Stem-loop predictions generated using the RNAstructure 4.6 software package (Mathews *et al.*, 2004).

J. Analysis of SINV inter-genic recombination

The ability for inter-genic recombination to generate full length SINV genomes that could be cell culture purified was examined. These experiments employed a SINV replicon and defective helper with similar topologies to those used to evaluate inter-genic recombination of CHIKV. The relative efficiency of SINV inter-genic recombination was determined by co-electroporating ten-fold serial dilution of SIN-FL-Help (pToto1411) RNA against a saturated 10µg per electroporation SinRep5 RNA (pSinRep5 (SR5)). A SINV Cherry expressing replicon was also generated, pSinRep5-Cherry and RNAs derived from this replicon co-electroporated with SIN-FL-Help (10µg per species)

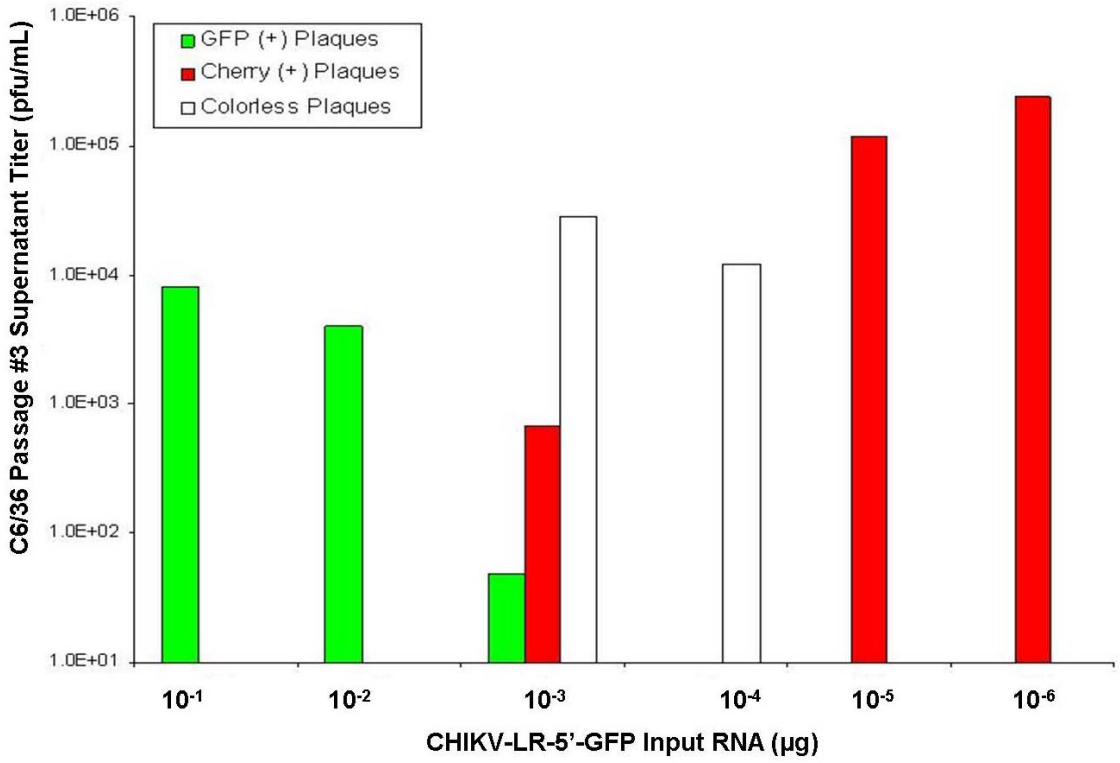


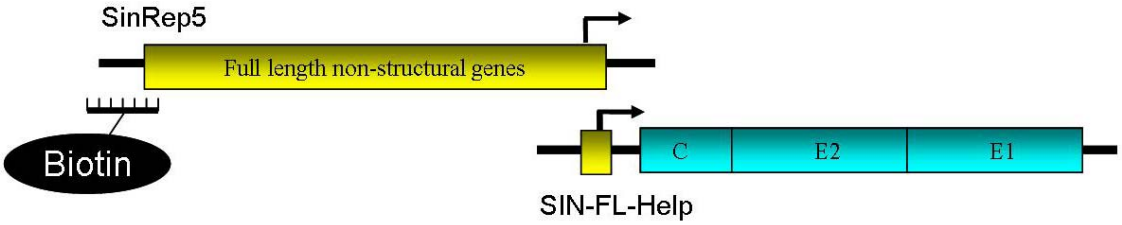
FIGURE 2.26 LIMIT OF RECOMBINANT CHIKUNGUNYA VIRUS RECOVERY WHEN COMPETING AGAINST SERIALLY DILUTED FULL LENGTH CHIKUNGUNYA GFP VIRUS. Titers are reported as plaque forming units (pfu)/mL

with infectious centers to verify co-infection efficiency within the experimental set, $5.0x10^6$/10µg each RNA.

Despite previous reports to the contrary, recombinant SINV was readily cell culture purified from these replicon/defective helper co-electroporations to a minimum co-infection efficiency of ~10^4. Clonal isolates of recombinant SINV were purified directly from electroporation supernatant samples and did not require additional amplification in C6/36 cells. This was not unexpected considering, that within these experiments; alphavirus genomes packaged in SINV protein coats were slightly more infectious for Vero cells. Recombinant SINV replicated efficiently in both vertebrate and arthropod cells with average titers of $3.47x10^7$-$8.6x10^8$pfu/mL being achieved by 48h

post-infection in C6/36 cells (**Figure 2.27**). Northern blot (SINV-5'UTR-BioProbe; **Table 2.3**) analysis of selected recombinants identified the presence of full-length-like SINV specific RNAs (**Figure 2.27, Lanes 5, 6, and 7**) indicating that the observed plaques were representative of full length recombinant virus and not packaged helpers as had been previously observed (Weiss & Schlesinger 1991).

Sequence analysis of two isolates from the lowest level of co-infection in which recombination was observed (~10^4) identified a mixed population of recombinants represented by at least two distinct genome topologies. SINV recombinant genome organization #1 resulted from a near-homologous RdRp template switch occurring within the inter-genic sequences of the replicon and defective helper genomes which was coincident with a duplication of only 26nt of inter-genic sequence (**Figure 2.28**). The replicative complex appears to have released the donor template within the inter-genic sequence immediately 5' to a potential stem-loop structure (33nt) followed by re-initiation on the acceptor template which was likely driven by sequence homologies with the nascent RNA. Whilst SINV recombinant genome organization #2 exhibited a more classic aberrant homologous template switch that resulted in the generation of a double subgenomic organization (**Figure 2.28**). This particular template switch appeared to have resulted from a release of the replicative complex from the donor template in the 3'UTR followed by re-initiation on the acceptor template ~400nt 5' of the defective helper SGP in the truncated nonstructural gene sequence without regard for RNA structure or homology. Interestingly, these two recombinant genomes displayed distinctly different plaque morphologies with the mono-subgenomic organization have a significantly ($P<.0001$) larger average Vero plaque size (4.85$\pm$7mm) than the dual-subgenomic organization (2.35$\pm$4mm) at 72h post infection. This observation suggested



H1411 : RsinRep5 RNA input ratio	Primary "co-infections"*	Isolates Analyzed (#/total)	Average Titer C6/36 P1 (pfu/mL)
H1411 10µg neg	--	n.d	--
RSR5 10µg neg	--	n.d	--
10µg : 10µg	~$1.0x10^6$	6/9	$8.6x10^8$
1µg : 10µg	~$1.0x10^5$	6/8	$4.13x10^8$
0.1µg : 10µg	~$1.0x10^4$	3/3	$3.47x10^7$
0.01µg : 10µg	~$1.0x10^3$	n.d.	--

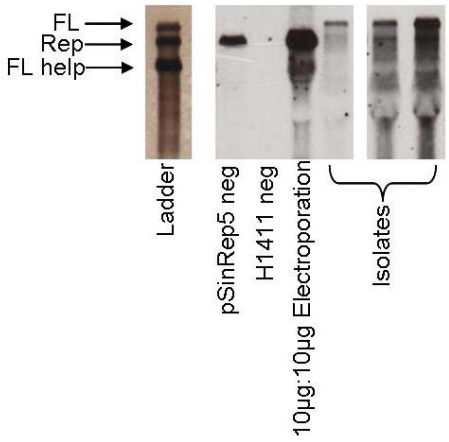


FIGURE 2.27 ANALYSIS OF SINDBIS VIRUS INTER-GENIC RECOMBINATION.
Average plaque assay titers for all dilutions in which putative recombinant CHIKV was detected following co-electroporation of SinRep5 (RSR5) and SIN-FL-Help (H1411) RNAs, including replicon and defective helper only negative controls, reported as plaque forming units (pfu)/mL. Minimum level of recombinant recovery highlighted in yellow. Northern blot analysis, hybridized using SINV-5'-UTR BioProbe, of selected SINV inter-genic recombinants. Lane 1: SINV RNA ladder, Lane 2: replicon only electroporation, Lane 3: defective helper only electroporation, Lane 4: H1411-RSR5 co-electroporation, and Lanes 5, 6, and 7: clonal recombinants.

that the recombinant genome containing the subgenomic promoter duplication was significantly attenuated for infectious spread at least in Vero cell culture.

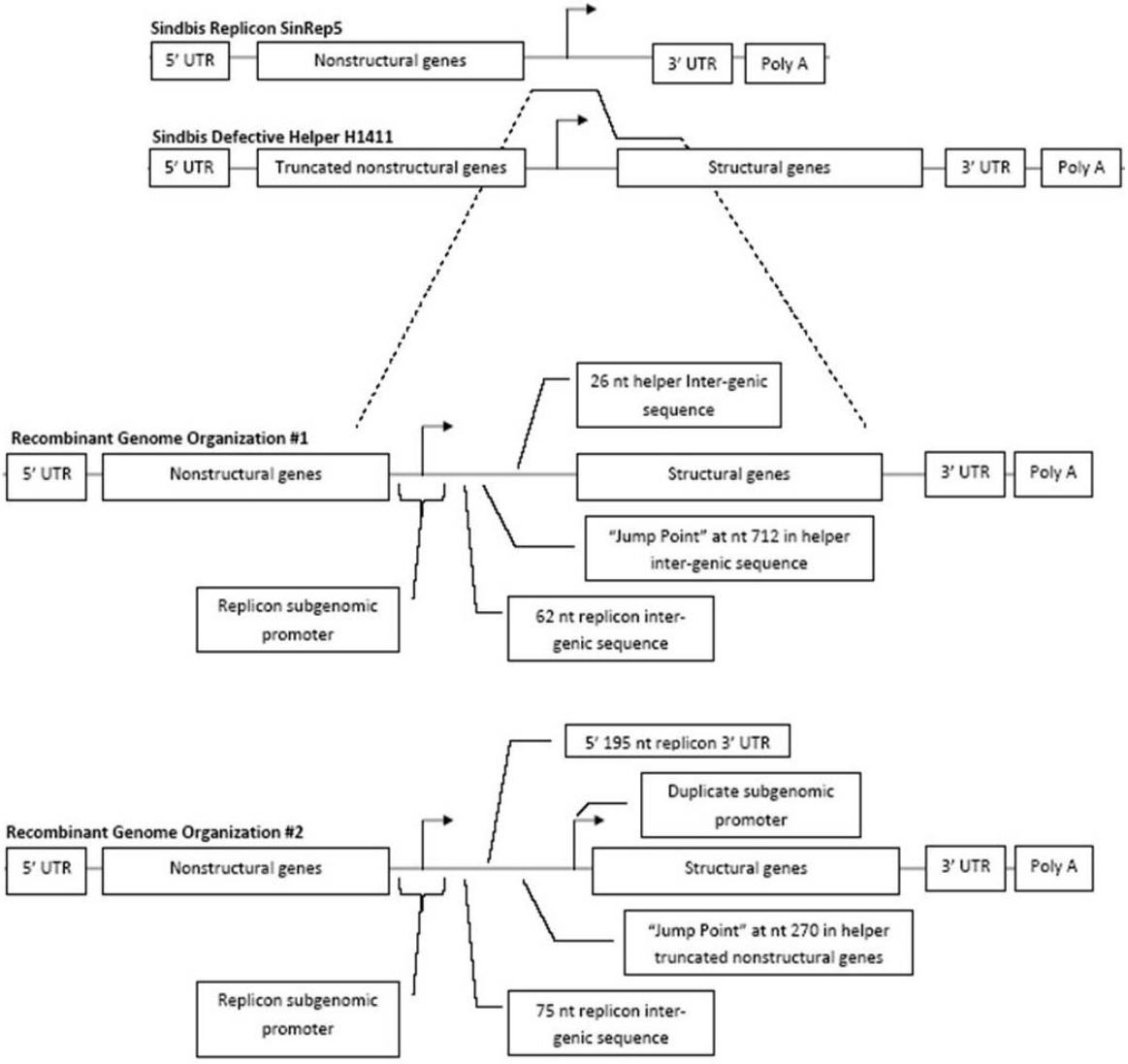


FIGURE 2.28 SINDBIS VIRUS INTER-GENIC RECOMBINANT GENOME ORGANIZATIONS. Schematic representation of parental replicon/defective helper recombinant cross (SinRep5 (RSR5) and SIN-FL-Help (H1411)) along with detailed sequence analysis of the cross-over region and resulting recombinant topologies.

K. Heterotypic recombination of CHIKV and SINV replicon/defective helper systems

Heterotypic recombination between distinct species of alphaviruses, as was observed for WEEV, has the potential to generate new and diverse viral species capable of adapting to previously unexploited biological niches. If such a CHIKV/SINV recombinant genome were to be generated from a single cross-over event the most likely topology would be a double-subgenomic organization, flanked by 5' and 3' UTRs derived from disparate parental viruses. As such expression of the structural genes would be driven by their native promoter. All defective helper genomes used in this dissertation are deficient for the expression of the viral RdRp and associated nonstructural proteins and are therefore only replicated and structural proteins expressed when these factors are supplied in *trans* by a complementing replicon genome. It was unknown if the CHIKV replicative machinery would replicate and/or express subgenomic RNAs from a SINV template and vice versa. Therefore I designed experiments to determine if CHIKV and SINV were capable of using each other's regulatory sequences toward designing recombinant crosses to investigate heterotypic recombination of CHIKV and SINV.

Primary analysis of genome compatibility involved the generation of CHIKV-GFP and SINV-Cherry replicons in which the 3'UTR had been replaced by the corresponding sequence from the heterologous virus; pCHIK-LR-GFP-SIN-3'UTR and pSinRep5-Cherry-CHIK-3'UTR. Both of these chimeric 3'UTR replicons were replication competent, expressed fluorescent proteins, and efficiently spread when co-electroporated with an appropriate defective helper genome. Based on these analyses it was determined that a CHIKV/SINV recombinants containing heterologous UTRs would be replication competent.

Next CHIKV/SINV mixed infection replicon/defective helper electroporations were performed to determine if these viruses were capable of heterotypic *trans* replication and protein expression. BHK-21 cells co-infected with RNAs from CHIK-LR-Cherry-Rep2 and SIN-FL-Help were fully competent for the generation of infectious foci, indicating that the CHIKV nonstructural proteins are capable of replicating and expressing subgenomic SINV RNAs and that the SINV structural proteins are in turn capable of packaging CHIKV genomes. However, co-electroporation of SinRep5-Cherry and CHIK-LR-FL-ST-Help RNAs resulted in a suicide infection without spread to neighboring Vero cells suggesting that these genomes are incompatible in either *trans* replication, subgenomic RNA expression, and/or packaging. Chimeric replication deficient reporter genomes were then constructed to evaluate the nature of this SINV replicon/CHIKV defective helper incompatibility. SinRep5 RNA was electroporated with the RNA generated from the following constructs; pCHIK-5'UTR-CHIK-SGP-GFP-SIN-SGP-Cherry-CHIK-3'UTR and pSIN-5'UTR-CHIK-SGP-GFP-SIN-SGP-Cherry-CHIK-3'UTR. Analyses of these reporters whilst co-infected with SINV and CHIKV replicons confirmed that while CHIKV is competent for the use of SINV regulatory sequences; SINV is completely deficient for use of both the CHIKV 5'UTR and SGP sequences (**Figure 2.29**). Based on the analysis it was determined that no additional molecular manipulation was required to investigate heterotypic recombination between a CHIKV replicon and a SINV defective helper. However, the development of a functional recombinant cross to investigate recombination between a SINV replicon and a CHIKV defective helper required the replacement of the CHIKV 5'UTR and SGP with the corresponding SINV sequences.

The potential for generation of a chimeric recombinant with CHIKV nonstructural and SINV structural genes was investigated by co-electroporating RNAs (10μg per

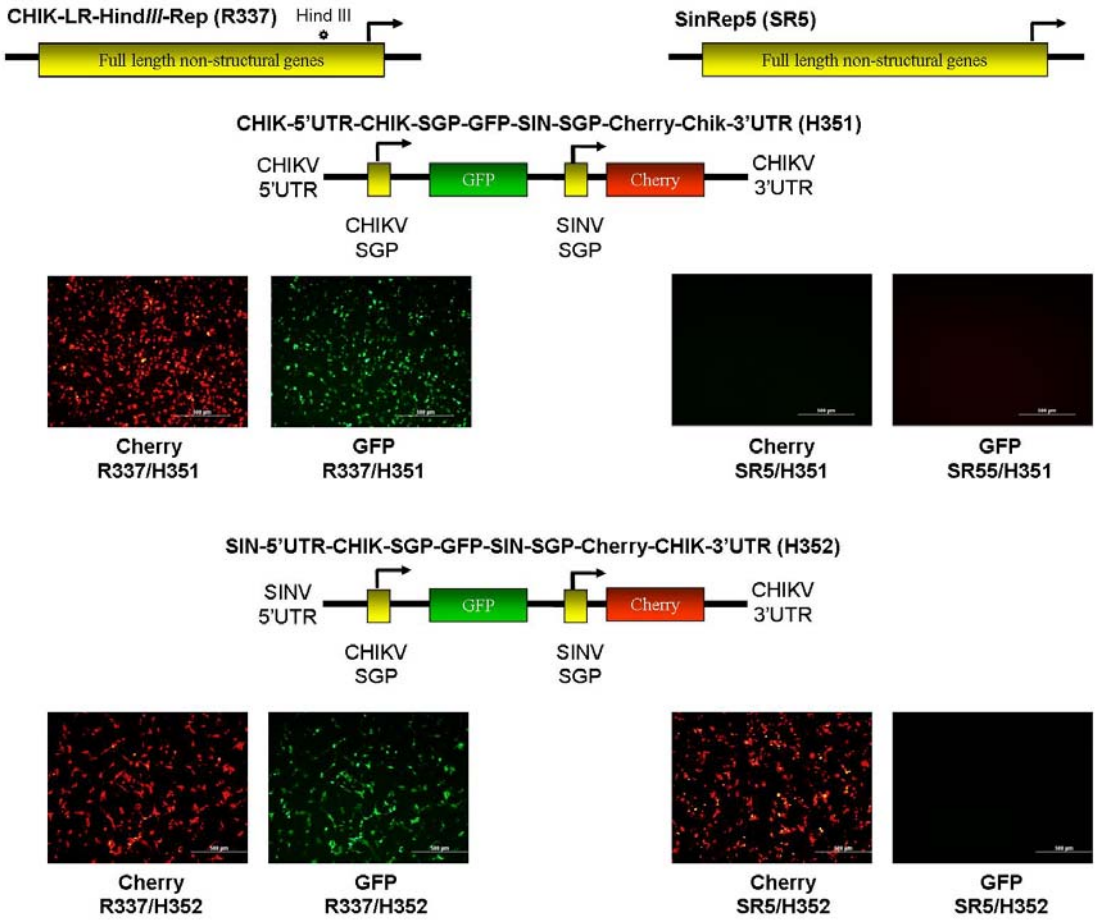
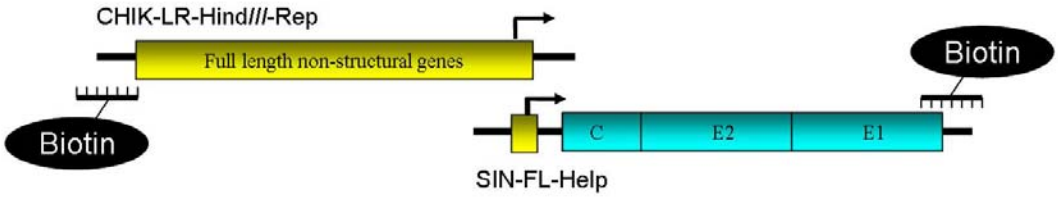


FIGURE 2.29 ANALYSIS OF THE ABIILTY OF SINDBIS VIRUS REPLICONS TO RECOGNIZE CHIKUNGUNYA VIRUS REGULATORY SEQUENCES
GFP-green fluorescent protein, CHIKV-chikungunya virus, SINV-Sindbis virus, UTR-untranslated region, SGP-subgenomic promoter.

species) CHIK-LR-Hind*III*-Rep and SIN-FL-Help in triplicate. The control cross CHIK-LR-Cherry-Rep2 and SIN-FL-Help was also included to allow for estimation of co-infection efficiency within the experimental set, $1.95x10^7$/10µg each RNA. Recombinant virus was efficiently purified from this co-electroporation supernatant without additional amplification in C6/36 cells, in fact passage of these samples in C6/36 cells before clonal

isolation in Vero cells resulted in decreased recombinant detection sensitivity. Putative heterotypic recombinants replicated in both Vero and C6/36 cells reaching average titers of $4.4x10^6$-$3.9x10^8$pfu/mL (**Figure 2.30**). Northern blot analysis was used to both confirm the presence of full length size viral genomes and identify the generation of chimeric genomes containing a CHIKV 5'UTR and a SINV 3'UTR. Briefly, duplicate gels/blots were performed and hybridized individually with either CHIKV-5'UTR BioProbe or SINV-3'UTR-BioProbe (**Table 2.3**). Additionally, cellular RNA extracted from BHK-21 cells electroporated with either CHIKV or SINV replicon RNA were included and confirmed that these oligonucleotide probes do not cross-hybridize at physiological concentrations (**Figure 2.30, Lanes 2 and 3**). Indeed 4/4 isolates examined in this way were found to be of full length size and were detected using both probes (**Figure 2.30, Lanes 6-9**). These data strongly suggested the generation of viable CHIKV/SINV heterotypic recombinant virus.

Sequence analysis of two isolates from replicate #3 identified two distinct but similar genome organizations. Both isolates were characterized by a template switch occurring between the donor 3'UTR (isolate #1 at nt72, isolate #2 at nt191) and acceptor inter-genic sequence (isolate #1 at 4nt 5' of capsid ATG start codon, isolate #2 at 25nt 5' of capsid ATG start codon) resulting in the generation of functional mono-subgenomic genomes (**Figure 2.31**). These template switches appear to have occurred without the assistance of any significant RNA secondary structures or sequence homologies, and interestingly resulted in the placement of the SINV ORF under control of the CHIKV SGP despite the fact that CHIKV appeared to be fully competent for efficient gene expression from a SINV SGP.



H1411 : R337 RNA input ratio	Primary "co-infections"*	Isolates Analyzed (#/total)	Average Titer C6/36 P1 (pfu/mL)
H1411 10µg neg	--	n.d	--
R337 10µg neg	--	n.d	--
10µg : 10µg Rep#1	~≥5.0x10^5	4/4	5.0x10^7
10µg : 10µg Rep#2	~≥5.0x10^5	2/2	4.4x10^6
10µg : 10µg Rep#3	~≥5.0x10^5	5/5	3.9x10^8

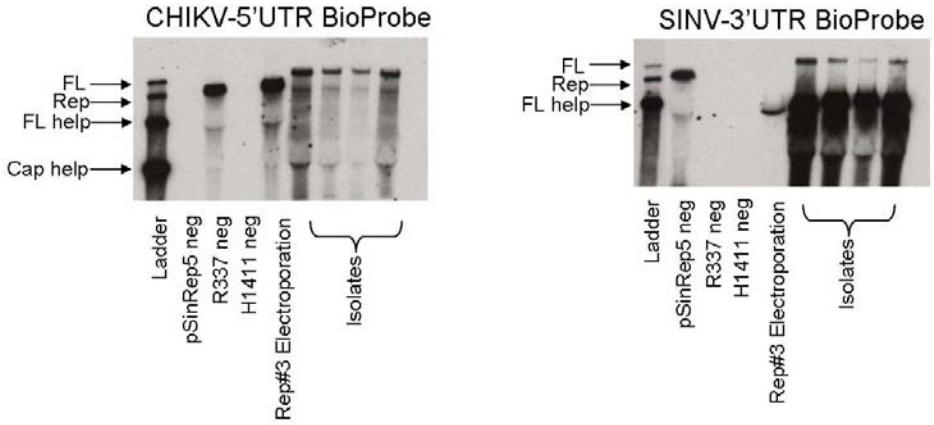


FIGURE 2.30 ANALYSIS OF HETEROTYPIC RECOMBINATION BETWEEN A CHIKUNGUNYA REPLICON AND SINDBIS DEFECTIVE HELPER.
Average plaque assay titers for all dilutions in which putative recombinant CHIKV was detected following co-electroporation of CHIK-LR-Hind*III*-Rep (R337) and SIN-FL-Help (H1411) RNAs, including replicon and defective helper only negative controls, reported as plaque forming units (pfu)/mL. Northern blot analysis, hybridized using CHIKV-5'-UTR and SINV-3'-UTR BioProbes, of selected CHIKV/SINV recombinants. Lane 1: CHIKV or SINV RNA ladder, Lane 2: SinRep5 replicon only electroporation, Lane 3: R337 replicon only electroporation, Lane 4: defective helper (H1411) only electroporation, Lane 5: R337-H1411 co-electroporation, and Lanes 6, 7, 8, and 9: CHIKV/SINV clonal recombinants.

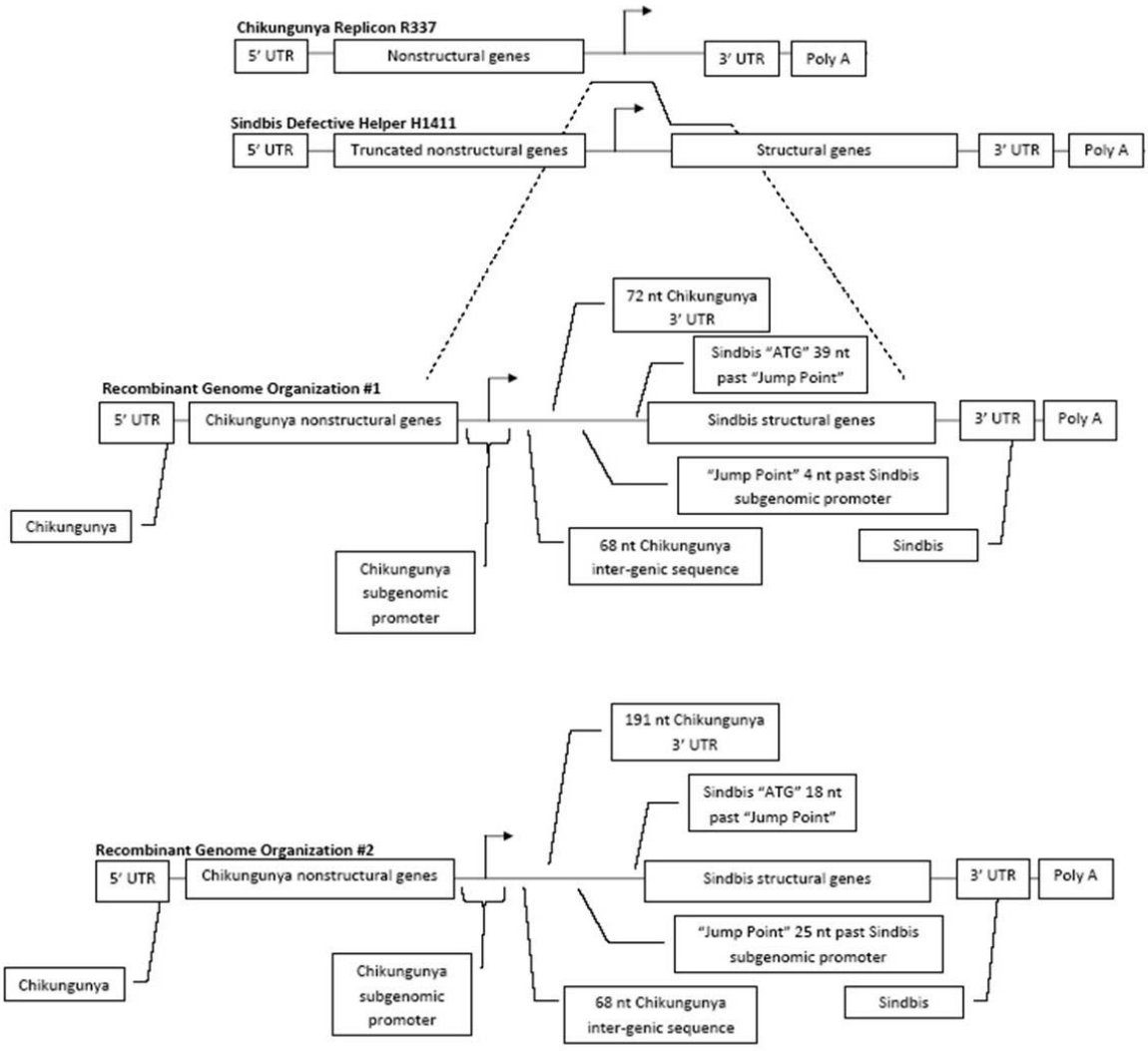


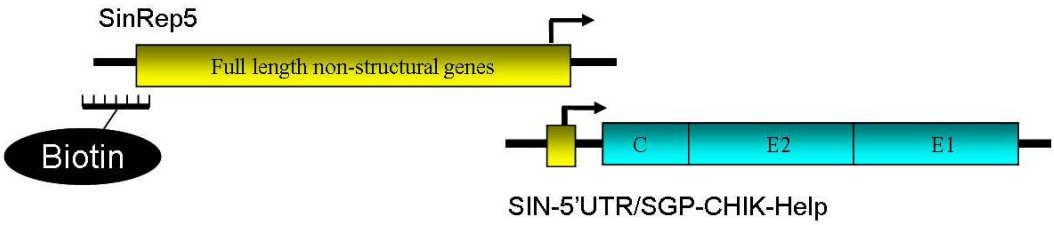
FIGURE 2.31 HETEROTYPIC CHIKUNGUNYA-SINDBIS VIRUS INTER-GENIC RECOMBINANT GENOME ORGANIZATIONS.
Schematic representation of parental replicon/defective helper recombinant cross (CHIK-LR-Hind*III*-Rep (R337) and SIN-FL-Help (H1411)) along with detailed sequence analysis of the cross-over region and resulting recombinant topologies.

Since the SINV replicative machinery was incompatible with CHIKV regulatory sequences (5'UTR and SGP) it was necessary to construct a hybrid defective helper genome comprised of the 5'UTR and SGP of SINV followed by the structural ORF and 3'UTR of CHIKV to generate a functional replicon/defective helper recombinant cross.

Insertion of these SINV sequences into the CHIKV backbone was necessary and sufficient to restore replication and subgenomic RNA expression. Analysis of the generation of a chimeric recombinant with SINV nonstructural and CHIKV structural genes was investigated by co-electroporating RNAs (10μg per species) SinRep5 and SIN-5'UTR/SGP-CHIK-Help in triplicate. Additionally, SinRep5-Cherry was electroporated with SIN-5'UTR/SGP-CHIK-Help to confirm co-infection within the experimental set, $1.0x10^7$/10μg each species. SINV/CHIKV heterotypic recombinants, competent for replication in both Vero and C6/36 cells were reproducibly generated and directly purified from electroporation supernatant samples without additional amplification. SINV/CHIKV recombinants replicated to high titers (average: $1.15\text{-}2.6x10^8$pfu/mL; **Figure 2.32**) by 48h post-infection in C6/36 cells and were confirmed to have full length size genomes by Northern blot analysis (SINV-5'UTR BioProbe (**Table 2.3**); **Figure 2.32, Lanes 6-8**). Sequence analysis of two SINV/CHIKV recombinants identified a single organization characterized by classic double-subgenomic topology coincident with a polymerase template switch releasing the donor replicon 8nt into the SINV inter-genic sequence followed by re-initiation on the acceptor defective helper in the SINV truncated nonstructural gene sequence 476nt 5' of the SGP (**Figure 2.33**). Furthermore, this topology was stable over five passages in Veros with no evidence of sequence evolution.

L. Mosquito infectivity of CHIKV/SINV and SINV/CHIKV heterotypic recombinants

CHIKV/SINV and SINV/CHIKV heterotypic recombinants were assayed individually for their relative abilities to orally infect *Ae. albopictus* mosquitoes. Mosquitoes were sampled (n=6) at day 0, and (n=8) at days 1, 3, 5, 7, post-infection with a bloodmeal comprised of either recombinant CHIKV/SINV (R337/H1411; bloodmeal titer 8.52 $\log_{10}TCID_{50}$/mL) or SINV/CHIKV (SR5/H355; bloodmeal titer 8.95



H355 : pSinRep5 RNA input ratio	Primary "co-infections"*	Isolates Analyzed (#/total)	Average Titer C6/36 P1 (pfu/mL)
H355 10µg neg	--	n.d	--
RSR5 10µg neg	--	n.d	--
10µg : 10µg Rep#1	~≥1.0x10^6	4/4	1.15x10^8
10µg : 10µg Rep#2	~≥1.0x10^6	6/6	2.6x10^8
10µg : 10µg Rep#3	~≥1.0x10^6	4/4	1.58x10^8

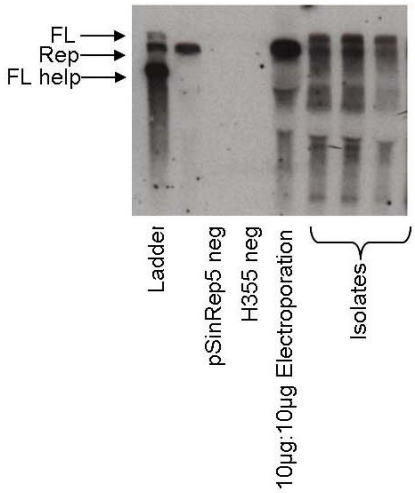


FIGURE 2.32 ANALYSIS OF HETEROTYPIC RECOMBINATION BETWEEN A SINDBIS REPLICON AND SINDBIS/CHIKUNGUNYA DEFECTIVE HELPER.
Average plaque assay titers for all dilutions in which putative recombinant CHIKV was detected following co-electroporation of SinRep5 (SR5) and CHIK-LR-EcoR*V*-Help (H338) RNAs, including replicon and defective helper only negative controls, reported as plaque forming units (pfu)/mL. Northern blot analysis, hybridized using SINV-5'-UTR BioProbes, of selected CHIKV/SINV recombinants. Lane 1: SINV RNA ladder, Lane 2: SinRep5 replicon only electroporation, Lane 3: R355 helper only electroporation, Lane 4: HSR5-R355 co-electroporation, and Lanes 6, 7, and 8: SINV/CHIKV clonal recombinants.

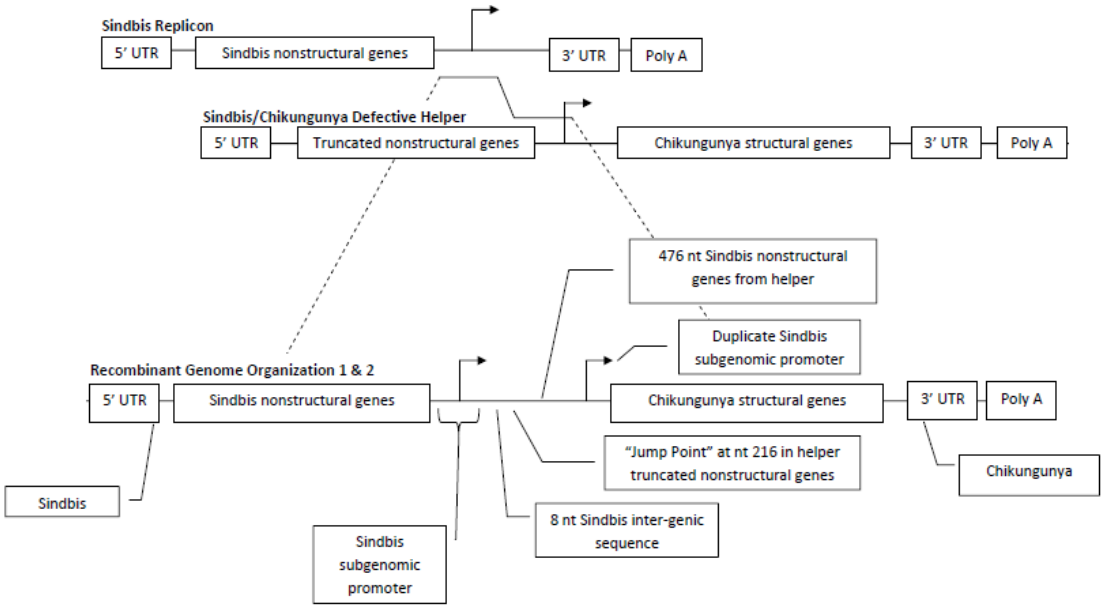


FIGURE 2.33 HETEROTYPIC SINDBIS-CHIKUNGUNYA VIRUS INTER-GENIC RECOMBINANT GENOME ORGANIZATIONS.
Schematic representation of parental replicon/defective helper recombinant cross (SinRep5 (SR5) and CHIK-LR-EcoR*V*-Help (H338)) along with detailed sequence analysis of the cross-over region and resulting recombinant topologies.

$\log_{10}$TCID$_{50}$/mL) and whole body titers assessed. Additionally, mosquito bodies and corresponding heads (n=20 per feed) harvested at fourteen dpi were analyzed individually to assess infection and dissemination rates respectively. Heterotypic recombinants were observed to be infectious for and replicate in *Ae. albopictus* mosquitoes (**Figure 2.34 A and B**) following oral exposure with comparable day fourteen infection rates observed for both CHIKV/SINV and SINV/CHIKV (**Figure 2.34 C**). However, while the heterotypic recombinant comprised of the nonstructural genes of SINV and structural genes of CHIKV was observed to disseminate to mosquito heads in 35% of mosquitoes assayed (**Figure 2.34 C**), the reciprocal heterotypic recombinant was not observed to disseminate.

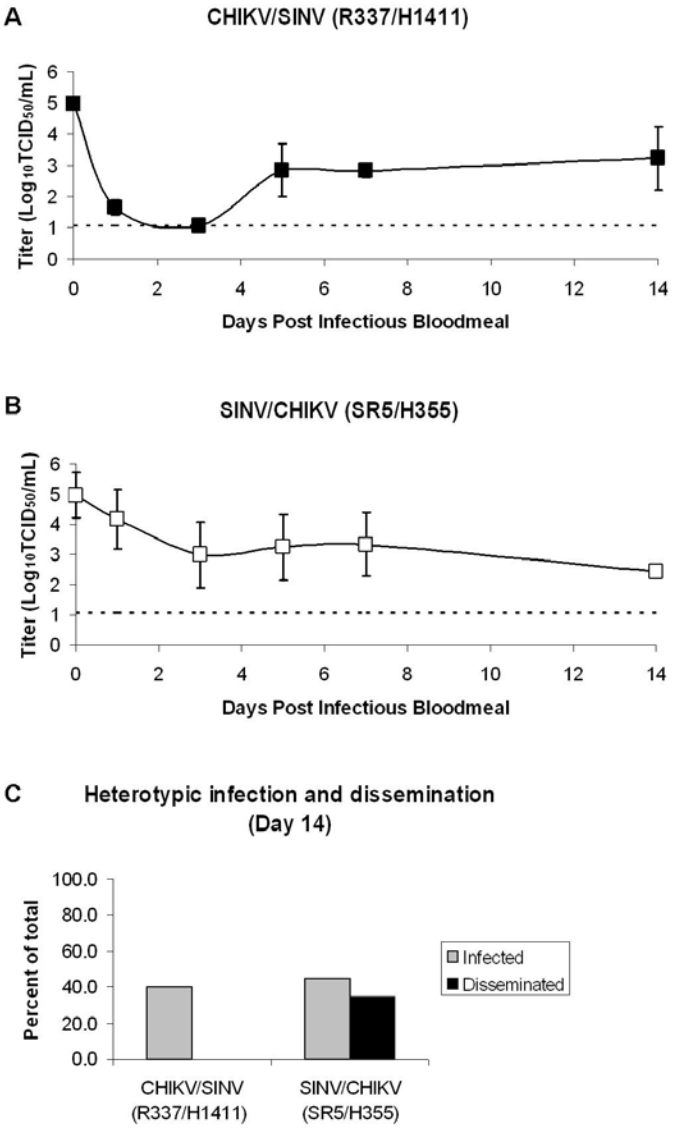


FIGURE 2.34 CHIKV/SINV AND SINV/CHIKV HETEROTYPIC MOSQUITO FEED DATA

A, Kinetic analysis of whole body titers of *Ae. albopictus* mosquitoes following oral ingestion of heterotypic recombinant CHIKV/SINV, average titers calculated from all positive individuals ± standard deviation; solid black squares (derived from CHIK-LR-Hind*III*-Rep (R337) and SIN-FL-Help (H1411). B, Kinetic analysis of whole body titers of *Ae. albopictus* mosquitoes following oral ingestion of heterotypic recombinant SINV/CHIKV, average titers calculated from all positive individuals ± standard deviation; empty black squares (derived from SinRep5 (SR5) and CHIK-LR-EcoR*V*-Help (H338)). C, Day fourteen infection and dissemination rates of CHIKV/SINV and SINV/CHIKV heterotypic recombinants. Limit of detection indicated where appropriate as a dotted line.

2.5 DISCUSSION

Seminal studies on RNA virus recombination (picornaviruses) employed variants with distinct and selectable growth characteristics followed by recovery of hybrid progeny after a high level co-infection recombinant cross (Hirst 1962, Ledinko 1963, Ledinko & Hirst 1961). This type of experimental system has several desirable characteristics for the study of recombination including; 1) the high plasticity of the viral genomes to allow for generation of phenotypic mutants, 2) distinct phenotypes to allow for ease of distinction of parental and recombinant genomes, 3) the ability to impart a selectable growth advantage to the recombinant progeny, and 4) suppression of the parental viruses growth kinetics to allow for detection of the recombinant population. Similar analyses of ts alphavirus (Atkins *et al.,* 1974, Burge & Pfefferkorn 1966, Pfefferkorn 1971) were unable to detect recombinant progeny likely due to lower efficiency as compared with picornaviruses. Furthermore, analyses of SINV replicon/defective helper systems (Schlesinger & Weiss 1994, Weiss & Schlesinger 1991) were unable to cell culture purify recombinant virus. In these studies the resulting recombinant genomes contained topologies that resulted in attenuation and decreased fitness compared to parental genomes. Therefore in the absence of recombination imparting some selective advantage, purification of putative SINV recombinant genomes was only achieved by biochemical purification using non-denaturing agarose gel electrophoresis.

First generation ΔFS replicons (described in this dissertation) were designed to use molecular strategies to impart some of the desirable characteristics of early picornavirus recombinant crosses. I hypothesized that truncation of the structural ORF would result in a complete loss of replicon packaging and, that genomes would only be

packaged in those cells in which recombination had occurred thereby significantly limiting the competition between recombinant and parental genomes. Insertion of GFP and Cherry coding cassettes into the CHIKV 5'/3' ΔFS confirmed these replicon topologies indeed resulted in a complete loss of infectious spread, which could be restored by supplementing the structural genes in *trans*. Unfortunately, co-electroporation ($\geq 10^7$) of these constructs did not result in the generation of detectable recombination progeny, likely due to the combined forces of low intra-genic recombination efficiency, reporter cassette instability, and the lack of an optimized cell culture purification protocol. Interestingly, co-electroporation of CHIKV ΔFS replicons encoding GFP or Cherry reporters and in-frame defective helper genomes was observed to result in the generation of detectable infectious foci that were initially thought to represent recombination. These foci were phenotypically distinct from full length virus in that they formed abortive infections which were characterized by infectious spread for three to five days followed by quiescence. This observation suggested that either these putative recombinants were highly unstable and were simply being degraded or that defective helper were being packaged either individually or as a bi-parte segmented genome as was previously observed for SINV (Frolov *et al.,* 1997, Frolova *et al.,* 1997, Weiss & Schlesinger 1991).

Analysis of CHIKV packaging was achieved by insertion of GFP reporter cassettes into defective helper genomes. I hypothesized that the use of a recombinant cross consisting of a CHIKV Cherry replicon and a CHIKV GFP defective helper would allow for rapid phenotypic distinction between packaged helper genomes and putative recombinant genomes. Using this system it would be expected that a foci of infection resulting from co-packaging would have a single "seed-cell" at its center which would be positive for both Cherry and GFP surround by a foci of Cherry only positive cells.

Conversely, a recombinant genome would contain the coding sequence for both reporters within a single covalently linked molecule. All cells in a foci generated by such a genome would be Cherry and GFP positive. The efficient packaging of alphavirus genomes has been hypothesized to result from the presence of both packaging signal sequences and the presence of *cis* acting elements that enhance replication and packaging (Frolova *et al.,* 1997, Volkova *et al.,* 2006). The observation of packaged CHIKV defective helper genomes was surprising considering that the ~7Kb deletion of nonstructural gene coding sequence engineered into these CHIKV genomes was rationally designed to eliminate any packaging signals or potential *cis* acting elements using previous analyses of SINV (nsP1 gene nt 945-1076), SFV (nsP2 gene nt 2767-2990), RRV (nsP2 gene three signal sequences nt 2902-3062, 2761-2905, and 1635-1929), and VEEV (sequences between nt 520-7290 and cis acting nsP1,2,3 precursor) (Frolova *et al.,* 1997, Volkova *et al.,* 2006). Nevertheless, CHIKV defective helper genomes containing a deletion of nt 373-7270 were packaged, as evidenced by GFP expression in cells infected with Cherry replicon/GFP helper co-electroporation supernatant. These data suggested that for CHIKV some, as of yet, unidentified packaging signal may exist in the remaining nonstructural/structural gene sequences. Therefore the observation of self-limited foci formation following first generation CHIKV replicon/defective helper recombinant crosses was likely explained as co-packaging of segmented genomes. As such these *trans*-complementing segmented genomes were capable of generating a foci of replicon genome expansion whose growth will cease when the "seed-cell" becomes exhausted or dies.

Co-electroporation of CHIK-LR-Cherry-Rep2 and CHIK-LR-5'-GFP-2A-Help reproducibly resulted in the generation of bi-colored recombinants presumably containing a dual subgenomic organization to a minimum co-infection of ~10^3. These bi-colored

recombinants were highly unstable and rapidly degraded into a mixed phenotypic population, following clonal isolation, comprised of variants capable of expressing both fluorescent proteins, only Cherry or GFP, and not competent for reporter expression. Unfortunately, sequence analysis of early bi-colored variants was not possible likely due to the high template length variability resulting from this rapid degradation. This post-recombination event evolution was expected given that previous analyses of SINV and VEEV recombinants have identified rapid recombinant sequence evolution toward recovery of the wt viral sequence (Pushko *et al.,* 1997, Weiss & Schlesinger 1991). Additionally, it was likely that the observed recombinant instability was exacerbated by the previously observed tendency of the parental constructs, used to generate my CHIKV replicons and defective helpers, to loose reporter cassette expression following serial passage in cell culture (Tsetsarkin *et al.,* 2006).

Interestingly, although these bi-colored recombinants appeared to be unstable, they were competent for the infection of *Ae. albopictus* mosquitoes following oral exposure as evidenced by the observation of Cherry and GFP positive midguts at seven dpi. However, neither bi- nor individually colored variants were detected by plaque assay from infected mosquitoes beyond three dpi indicating that when competed as a mixed population the virus most like the wt virus (the colorless variant) was the most fit *in vivo*. Since it was not possible to generate a pure population of bi-colored recombinants, it remains unknown if this virus would have been capable of midgut escape, salivary gland infection, or transmission. Perhaps the most interesting finding with regard to this mosquito experiment was that in a natural host the virus most like the wt virus was the most fit for vector infection and subsequent transmission. The replicative disadvantage of the bi-colored variant arises from the need to replicate and encapsidate ~2Kb extra coding sequence as compared to the colorless variant. This

increase in genome size (>15%) was likely to result in decreased replication kinetics as well as the need to express two subgenomic or 26S-like RNAs which should significantly attenuate this virus as compared to wt. All observations of alphavirus recombinant topologies both in previous studies and reported in this dissertation were characterized by aberrant events coincident with sequence duplications likely resulting in attenuation. For these recombinants to be successful they would have to out compete the abundant parental viruses population. Given the low abundance of recombinants and their attenuated phenotype, this seems highly unlikely and may be largely responsible for the rarity of recombinant detection in nature.

Due to the unstable nature of reporter gene expression cassettes, detailed analysis of the sequences and conditions which favor CHIKV and SINV recombination were conducted using replicon/defective helper systems without fluorescent protein cassettes with systems coding reporters included as efficiency controls only. Co-electroporation of replicon/defective helper sets was reproducibly observed to result in co-infection levels of $>10^6$ (one fifth of electroporation mixture). Additionally, co-electroporation of serial ten-fold dilutions of *in vitro* transcribed RNAs allowed for evaluation of the minimum co-infection required to detect viable potential recombinant genomes. CHIKV recombinant genomes were readily and reproducibly purified from all recombinant crosses with the exception of the dual (bi-parte) helper cross. The efficiency of CHIKV recombination appeared to be related to the functional constraints on the sequence participating in the event with inter-genic recombination being ~100 fold more efficient (minimum co-infection: $\sim10^3$) than intra-genic (minimum co-infection: $\sim10^5$). This was not surprising considering that any recombination event occurring within an ORF must be completely homologous or comprise a functional in-frame deletion, duplication, or insertion.

Although both inter- and intra-genic recombination events resulted in sequence duplications these topologies were stable during serial passage in cell culture. Most interesting was the serial passage stability of the intra-genic recombinant organization, comprised of duplication the 5' 34aa of E3 and 3' 20aa of capsid flanked by full length capsid and E3 coding sequences. This stable insertion suggested that it may be possible to insert exogenous genes of interest into this position (between capsid and E3) provided the appropriate cleavage sequences are maintained. This organization warrants further investigation as it may provide increased stability of alphavirus reporter constructs while maintaining the important viral regulatory and enhancer sequences in their native orientation.

Analysis of recombination using a CHIKV system containing in-frame structural deletions revealed intermediate characteristics. The efficiency of recombination using this cross was comparable to as observed using the ΔFS system (~10^5 co-infections required to generate full length recombinants) however, the recombinant topology was a double subgenomic organization similar to intra-genic crosses. This was the only topology that was unstable and was observed to completely revert to the CHIKV wt sequence after five serial passages in Vero cells. This instability was similar to that which was observed for bi-colored recombinants, reported here, and for previous analyses of SINV and VEEV recombinants (Pushko *et al.,* 1997, Weiss & Schlesinger 1991).

Contrary to previous reports full length SINV recombinants were readily and reproducibly purified in cell culture to a minimum of ~10^4 co-infections. It was possible my increased detection sensitivity may have resulted from differences in RNA transfection efficiencies and/or viral infectivity for the cell types used during purification. My experiments employed electroporation in BHK-21 cells and purification in Vero cells

while previous analyses used cationic lipid transfection and purification in chicken embryonic fibroblast cells (Weiss *et al.,* 1989). Indeed within my experimental set it was determined that, in general, detection of CHIKV recombinants required a single amplification in C6/36 cells while similar passage of SINV resulted in a loss of sensitivity. Sequence analysis of SINV recombinants identified two distinct genome organizations characterized by a near homologous event occurring within the inter-genic sequence and a more classic double subgenomic organization. It should be noted that since all recombinant genome detection required some level of cell culture passage it is possible that the near homologous event could represent post-recombination sequence evolution as previously observed (Weiss & Schlesinger 1991).

In the above studies I have shown that homotypic recombination of both CHIKV and SINV replicon/defective helper systems reproducibly result in the generation of viable full length alphavirus genomes. Although this type of recombination was highly favored because of the high levels of sequence homology between the parental genomes homotypic recombinants in nature would largely go undetected and would only generate minimal diversity. The ability for recombination to generate a viable chimeric genome may be governed by sequence compatibilities that are not yet fully understood. A number of chimeric alphavirus genomes have been synthetically generated including CHIKV/O'nyong nyong, SINV/RRV, and SINV/VEEV and the relative viability of these constructs evaluated (Kuhn *et al.,* 1996, Paessler *et al.,* 2003, Schoepp *et al.,* 2002, Strauss & Strauss 1997, Vanlandingham *et al.,* 2006).

In general, substitution of the alphavirus structural cassette has resulted in the generation of a viable chimera provided the native regulatory sequences (UTRs, SGP, and any enhancer sequences) remain intact. Though this chimerization was usually coincident with varying degrees of attenuation and as such has been employed as a

vaccine strategy (Paessler *et al.,* 2003, Schoepp *et al.,* 2002). Reciprocal chimeras were not always equivalent, for example a SINV/RRV capsid chimera was observed to have significantly higher growth kinetics as compared to the reverse RRV/SINV capsid chimera (Strauss & Strauss 1997). Similar non-reciprocal compatibility was observed for SINV/SFV glycoprotein structural chimeras (Smyth *et al.,* 1997). Nevertheless, insertion of an entire heterotypic structural ORF generally results in a viable replication competent chimera capable of packaging and infectious spread.

Within the experiments I report here similar compatibilities and non-reciprocity was observed. The CHIKV and SINV structural proteins appear to be fully competent for packaging of each others genomes to titers $>10^6$ virus like particles/mL suggesting that successful genome packaging does not solely rely on the presence of primary signal sequences. Additionally, CHIKV and SINV replicon genomes containing reciprocal 3'UTR swaps were fully replication competent. However, although CHIKV was fully competent for the use of the SINV 5'UTR and SGP in *trans* replication and subgenomic RNA expression the converse did not hold. Therefore, evaluation of heterotypic recombination between a SINV replicon and a CHIKV defective helper required additional molecular manipulation (see Results: Heterotypic recombination of CHIKV and SINV replicon defective helper systems).

I observed heterotypic recombination to reproducibly result in the generation of viable chimeric CHIKV/SINV and SINV/CHIKV genomes capable of replication, transcription, translation, assembly, and release. Interestingly, although the CHIKV replicative complex was fully competent for expression of subgenomic RNAs from a SINV SGP, CHIKV/SINV recombinants were characterized by a mono-subgenomic organization in which the SINV structural ORF was placed under the control of the native CHIKV SGP. SINV/CHIKV recombinants on the other hand exhibited a more classic

double subgenomic organization. Recombinants from both crosses were observed to replicate efficiently in both Vero and C6/36 cells with titers of $\geq 10^6$ pfu/mL observed by 48h post C6/36 infection. It was difficult to say if these CHIKV/SINV and SINV/CHIKV would be selected for in nature should they occur. Although these heterotypic recombinants were viable they do not appear to have any identifiable selective advantage that would allow them to over compete their respective wt full length parental viruses in a natural transmission cycle.

Since analysis of ts full length alphavirus mutants has been unable to generate detectable recombinants all successful alphavirus recombination experiments, described in this dissertation and elsewhere, utilize replicon/defective helper systems to facilitate both generation and detection. However, these genomes are highly manipulated and although they provide information about the possibility for such events to occur they provide little information about the relative success of such a recombinant. In a natural co-infection circumstance we would expect two full length alphaviruses to be simultaneously replicating and should a recombination event occur, that recombinant progeny would have to compete against both parental genomes. Therefore I sought to determine the relative ability of a recombinant CHIKV-Cherry to be purified when titrated against a full length CHIKV-GFP. Interestingly recombinant virus was first detected at a initial input of $\leq 10^3$ primary full length CHIKV-GFP infections which correlates exactly with the lower limit of inter-genic recombination efficiency. Taken together these data suggest that the low recovery of naturally occurring recombinants does not result from molecular impediments to template switching events. It is far more likely that recombination occurs very frequently, at least for alphaviruses, but our ability to detect these events is governed by the very complex interplay occurring within a co-infected organism where super-infection resistance, number of co-infected cells,

recombination efficiency, immune responses, and relative titers of parental and recombinant genomes released all contribute to the probability of recombinant recovery. In other words, fitness rather than mechanics of recombination (or mutation) drives evolution.

2.6 KEY OBSERVATIONS

- Mutations engineered into replicons and defective helpers (ie. unique restrictions sites, expression cassettes ect.) did not appear to significantly impair CHIKV replication.

- "Tagging" defective helper genomes with fluorescent proteins allowed for convenient identification of "co-infected" cells following electroporation and of helper packaging and passage carry over, but confounded analysis of putative recombinant genomes.

- Co-electroporation efficiently and reproducibly resulted in co-infection rates $>10^6$.

- Co-electroporation of serial ten-fold dilutions of *in vitro* transcribed RNAs allowed for evaluation of the minimum co-infection required to detect viable putative recombinant genomes.

- Co-electroporation of replicon and helper genomes efficiently generated full-length genomes of variable phenotype that could be purified in cell culture.

- Although a mixed population of CHIKV plaque phenotypes was initially detected following co-electroporation of Cherry-replicons and GFP-2A-defective helpers:
  - This likely resulted from sequence evolution during cell culture passage.
  - Bi-colored recombinants were to be capable of mosquito infection but are likely out-competed by non-colored derivative genomes.

- CHIKV recombinant progeny were readily purified from all recombinant crosses.

- As previously observed the use of a bipartite or "dual" helper system abrogated detectable recombinant events that generate full-length virus.

- The efficiency of CHIKV recombination was related to specific sequence constraints.
  - Inter-genic sequence: minimum co-infection: ~10^3.
  - Intra-genic sequence: minimum co-infection: ~10^5.

- Contrary to previous reports recombinant full length SINV could be purified in cell culture following co-transfection of SINV replicon and full length defective helper genomes.

- CHIKV replicons was fully capable of utilizing SINV regulatory sequences for replication and protein expression while the converse was not true.

- Heterotypic recombination between CHIKV and SINV was observed to result in generation of viable chimeric full-length alphavirus genomes.

- All crossover events were aberrantly homologous but functional and stable over five passages in Vero cells with the exception of the CHIKV in-frame deletion cross which reverted to the CHIKV wt sequence.

CHAPTER 3: ANALYSIS OF YELLOW FEVER VIRUS 17D HOMOTYPIC RECOMBINATION

3.1 INTRODUCTION

Viruses belonging to the family Flaviviridae have long been known to be competent to participate in recombination. The seminal observation of flavivirus recombination involved detection, by Northern blot analysis, of cellular ubiquitin sequences which had been incorporated into the bovine viral diarrhea virus (BVDV; genus *Pestivirus*) genome (Meyers *et al.,* 1989). At this time it was known that the development of pathogenic mucosal disease was always characterized by the simultaneous presence of two distinct BVDV biotypes classified as cytopathic (cp) and non-cytopathic (ncp) based on cell culture phenotype (Baker 1987). Fetal bovine infection *in utero* with ncp-BVDV can result in the establishment of immunotolerance resulting in a persistent life-long infection coincident with continuous virus replication (Baker 1987). Initial experimental evidence suggested that mucosal disease etiology was due to superinfection of immunotolerant carriers of ncp-BVDV with cp-BVDV strains (Bolin *et al.,* 1985, Brownlie *et al.,* 1984). Serologic analysis of naturally occurring cp/ncp-BVDV pairs, isolated from fatal bovine infections, indicated that within an individual animal, isolates representing both biotypes were highly antigenically related (Corapi *et al.,* 1988). The only detectable difference between BVDV pairs was the expression of the 80KDa nonstructural protein 3 cleavage product (NS3; p80) (Meyers *et al.,* 1992).

Sequence analysis of virus pairs, isolated from fatal bovine infections, identified recombination-driven sequence duplications, deletions, insertions and rearrangements

within ncp-variants. However, >99% sequence identity was detected between matched cp and ncp variants when the non-recombinant regions were compared (Kummerer *et al.,* 2000, Meyers *et al.,* 1991, Meyers *et al.,* 1991, Meyers *et al.,* 1992). These recombinant sequences facilitate expression of NS3 which is cleaved from precursor NS2-3 (remains uncleaved in ncp-variants). This cleavage imparts an apoptosis inducing phenotype (via an unknown mechanism) to cp-BVDV variants resulting in mucosal disease (Gamlen *et al.,* 2010). Furthermore, molecular characterizations of isolates derived from animals exhibiting post-vaccination mucosal disease suggest that recombination can occur between ncp-BVDV wt and cp-BVDV vaccines in persistently infected animals (Becher *et al.,* 2001, Ridpath & Bolin 1995). However, cell culture passage of cp-BVDV suggests that these recombinants may be unstable and can revert to ncp-BVDV variants via homologous and non-homologus recombination (Baroth *et al.,* 2000, Gallei *et al.,* 2005).

Hepatitis C virus (HCV; genus *Hepacivirus*) is capable of initiating a persistent hepatatropic infection in humans. Extreme heterogenecity has been observed with respect to HCV (many variants displaying only 68-79% identity (Blackard & Sherman 2007, Simmonds *et al.,* 1993)) leading to the classification of six distinct genotypes each comprised of multiple subtypes, based on NS5 sequence analysis (Simmonds *et al.,* 1993). This sequence heterogenecity coupled with the extensive documentation of dual infections (co-infection and superinfection; (Blackard & Sherman 2007)) greatly increases the probability of generation and detection of HCV inter and intra-typic recombinants. The seminal observation of HCV recombination involved the detection of a 2k/1b recombinant (RF1_2k/1b) in human serum samples collected in St. Petersburg, Russia (Kalinina *et al.,* 2002). Sequence analysis identified the cross-over point as having occurred within the NS2-3 sequence (nt 3175-3176) with template

disassociation/reassociation potentially being guided by stem-loop structures flanking a highly conserved sequence (Kalinina *et al.,* 2004, Kalinina *et al.,* 2002). This recombinant has been repeatedly isolated from human serum samples across a wide geographic range, including: Russia, Ireland, Estonia, and Uzbekistan (Kurbanov *et al.,* 2008, Legrand-Abravanel *et al.,* 2007, Moreau *et al.,* 2006, Viazov *et al.,* 2010). Sequence analysis of these related isolates suggests they are all derived from a common ancestor which likely recombined 50-80 years ago and subsequently spread over a large geographic range (Kurbanov *et al.,* 2008).

Three other distinct inter-typic recombinant viruses have been identified: a 2i/6p Vietnamese isolate (Noppornpanth et al., 2006), a 2b/1b Philippine isolate (Kageyama et al., 2006) and a 2(unknown subtype)/5a French isolate (Legrand-Abravanel et al., 2007). Interestingly all three of these inter-typic recombinants exhibited evidence of template switching in the NS2-3 junction, suggesting this may be a critical sequence for chimeric recombinant viability or that this is the only region that will tolerate a cross-over on a biological level. With regard to intra-typic recombination, there appears to be more flexibility in cross-over selection, as template switches have been identified to occur in the envelope (E1/E2), NS5A (protein kinase R binding domain), and NS5B sequences, likely resulting from greater compatibilities between more closely related viruses (Colina *et al.,* 2004, Cristina & Colina 2006, Moreno *et al.,* 2006). Since interferon (INF) resistance is mediated by sequences in the E2 and NS5A protein, recombination has serious implications with respect to anti-HCV therapeutic strategies. Studies of the effects of recombination on INF resistance are lacking, although *in vivo* analysis indicates that RF1_2k/1b remains sensitive to INF therapy, however this will have to be evaluated on a case by case basis.

Until recently, recombination in the genus *Flavivirus*, has been substantiated solely by phylogenetic sequence analyses. Studies performed in this way have suggested recombinant intra-typic dengue viruses (DENV) exist across all four serotypes (Carvalho *et al.,* 2010, Chen *et al.,* 2008, Holmes *et al.,* 1999, Perez-Ramirez *et al.,* 2009, Tolou *et al.,* 2001, Twiddy & Holmes 2003, Worobey *et al.,* 1999). Cross-over regions, in general, appear to be localized to the 5' end of the genome (premembrane (prM), envelope (E), and nonstructural gene 1 (NS1)) (Carvalho *et al.,* 2010, Holmes *et al.,* 1999, Perez-Ramirez *et al.,* 2009, Tolou *et al.,* 2001, Twiddy & Holmes 2003, Worobey *et al.,* 1999) however, one report has identified recombination in the DENV 1 NS3 gene sequence (Chen *et al.,* 2008).

Perhaps the most interesting aspect of these analyses has been the identification of DENV genomes that appear to be mosaics resulting from multiple homologous recombinant events (Perez-Ramirez *et al.,* 2009, Tolou *et al.,* 2001, Twiddy & Holmes 2003, Worobey *et al.,* 1999). In fact one report suggested that a strain of DENV 1 has been sequenced that contains three recombinant regions coincident with six homologous polymerase jumps (Chen *et al.,* 2008). Similarly intra-typic recombination has been reported for Japanese encephalitis virus (JEV) and St. Louis encephalitis virus (SLEV) (Twiddy & Holmes 2003) with cross-over sequences localized to the E gene coding sequence. Additionally, a recent report by Pickett and Lefkowitz (2009) has also identified a recombinant West Nile virus (WNV) sequence with the cross-over occurring in the NS5 polymerase gene (Pickett & Lefkowitz 2009).

Initial reports of flavivirus recombination met with some skepticism, due to a lack of empirical data to substantiate recombinant events inferred from bioinformatic sequence analysis (de Silva & Messer 2004, Hombach *et al.,* 2004, Murphy *et al.,* 2004). Because the potential for flavivirus-flavivirus co-infection interactions continues to rise

(See: Introduction Section 1.4), investigations into the potential for the generation of wild-type (wt)/wt and wt/vaccine recombinants is vital to our understanding of, and ability to, predict how these viruses are likely to evolve in nature. Therefore, experiments described in this chapter were designed to evaluate the potential of yellow fever virus (YFV) 17D to undergo homologous or aberrantly homologous recombination.

3.2 AIMS AND HYPOTHESES

The **aim of these studies** was to use the yellow fever virus live attenuated vaccine 17D reverse genetics and alphavirus optimized conditions to evaluate the potential for flavivirus homotypic recombination. The hypotheses of these studies were as follows:

A. *In vitro 17D recombination will be observed but will likely be inefficient as compared to previous reports of picornavirus and alphavirus recombination.*
B. *Rationally engineered complementary in-fame deletions of the envelope gene coding sequence will generate 17D replicon genomes incapable of infectious spread following electroporation.*
C. *YFV 17D recombinant virus will be recovered following co-infection of complementary 5' and 3' envelope gene deletion mutant replicons.*
D. *Aberrantly homologous recombination will occur within the complementary sequence located between the two in-frame envelope deletions and will be characterized by in-frame insertions, duplications, or deletions.*
E. *The generation of 17D envelope deletion mutants containing insertions of Cherry or GFP – FMDV2A reporter cassettes will allow for estimation of co-infection efficiency.*
F. *Supplementation of the YFV 17D structural genes in trans under the control of a non cytopathic Venezuelan equine encephalitis virus (VEEV) replicon will provide efficient means of replicon genome packaging.*

G. Infection of arthropod and vertebrate cells with YFV Asibi will render them resistant to superinfection with YFV 17D.

3.3 MATERIALS AND METHODS

A. Cells

Baby hamster kidney (BHK-21) cells, African green monkey kidney (Vero) cells, and *Aedes albopictus* (C6/36) cells were maintained as previously described (Chapter 2: 2.3 Materials and Methods: Section A) *Aedes albopictus* ($C_7$10) cells (generously provided by Dr. Ilya Frolov) were grown in Dulbecco's minimum essential medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1% penicillin streptomycin (pen-strep), 1% L-glutamine (L-glu; Cellgro® Mediatech, Inc.) and maintained at 30ºC in the presence of 5% CO_2. Vero cells used for electroporation (generously provided by Dr. Scott Weaver) were grown in minimum essential medium (MEM; Gibco) supplemented with 5% FBS and 1% pen-strep and maintained at 37ºC

B. Plasmid Constructs

All molecular cloning methodologies involved in the generation and evaluation of all full length, replicon, and helper reverse genetics systems or infectious clones (i.c.s) were as described (Chapter 2: Materials and Methods: Sections B-D). Oligonucleotide primers used in the PCR amplification and construction of flavivirus i.c.s are listed in **Table 3.1** and **Table 3.2**.

All plasmid constructs used or generated for the experiments contained in this chapter are listed in **Table 3.3**. All YFV 17D plasmid constructs were generated or derived in some way from the parental YFV 17D i.c. (pACNR/FLYF-17D; (Bredenbeek *et al.,* 2003)) which is comprised of the YFV 17D-204 full length viral sequence cloned

TABLE 3.1 SPECIFIC PRIMERS INVOLVED IN THE AMPLIFICATION, CLONING, AND SEQUENCING OF YELLOW FEVER VIRUS CONSTRUCTS.

Primer	Sequence	Position
17D-F1	5'-AAGTGCCACCTGACGTGTCG	N/A
17D-Begin-R	5`-CTATAGTGTCACCTAAATCGT	N/A
17D-Begin-F	5`-AGTAAATCCTGTGTGCTAA	1-19
17D-F8	5`-ACGCCGTTCCCATGATGTTC	415-34
17D-R8	5`-ATTAGGAATTGCACAGTCA	435-453
17D-BspE-R	5`-TGAGATTGGGACAGTTGTA	620-638
p169 R	5`-TAGTGAGATGTCCAATGAAGG	1088-1108
p169 F	5`-ATGAGTTTGTTTGAGGTTG	1343-1364
17D-F4	5`-AGGTGCAAACTGCGGTGGAC	1521-1540
17D-R2	5`-GCCATGGCAGGGTCAAGTC	1608-1626
YF-E3-R	5`-ATTGAGCTTCCCTCTTTGTG	2142-2161
YF-E3-F	5`-AGGAAAGTTGTTCACTCAG	2162-2180
YF E 22aa F	5`-ACAATGTCCATGAGCATGAT	2387-2406
17D-Mlu F	5`-GAAAGTCCAGGAAAGAATGCC	2862-2882
17D-R3	5`-AAGACTGCGTCCATGTACAC	2951-2970
17D-R10	5`-AGGGATATGATTGTGAGAGC	3207-3226
17D-F9	5`-CGCATGAAAGCCATCTGGTG	3471-3490
17F-F10	5`-GAGTATCCCAGTGAATGAG	4180-4198
17D-R9	5`-GCACTCCCACTAGACCAGCT	4210-4229
17D-F3	5`-CCTCGCTGGCCTTGGTTG	4480-4497
17D-Sap R	5`-ACATCCCCACTTCTCCTAGC	4562-4581
17D-F7	5`-GCTTGCGCACTCTTGTGTTG	5229-5248
17D-NgoM F	5`-TGTCTCCCAAAGGCATCAGT	6654-6673
17D-R4	5`-GAGATGTGAGTGGGTTTGAC	6734-6753
17D-F5	5`-TGATGAGGAGAATGAGGCGT	8355-8374
17D-R5	5`-CTTCTATGGCCTCTTTGTCC	8455-8474
17D-Cla-F	5`-GGCTCACTGAGCACGGATGT	9585-9604
As-Cla-F	5`-CTCACTGAGCACGGATGTA	N/A
17D-ClaI R	5`-CATGTTGTGCGTCCTTGTG	10014-10032
17D-3`UTR-F	5`-TCTGAAACACCATCTAACAGG	10342-10371
As-3`UTR -R	5`-GTTTGTATCCCGGTTATTCC	10370-10389
17D-F6	5`-GCCTCCGCTACCACCCTC	10666-10683
17D-R6	5`-TCCCTGGCGTCAATATGGTC	10748-10767
YF-Qi	5`-CGGGAAACGTCTTGCTCGA	N/A
17D-Vec-R	5`-GCCACGTTGTGTCTCAAAATC	N/A
17D-RT	5`-CGAGTGGTTTTGTGTTTGT	N/A

Positions are relative to the YFV 17D sequence. N/A-not applicable.

into the low-copy-number plasmid pACNR1181. This construct was further modified in a two step cloning process. p17D-494C was generated via two fragment cloning (vector (V) pACNR/FLYF-17D digested with Not*I*, Apa*I*, and calf intestinal phosphatase (CIP)

TABLE 3.2 SPECIFIC PRIMERS INVOLVED IN AMPLIFICATION OF NON-VIRAL SEQUENCES, INSERTION OF RESTRICTION SITES, FUSION PCR, SITE DIRECTED MUTAGENESIS, AND PACKAGING CONSTRUCT GENERATION.

Name	Sequence
Asibi-RBZ-R	5`-GAGATGCCATGCCGACCCAGTGGTTTTGTGTTTTTCA
Asibi-RBZ-F	5`-TGAAAAACACAAAACCACTGGGTCGGCATGGCATCTC
Asibi-RBZ-Nru-R	5`-CCATCTCGCGAGATCTTGATGAGTTTG
17D-F2	5'-ACGGGTGGAGTGACCTTGGTCCGGAAAAACAGATGGTTGC
17D-R1	5'-GCAACCATCTGTTTTTCCGGA CAAGGTCACTCCACCCGT
YF-St-Cla*I* F	5'-CACTCATCGATGTCTGGTCGTAAAGCTC
YF-St-BsrG*I* R	5'-GTGAGTGTACACTACGCCCCAACTCCTAGAGAC
p707 Apa*I* F	5'-AGCAGTGTTAAATCATTCAG
p707 Cla*I* R	5'-GAGTGATCGATTGGCGGCAAGCTCTAGAC
YF-NruI R	5'-TGTCGCGAGTGGTTTTGTGTTTTTCATCCAAGGTCTG
YF-Nar R	5'-TTGATCGGCGCCAACTCCTAGAGACAAAAACA
p173-R1	5'-ACAAAATAAACCCTCCACAG
p173-F1	5'-TTATTCCAGAGTACGATGCC
GFP-5end-R	5'-TCCTCGCCCTTGCTCACCAT
GFP-3end-F	5`-GCATGGACGAGCTGTACAAG
17D-GFP-F	5'-CGCTCCTTGTCAAACACCATGGTGAGCAAGGGCGAGGA
17D-GFP-R	5'-TCAGCAGGTCGAAGTTAAGCTTGTACAGCTCGTCCATGC
Cherry-Pst-F	5'-GTGACCGTGACCCAGGACTC
Cherry-Pst-R	5'-GCGCAGCTTCACCTTGTAG
155 (YF-GFP)-R	5'-TTGTTTGACAAGGAGCGAACGCCGCGCCTCACCATG
155 (YF-GFP)-F	5'- TGGTGAGGCGCGGCGTTCGCTCCTTGTCAAACAA
p170 R	5'- CAACCGAAGTGAAGAACCCTACTATCACTGGAATCCT
p170 F	5'- AGGATTCCAGTGATAGTAGGGTTCTTCACTTCGGTTG

and insert (I_1) a pACNR/FLYF-17D derived fusion PCR amplicon (primers (pr) 17DF1/17DR1 and 17DF2/17DR2) digested with Not*I* and Apa*I*) to achieve site directed mutagenesis at nt 495 to allow for incorporation of a unique BspE*I* restriction site to facilitate ease of cloning into the structural genes. Incorporation of this silent mutation did not result in any change in viral replication kinetics (K. Tsetsarkin unpublished data). A Hepatitis Δ rybozyme (RBZ) sequence was then sub-cloned into p17D-494C via two fragment ligation (V p17D C494 digested with Xba*I*, Xho*I*, and CIP and I_1 p17D-GFP-

RBZ i.c. (p1612; generously provided by Dr. Ilya Frolov) digested with Xba*I* and Xho*I*) directly 3' to the YFV sequence to generate p17D-RBZ i.c. (p172).

Incorporation of this RBZ facilitates precise cleavage of the viral 3' untranslated region (UTR) resulting in a >100 fold increase in electroporation efficiency of these YFV i.c.s (Frolov and McGee unpublished observation). Additionally, the parental YFV Asibi i.c. (pYFV-As i.c. (p12.7)) as described by (McElroy *et al.,* 2005) was also modified by incorporation of this RBZ sequence. pYFV-As-RBZ i.c. (p178) was constructed by two fragment ligation of V p12.7 digested with Xba*I*, Not*I*, and CIP and I_1 pYFV-As-GFP-RBZ (p159; see below) digested with Xba*I* and Not*I*.

Two distinct 17D deletion mutant virus variants designated 17D 5' ΔE and 17D 3' ΔE respectively, were constructed to permit evaluation of YFV heterotypic recombination. Each of these mutants contained an in-frame deletion in the E protein coding sequence with 636 nt of homologous coding sequence between the two respective deletions (**Figure 3.1**). A 5' deletion of nt 1109-1324 was engineered into p172 to generate p17D-5'-ΔE-Rep (p169) via three fragment ligation of V p172 digested with BspE*I*, Mlu*I* and CIP, I_1 PCR amplicon of p172 using pr 17DF8/p169R digested with BspE*I* and phosphorylated using T4 polynucleotide kinase (PNK), and I_2 PCR amplicon of p172 using pr P169F/17DR3 digested with Mlu*I* and phosphorylated using T4 PNK. A complementary 3' deletion of nt 1984-2236 was also engineered into p172 to generate p17D-3'-ΔE-Rep (p170) via two fragment ligation of V p172 digested with Apa*I*, Mlu*I*, and CIP and I_1 fusion PCR amplicon of template p172 using pr 17DF4/p170R and p170F/17DR3 digested with Apa*I* and Mlu*I*.

p17D-GFP-RBZ i.c. contains a 5' GFP reporter cassette fused within the viral ORF comprised of 25 amino acids (aa) of the 17D capsid sequence followed by the sequences for GFP and a foot-and-mouth disease virus 2A protease immediately fused to

TABLE 3.3 SPECIFIC PLASMID CONSTRUCTS USED IN THESE STUDIES.

Plasmid Number	Plasmid Name	Description
N/A	pACNR/FLYF-17D i.c.	Parental YFV 17D-204 full length i.c. as described by Bredenbeek *et la.*, 2003.
N/A	p17D-494C i.c.	pACNR/FLYF-17D modified by site directed mutagenesis to incorporate unique BspE*I* site at nt 495 (K. Tsetsarkin unpublished).
p12.7	pYFV-As i.c.	Full length YFV Asibi i.c. as described by McElroy *et al.*, 2005.
p128	pCHIK-2-Helper	Intermediate cloning vector (K. Tsetsarkin unpublished).
p155	pYFV-As-GFP i.c.	pYFV-As-RBZ i.c. modified to include a 5' GFP-2A expression cassette (K. Tsetsarkin unpublished).
p159	pYFV-As-GFP-RBZ i.c.	pYFV-As-GFP i.c. modified to include a 3' Hepatitis Delta rybozyme sequence (C. McGee unpublished).
p169	p17D-5'-ΔE-Rep	p17D-RBZ i.c. modified to include a deletion of nt 1109-1324 (C. McGee unpublished).
p170	p17D-3'-ΔE-Rep	p17D-RBZ i.c. modified to include a deletion of nt 1984-2236 (C. McGee unpublished).
p171	N/A	Intermediate cloning construct (C. McGee unpublished).
p172	p17D-RBZ i.c.	p17D-494C modified to include a 3' Hepatitis Delta rybozyme sequence (C. McGee unpublished).
p173	pVEEV-YFV-17D-Help	pVEEV-GFP non-cytopathic replicon modified to express YFV 17D structural genes C, prM, and E (C. McGee unpublished).
p174	p17D-3'-ΔE-GFP-Rep	p17D-3'-ΔE-Rep modified to include a 5' GFP-2A expression cassette (C. McGee unpublished)
p177	p17D-5'-ΔE-Cherry-Rep	p17D-5'-ΔE-Rep modified to include a 5' Cherry-2A expression cassette (C. McGee unpublished).
p178	pYFV-As-RBZ i.c.	pYFV-As i.c. modified to include a 3' Hepatitis Delta rybozyme sequence (C. McGee unpublished).
p179	p17D-Cherry –RBZ i.c.	Full length YFV 17D-204 i.c. modified to include a 5' Cherry-2A expression cassette (C. McGee unpublished).
p707	pVEEV-GFP	VEEV non-cytopathic replicon with puromycin resistance gene as described by Petrakova *et al.*, 2005.
p1612	p17D-GFP-RBZ i.c.	Full length YFV 17D-204 i.c. modified to include a 5' GFP-2A expression cassette as described by Shustov *et al.*, 2007.

As-Asibi, C-capsid, Cherry-cherry fluorescent protein, E-Envelope, GFP-green fluorescent protein, Rep-replicon, Help-Helper, i.c.-infectious clone, nt-nucleotide, prM-premembrane, RBZ-Hepatitis Delta rybozyme, VEEV-Venezuelan equine encephalitis virus, YFV-yellow fever virus, Δ-deletion, 2A-foot-and-mouth disease protease.

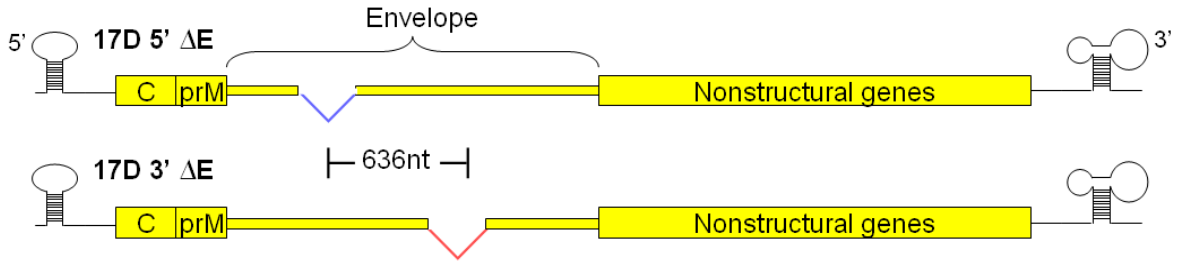


FIGURE 3.1 SCHEMATIC REPRESENTATION OF YELLOW FEVER VIRUS 17D 5' AND 3' DELETION MUTANT REPLICON GENOMES.
17D 5' ΔE contains an in-frame deletion of nt 1109-1324 of the envelope coding sequence (relative to the p17D i.c. sequence) shown in blue. 17D 3' ΔE contains an in-frame deletion of nt 1984-2236 of the envelope coding sequence shown in red. Alignment of these two replicon genomes allows for the identification of 636nt of completely homologous sequence overlap between these two staggered deletions. Only a recombination event occurring within the overlap will restore the complete envelope coding sequence resulting in the generation of a full length 17D virus.

a human codon optimized full length 17D capsid sequence as previously described (Shustov *et al.*, 2007). Insertion of reporter cassettes into flavivirus (YFV and WNV) genomes in this facilitates efficient exogenous gene expression *in vitro* and *in vivo* with increased stability as compared to classic 3' insertions (McGee *et al.*, 2009, Shustov *et al.*, 2007). Similar reporter cassettes expressing either GFP or Cherry were engineered into other YFV constructs including 17D, Asibi, and 17D 5'/3' ΔE replicons. GFP was engineered into p170 to generate p17D-3'-ΔE-GFP-Rep (p174) by two fragment ligation of V p170 digested with Not*I*, Nsi*I*, and CIP and I_1 p1612 digested with Not*I* and Nsi*I*. p17D-5'-ΔE-Cherry-Rep (p177) was constructed via three fragment ligation of V p169 digested with Not*I*, BspE*I*, and CIP, I_1 fusion PCR amplicon of p1612 and p191 (pCHIK-LR-Cherry 226A; K. Tsetsarkin) using pr 17DF1/17D-GFP 5'endR and 17D-GFP-F/CherryPstR digested with Not*I* and Pst*I*, and I_3 fusion PCR amplicon of p1612 and p191 using pr CherryPstF/17D-GFP-R and GFP3'endF/17DBspER digested with Pst*I* and BspE*I*. The Cherry fluorescent gene reporter sequence was also inserted into p172 to

generate a full length 17D Cherry virus designated p17D-Cherry-RBZ i.c. (p179) by two fragment ligation of V p172 digested with Not*I*, BspE*I*, and CIP and I_1 p177 digested with Not*I* and BspE*I*. A YFV Asibi wt i.c. containing the sequences for GFP and RBZ (pYFV-As-GFP-RBZ i.c.) was generated by sequential two step cloning. The intermediate construct pYFV-As-GFP i.c. was generated by two fragment ligation of V p12.7 digested with Not*I*, BspE*I*, and CIP and I_1 fusion PCR amplicon of templates p1612 and 12.7 using pr 17DF1/155 (YF-GFP)-R and 155 (YF-GFP)-F/17D BspE R digested with Not*I* and BspE*I*. The RBZ sequence was then inserted into p155 to generate pYFV-As-GFP-RBZ i.c. (p159) by two fragment ligation of V p155 digested with XbaI, NruI, and CIP and I_1 fusion PCR amplicon of sequences from p12.7 and p1612 using pr 17DF6/Asibi-RBZ-R and Asibi-RBZ-F/Asibi-RBZ-Nru-R digested with Xba*I* and Nru*I*.

Additionally, a YFV 17D packaging construct was constructed using the VEEV-GFP non-cytopathic replicon (p707; generously provided by Dr. Ilya Frolov (previously described by (Petrakova *et al.*, 2005)). pVEEV-YFV-17D-Help was generated by two step sequential cloning. First, sequences from p172 and p707 were sub-cloned into the intermediate cloning vector p128 (CHIK-2-Helper; K. Tsetsarkin) by three fragment ligation of V p128 digested with Cla*I*, Not*I*, and CIP, I_1 PCR amplicon of p172 using pr YF-St-Cla*I*F/YF-St-BsrG*I*R digested with Cla*I* and BsrG*I*, and I_2 p707 digested with BsrG*I* and Eag*I* to generate p171. Following sequence confirmation of PCR amplified regions, pVEEV-YFV-17D-Help (p173) was generated by three fragment ligation of V p707 digested with Apa*I*, Eag*I*, and CIP, I_1 PCR amplicon of p707 using pr p707 Apa*I*F/p707 Cla*I*R digested with Apa*I* and Cla*I*, and I_2 p171 digested with Cla*I* and Eag*I*.

C. Transcription and transfection of plasmid derived viral RNAs

In vitro transcription and purification of viral RNAs was performed using the SP6 mMESSAGE mMACHINE kit followed by LiCl precipitation as previously described

(Chapter 2: Materials and Methods: Section D). RNA transfections, via electroporation, and the quantification of efficiency by infectious centers assay (specific infectivity) were as described (Chapter 2: Materials and Methods: Section D). Electroporation of BHK-21 cells was as previously described. Preparation of $C_7$10 and Vero cells for electroporation was as described for BHK-21 cells. Following preparation, $C_7$10 cells were electroporated in a 2mm gap cuvette and pulsed twice at (1500 Volts; 25μFaradae) and Vero cells were electroporated in a 4mm gap cuvette (700μL total volume) and subjected to three 10ms pulses (250 Volts; 1s intervals).

D. Protocols involved in the analysis and conformation of alphavirus recombination

The protocol for the attempted generation and purification of YFV 17D recombinants following co-electroporation of 5'/3' deletion mutants was as previously described (Chapter 2: Materials and Methods: Section E).

E. Analysis of viral replication kinetics

Post-electroporation growth curves were performed in duplicate for all YFV full length and replicon genomes in either BHK-21 cells or in BHK-21 cells previously transfected with the VEEV helper p173 (**Figure 3.2**). Generation of VEEV-YFV-17D-Help BHK-21 packaging cells was achieved by electroporation of 10μg *in vitro* transcribed RNA into ~$1x10^7$ BHK-21 cells, followed by antibiotic selection using 5μg/mL puromycin beginning 24h post electroporation. Generally, cells underwent a growth arrest for approximately three days post-electroporation after which time normal BHK-21-like growth kinetics were observed. Following electroporation, cells were resuspended in a total volume of 5.0mL BHK-21 cell maintenance medium and plated in a T-25 tissue culture flask. Samples were then harvested (500μL) every 24h post-electroporation until 100% cytopathic effect (CPE) was observed. Growth curve samples

were titrated by plaque assay on Vero cells using a methyl-cellulose overlay (Chapter 2: Materials and Methods: Section E). Plates were either scored, at four days post-infection (dpi), by Cherry or GFP fluorescence or fixed in acetone:phosphate buffered saline (PBS; 3:1) solution and visualized by indirect immunofluorescence (IFA). Plaque assays of 17D deletion mutant viruses were performed using Vero cells that had been transfected with VEEV-YFV-17D-Help to allow for the generation of infectious foci.

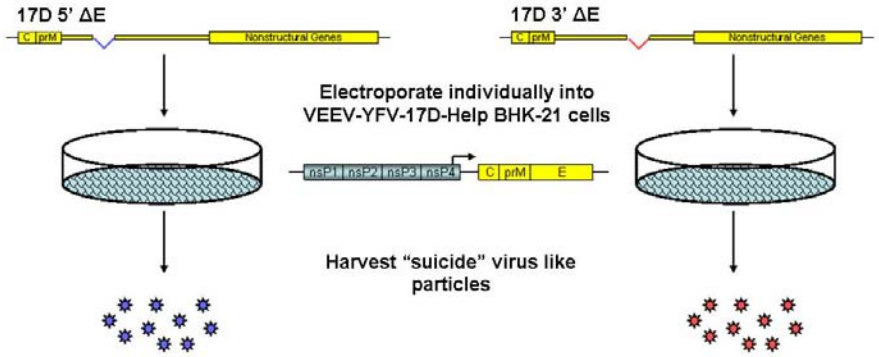


FIGURE 3.2 PROTOCOL FOR THE PACKAGING OF YELLOW FEVER VIRUS 17D DELETION MUTANT REPLICONS.

Indirect immunofluorescence assay

All YFV assay plates that required detection by immunological means were fixed by removal of the methyl-cellulose overlay, rinsed several times with 1X PBS, and fixed for at least 20m with ice cold acetone:PBS (3:1) solution. The fixative was then removed and the monolayers allowed to air dry, followed either by immediate staining or storage at -20ºC. Plates that were stored at -20ºC were allowed to come to room temperature and air dry before staining. Following a brief rehydration with 1X PBS, monolayers were incubated for 40m with agitation at 37ºC with a solution of 1X PBS containing a 1:500 concentration of a YFV NS1 specific murine monoclonal antibody designated 8b3a (S. Higgs) and a 1:200 concentration of a 0.5% Evans blue solution. This primary antibody

concentration was determined empirically and was observed to result in optimal fluorescence for all YF full length and replicon viruses (17D and Asibi). Plates were then rinsed twice with 1X PBS followed by incubation with 1:500 goat-anti mouse IgG human absorbed secondary antibody (Alexa Fluor® 488 Dye; Invitrogen, Carlsbad, CA) and 1:200 0.5% Evans blue solution in 1X PBS for 40m with agitation at 37ºC. Cellular monolayers were then washed twice with 1X PBS and overlayed with 90% glycerol + 10% PBS, pH 8.6, 2.5% w/v 1,4-diazabicyclo[2.2.2]octane mounting solution. Immuno-stained YFV foci were then visualized using a Olympus IX-70 epifluorescence microscope followed by storage at 4ºC in the dark.

F. Analysis of YFV 17D/Asibi superinfection resistance

The ability of cells (Vero and C710) to become resistant to superinfection with 17D-Cherry or CHIK-LR-5'-Cherry following primary infection with YFV-As-GFP was assessed. The basic experimental protocol for these experiments is depicted in **Figure 3.3**. Cells were electroporated with 10µg YFV-As-GFP RNA and allowed to incubate at the appropriate temperature in their corresponding maintenance medium for six days post-electroporation. At this time cells were visually examined to insure ~100% were GFP fluorescence positive. YFV-As-GFP infected cells were then seeded into T-25 cell culture flasks and incubated an additional 24h to allow for attachment. Following attachment, cells were superinfected with either 17D-Cherry-RBZ or CHIK-LR-5'-Cherry at a multiplicity of infection (moi) of ~0.1 in 1.0mL media at room temperature for 1h with agitation. Additionally, mock electroporated Vero and $C_7$10 cells were also similarly infected as non-superinfection controls. Cell monolayers were then washed three times with sterile 1X PBS, 5.5mL of the appropriate maintenance medium added, and 500µL aliquots sampled and replaced at 0, 24, 48, 72, and 96h post infection. The presence of 17D Cherry or CHIK-LR-5'-Cherry was then quantified in superinfection

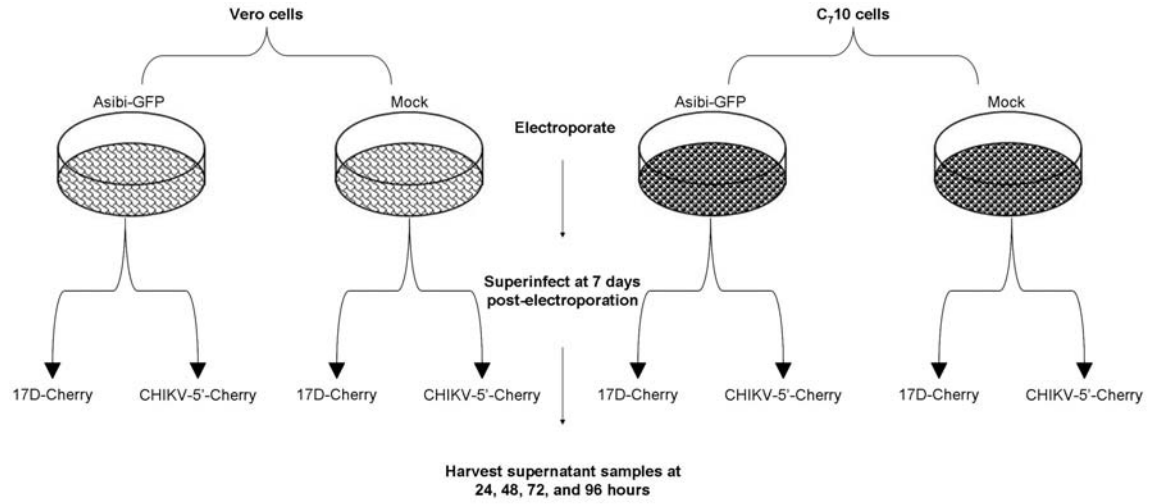


FIGURE 3.3 BASIC EXPERIMENTAL PROTOCOL FOR THE ANALYSIS OF SUPERINFECTION RESISTANCE BETWEEN YELLOW FEVER VIRUSES ASIBI AND 17D

supernatant samples by virus titration as previously described (Higgs 1997). Briefly, ten-fold serial dilutions of virus samples were performed in a 96-well plate format and seeded with Vero cells. Titration plates were then stored at 37ºC and scored for Cherry fluorescence at 48h (CHIK-LR-5'-Cherry) to 96h (17D-Cherry-RBZ) post infection. Titers were calculated as $\log_{10}$ tissue culture infectious dose 50 ($\log_{10}$ $TCID_{50}$) per mL.

3.4 RESULTS

A. *In vitro* analysis of YFV 17D and Asibi full length and replicon growth kinetics

All YFV full length and replicon viruses had specific infectivity values within a comparable range (**Table 3.4**) indicating that all molecular manipulations resulted in the generation of stable infectious YFV RNAs capable of initiating viral replication. All YFV variants were analyzed for their ability to grow and produce infectious particles following electroporation in BHK-21 or VEEV-YFV-17D-Help BHK-21 packaging cells. Samples were titered by plaque assay and scored by either fluorescent reporter protein expression or following IFA. YFVs 17D and Asibi containing a 5' intra-ORF reporter

expression cassette were replication competent following electroporation in BHK-21 cells. However, insertion of GFP or Cherry into YFV ORF in a 5' orientation resulted in

TABLE 3.4 SPECIFIC INFECTIVITY OF YFV VIRUSES AND REPLICONS

Yellow fever virus/replicon	Plasmid	Specific infectivity
17D	p172	$1.0x10^6$/µg RNA
17D GFP	p1612	$1.8 x10^6$/µg RNA
17D Cherry	p179	$1.6 x10^6$/µg RNA
Asibi	p178	$1.5 x10^6$/µg RNA
Asibi GFP	p159	$9.0 x10^5$/µg RNA
17D 5' ΔE	p169	$9.0 x10^5$/µg RNA
17D 5' ΔE Cherry	p177	$1.2 x10^6$/µg RNA
17D 3' ΔE	p170	$9.5 x10^6$/µg RNA
17D 3' ΔE GFP	p174	$1.2 x10^6$/µg RNA

a ~24h lag in replicative kinetics and an approximately ten fold decrease in peak titers (**Figure 3.4 A and B**). Furthermore, RT-PCR analysis of Asibi GFP, using pr 17D Begin F/17DR8 which flank the inserted sequence, indicated that at least in YFV Asibi, this reporter cassette is relatively unstable resulting in a near complete loss of exogenous sequence after a single passage in C6/36 cells (**Figure 3.5**). Interestingly this trend did not hold for the YFV 17D 5' and 3' envelope deletion replicons. For these replicons, similar growth kinetics and peak titers were observed for 17D 5' ΔE regardless of the presence or absence of the Cherry coding cassette, while 17D 3' ΔE displayed slightly lower peak titers than its respective GFP containing replicon. It should be noted however, that 17D 5' ΔE and 17D 3' ΔE growth curve samples were subjected to an extra freeze thaw cycle before titration which may have resulted in a small loss of titer. Nevertheless, 17D deletion mutant replicons were observed to have similar growth kinetic profiles as compared to full length constructs, reaching peak titers by two to three days post-electroporation albeit $\geq$100-fold lower.

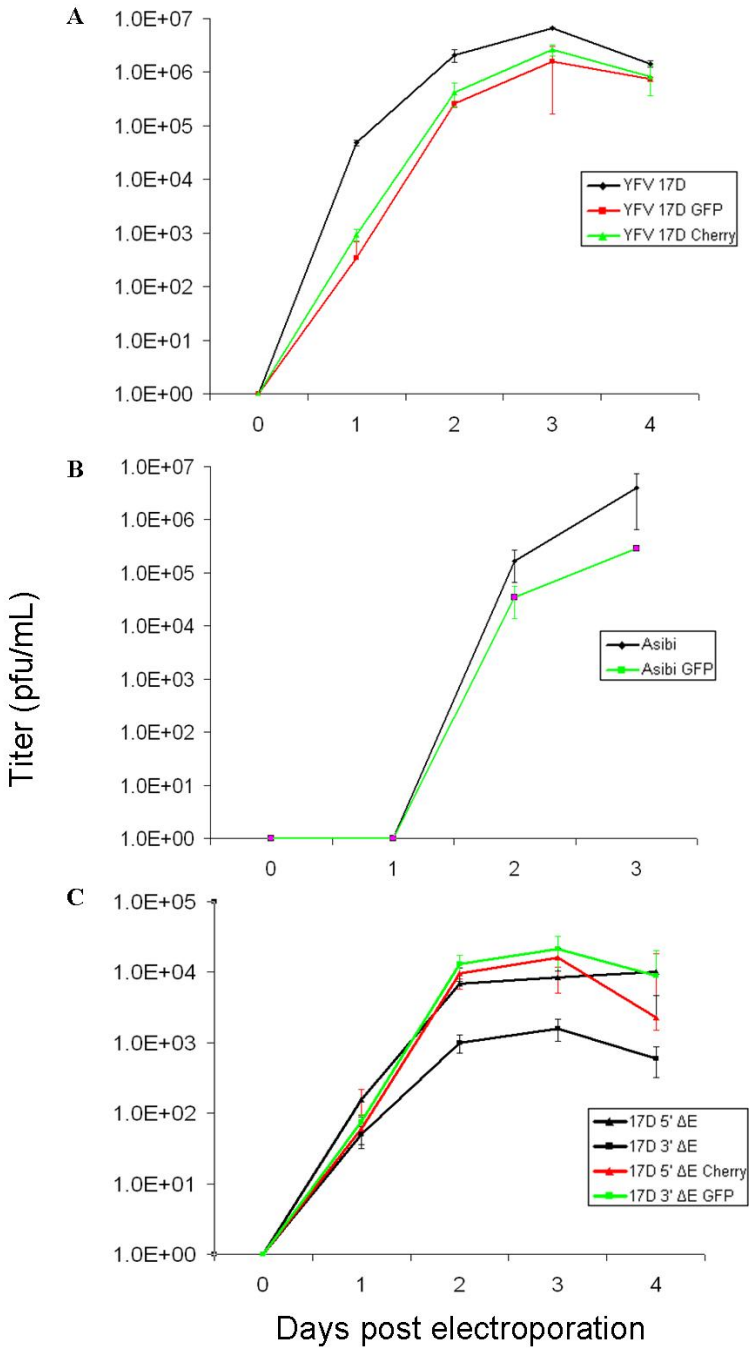


FIGURE 3.4 POST ELECTROPORATION IN VITRO ANALYSIS OF YFV 17D AND ASIBI FULL LENGTH AND REPLICON GROWTH KINETICS.
Comparison of BHK-21 growth kinetics of YFVs A) 17D, 17D GFP, and 17D Cherry; B) Asibi and Asibi-GFP; C) 17D 5' ΔE, 17D 3' ΔE, 17D 5'ΔE GFP, and 17D 3' ΔE Cherry. pfu-plaque forming units.

B. Optimization for detection and analysis of YFV 17D deletion mutant recombination

Parallel electroporation of 17D 5' ΔE Cherry and 17D 3'Δ GFP on BHK-21 and BHK-21 VEEV-YFV-17D-Help cells confirmed that the engineered sequence deletions resulted in the generation of suicide 17D genomes and that these genomes were only capable of spreading when full length E was supplemented in *trans* (**Figure 3.6**). Previous analysis of alphavirus recombination using chikungunya virus (CHIKV) and Sindbis virus (SINV) suggested that the conditions for cell culture purification of putative recombinants was highly virus specific (Chapter 2: CHIKV was ≥50 fold more infectious of C6/36 cells while SINV was slightly more infectious for Vero cells). As such, experiments were performed to determine the optimal passage protocol for detection of a full length YFV 17D against a saturated background of ΔE replicons. Ten-fold serial dilutions of purified YFV 17D Cherry RNA (range: 10^{1}-10^{-6}) were co-electroporated in BHK-21 cells against 10ug each 17D 5' ΔE and 17D 3' ΔE. One-fifth of the total volume of each electroporation (~$2.0x10^{2}$ cells in 2.0mL total volume BHK-21 maintenance medium) was then plated in a single well of a six-well plate. At 24, 48, 72, and 96h post-electroporation, total supernatant was harvested and replaced with sterile BHK-21 medium. Five hundred microliters were then used to inoculate C6/36 and Vero cell ~80% confluent monolayers grown in six-well

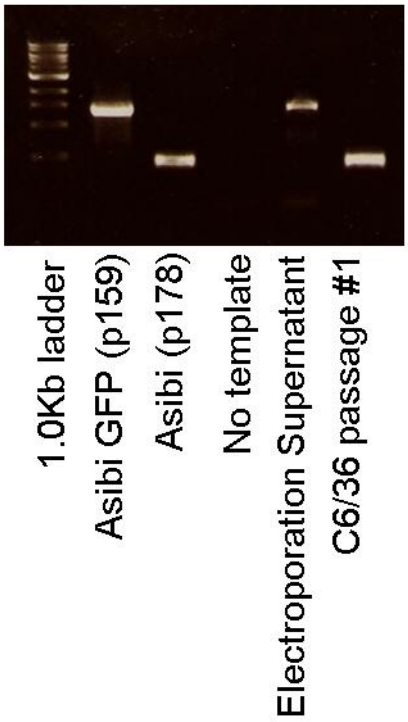


FIGURE 3.5 RT-PCR ANALYSIS OF ASIBI GFP STABILITY.

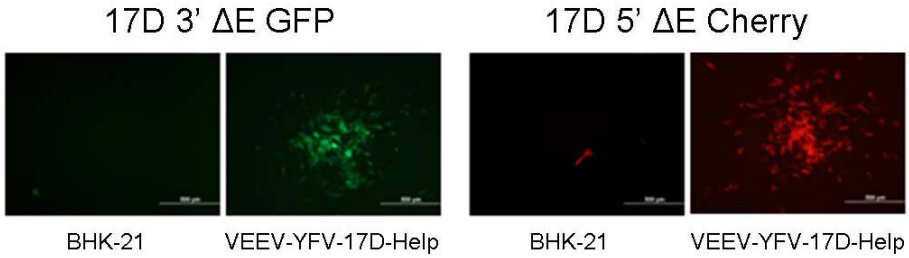


FIGURE 3.6 CONFIRMATION OF THAT IN-FRAME ENVELOPE DELETIONS RESULT IN GENERATION OF SUICIDE GENOMES THAT CAN BE SUPPLEMENT IN *TRANS*.

plates. Vero and C6/36 plates were examined daily for up to four dpi for signs of expanding Cherry foci indicating recovery of full length 17D Cherry. Analyses of detection sensitivity performed in this way indicated that Vero cells were ~100-fold more sensitive than C6/36 cells for the detection of 17D Cherry when titrated against a saturated background of envelope deletion replicons. Furthermore, it was determined that optimal detection was achieved at 72h post-electroporation to a sensitivity of 10^{-3} μg 17D Cherry RNA input (equivalent to ~50 primary full length infections). To see if detection sensitivity could be further increased, 500μL of electroporation supernatant samples from 17D Cherry dilutions 10^{-3}-10^{-5} were used to infect $1.0x10^{7}$ Vero cells in suspension (as described Chapter 2: Materials and Methods: Section E) followed by plating in 96-well plates. Analysis in this way increased the sensitivity of 17D Cherry detection to a dilution of 10^{-4} (~5 primary infections). Therefore, based on these analyses, it was decided that all attempts to recover 17D recombinant virus would involve co-electroporation of the recombinant cross (envelope deletion replicons) followed by a 72h incubation at the appropriate growth temperature, and then attempted recovery by direct infection of $1.0x10^{7}$ Vero cells. Due to the non-cytopathic nature of YFV 17D when inoculated at low moi, at 72h post-infection all medium was removed from $1.0x10^{7}$ Vero plates and placed in correspondingly labeled 96-well plates and stored at -80ºC.

Plates were then fixed, stained by IFA, and all wells visually examined for the presence of YFV 17D infectious foci. 17D 5' ΔE and 17D 3' ΔE co-electroporation recombinant crosses (10μg per RNA species) were performed in triplicate in both BHK-21 and $C_7$10 cells. 17D 5' ΔE Cherry and 17D 3' ΔE GFP were also co-electroporated (10μg per species) to allow for estimation of co-infection efficiency. Despite achieving estimated YFV replicon co-infection levels of ~$2.4x10^6$ in BHK-21 cells and ~$1.05x10^5$ in $C_7$10 cells, no evidence of YFV 17D recombination was detected.

C. Analysis of YFV superinfection resistance in Vero and $C_7$10 cells

Superinfection resistance studies were performed to determine if primary infection with YFV Asibi would influence the replication of a secondary infection with YFV 17D. Primary infections were performed by electroporating RNA derived from a GFP expressing Asibi i.c. (Asibi-GFP); this virus was chosen because it allowed for visual estimation of the percentage of cells positive for primary YFV infection. At seven days post-electroporation ($\geq$95% of cells positive for GFP expression) cells were superinfected with virus derived from a 17D i.c. competent for the expression of Cherry fluorescent protein (17D-Cherry). This virus was chosen for these studies because the expression of Cherry would allow for easy distinction between 17D and Asibi during virus titration. Superinfection growth curves were also performed using Asibi-GFP infected cells and an unrelated alphavirus, CHIK-LR-5'-Cherry. These infections were included to verify that any changes in 17D replication kinetics (if observed) were due to the establishment of YFV specific superinfection resistance and not a result of Asibi infection resulting in the induction of a non-specific antiviral state. Additionally, control (mock) electroporated Vero and $C_7$10 cells were also infected with either 17D-Cherry or CHIK-LR-5'-Cherry in parallel. The basic experimental design for these experiments is depicted in **Figure 3.3**.

Vero cells previously electroporated with Asibi-GFP were slightly less permissive for 17D-Cherry replication than mock electroporated cells. Average titers of 17D-Cherry harvested from Vero cells that were also infected with Asibi-GFP were approximately 0.5-1.0 $\log_{10}TCID_{50}$/mL lower than from corresponding mock electroporated Vero cells at all times post-superinfection (**Figure 3.7 A**). In $C_7$10 cells 17D-Cherry was not detected (limit of detection 1.0 $\log_{10}TCID_{50}$/mL) in Asibi-GFP infected cells beyond 24h post-infection, with titers at this time point likely representing residual viral inoculum. Average titers of 17D-Cherry harvested from $C_7$10 cells harboring a primary Asibi-GFP were significantly ($P<.05$) lower at two, three, and four dpi as compared to corresponding time points harvested from mock electroporated $C_7$10 cells. Infact primary infection with Asibi, in $C_7$10 cells, appeared to result in the generation of a cellular environment that was completely refractory to superinfection with/replication of 17D (**Figure 3.7 B**). As expected, previous infection with Asibi-GFP did not have any significant untoward effect on CHIK-LR-5'-Cherry replication in either cells line used (**Figure 3.7 C and D**).

3.5 DISCUSSION

I designed these studies to provide empirical evidence of flavivirus recombination using YFV 17D replicons bearing complementary in-frame E coding sequence deletions. Electroporation of these constructs into BHK-21 cells both with and without a VEEV packaging construct, capable of supplementing functional envelope in *trans*, verified the viability and "suicide" phenotype of these replicons. Interestingly, replicon particle titers harvested from packaging cells were $\geq$1000-fold less than as previously reported for YFV 17D replicon genomes (Shustov *et al.,* 2007). Both of the deletion mutant replicons (17D 5' ΔE and 17D 3' ΔE) are competent for the expression of an E protein that has a large internal deletion. 17D 5' ΔE contains an 80aa deletion within E dimerization domain (DII) including the fusion loop (aa 98-110), while the majority of the receptor binding

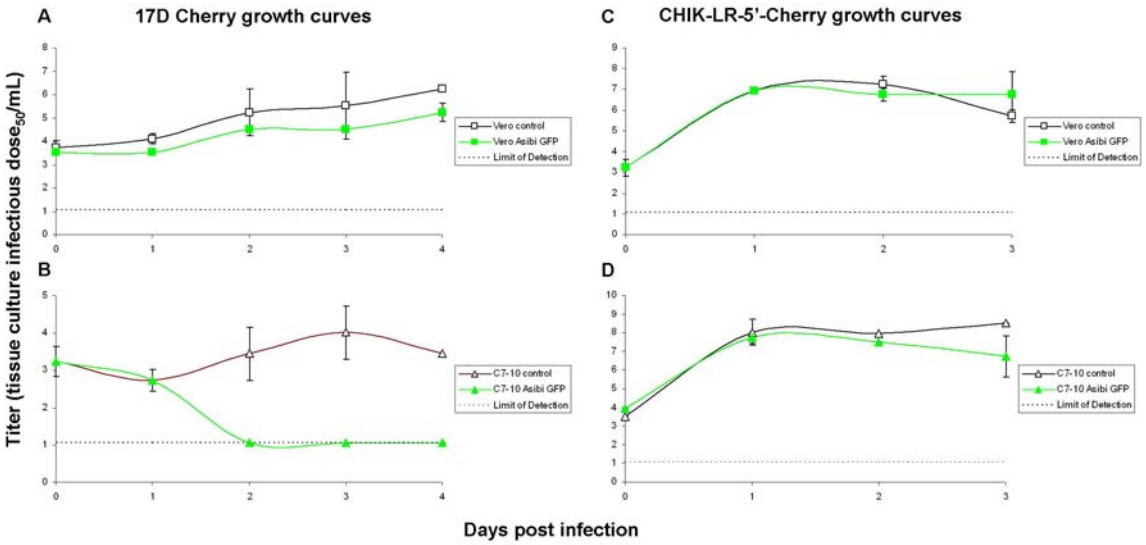


FIGURE 3.7 SUPERINFECTION OF ASIBI GFP WITH 17D CHERRY AND CHIKUNGUNYA 5' CHERRY VIRUSES.
Vero and $C_7$10 monolayers with and without Asibi GFP infection were superinfected with either 17D Cherry (A and B) or CHIK-LR-5'-Cherry (C and D) followed by kinetic analysis of replication of the superinfecting virus.

domain (89aa of E DIII) has been removed from 17D 3' ΔE (**Figure 3.8**). Although major functional domains have been removed from these truncated E proteins, these truncated envelope genes have been designed in such a way that truncated proteins should be expressed and maintain the correct transmembrane topologies of the native 17D E protein. As such, it was possible that these truncated E proteins may have been incorporated into replicon containing virus like particles (VLP). If this were to happen, these particles would have significantly impaired infectivity due to their inability to interphase with cellular receptors and/or initiate fusion of the viral and endosomal membranes. As such, I hypothesize that the observed disparity in 17D VLP titers as compared to previous reports may have resulted from expression and incorporation of these truncated E proteins into replicon bearing VLPs thereby decreasing particle

infectivity. A second possible explanation for these titer discrepancies may be that the presence of truncated E protein may in some way disrupt particle assembly.

Nevertheless, co-electroporation of 17D 5' ΔE-Cherry and 17D 3' ΔE-GFP RNAs was observed to result in high level co-infection in BHK-21 (~10^6) cells as indicated by cells positive for the expression of both GFP and Cherry. Despite this artificially high level of co-infection at the cellular level no recombinant full length YFV 17D was detected. As such, these data strongly argue that even if acute robust post-vaccination replication were to occur within a vaccinee recombination is unlikely. This conclusion is further supported by a recent paper by Taucher *et al.,* (2009). In these studies, persistent high level co-infection of *trans*-complementing flavivirus replicon genomes was required to yield recombinant JEV (Taucher *et al.,* 2009). These recombinant crosses employed replicons designed similarly to YFV E deletion mutants described in this dissertation, in that they contained various in-frame deletions of the structural gene coding sequence. JEV recombinants were isolated from this system only following three to five rounds of serial high moi passage in BHK-21 cells. Briefly, *trans*-complementing JEV genomes were co-electroporated, harvested, and re-inoculated onto fresh BHK-21 mono-layers with limiting dilution assays performed to detect recombinants following each passage. Additionally, recombinants were aberrantly homologous and contained sequence duplications coincident with cross-overs occurring outside the predicted sequence (region located between structural protein deletions) which resulted in attenuated *in vitro* replication as compared to wt.

It has been hypothesized by McGee *et al.,* (2008) that because flaviviral infection in a mosquito/tick is persistent, in nature recombination would most likely occur in the arthropod vector (McGee *et al.,* 2008). Indeed the persistent replication in a mosquito vector can be quite long-lived as *Ae. aegypti* mosquitoes experimentally infected with

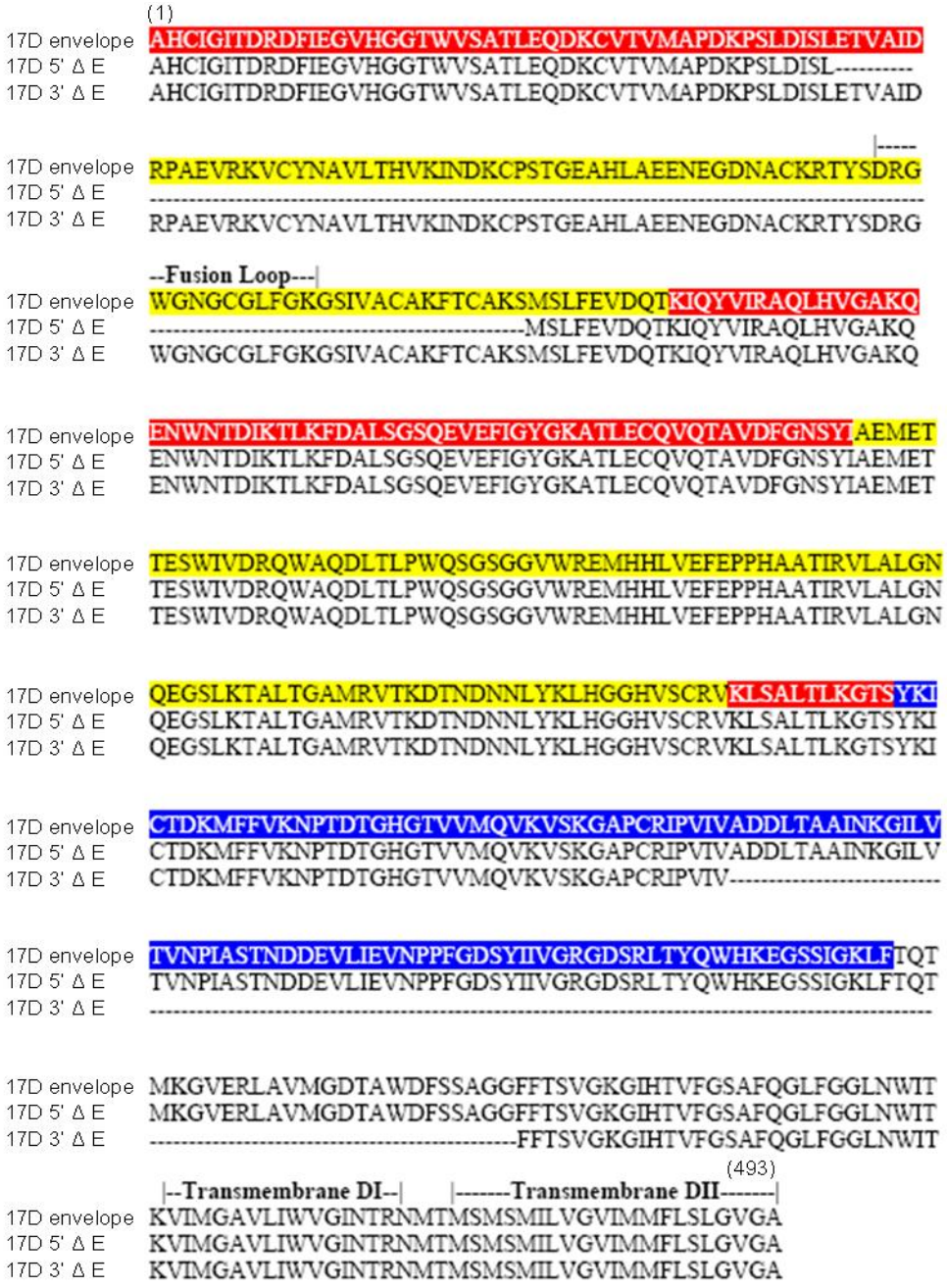


FIGURE 3.8 AMINO ACID SEQUENCE ALIGMENT OF YELLOW FEVER VIRUS 17D AND DELETION MUTNANT ENVELOPE PROTEINS.
Domains I, II, and III are depicted in red, yellow and blue respectively. Sequence deletions are indicated with dashed lines. Transmembrane domains (D) and fusion loop are also denoted.

Asibi have been observed to fatally infect monkeys for up to 168 days post-infectious bloodmeal (Philip 1962). Since 17D and 17D chimeric vaccines exhibit an impaired ability to escape the mosquito midgut, recombination events occurring between a vaccine and wt flavivirus would have to occur with co-infected midgut epithelial cells. The mosquito midgut is estimated to be comprised of ~10^4 epithelial cells. Analysis by Smith *et al.,* (2008) suggest that only a small sub-population of these cells, perhaps 100, are susceptible to primary infection with Venezuelan equine encephalitis virus (Smith *et al.,* 2008), a conclusion which has been further supported by the observations of relatively low numbers of primary WNV midgut infections regardless of input titer (McGee *et al.,* 2009, Scholle *et al.,* 2004). Taken together, these data suggest that for any given mosquito-borne virus only a small sub-population of midgut epithelial cells may be susceptible to primary infection, although the actual population of susceptible cells may be different for each virus. Thus my achieved levels of co-infection described here, specifically with regard to arthropod cells (electroporation of $C_7$10 with 17D 5' ΔE-Cherry and 17D 3' ΔE-GFP RNAs reproducibly resulted in $\geq 10^5$ co-infections), are highly significant as they suggest that even if every cell within the mosquito gut were simultaneously co-infected with two distinct flaviviruses a recombinant virus would still not likely be produced.

Intra-mosquito reports of flavivirus superinfection resistance are scarce. One report by Sabin (1952) suggests that *Ae. aegypti* mosquitoes harboring a primary infection with DENV become resistant to superinfection with YFV (Sabin 1952). However because no data regarding the number of individual mosquitoes assayed were provided the extent of this resistance is unclear. Intra-vector superinfection resistance to LaCrosse virus (Family Bunyaviridae, genus *Orthobunyavirus*) in *Ae. triseriatus* mosquitoes has been repeatedly demonstrated (Beaty *et al.,* 1985, Borucki *et al.,* 1999,

Sundin & Beaty 1988). The establishment of this interference appears to be time dependent since mosquitoes only become refractory to secondary infection if oral exposure is separated by >48h. A similar time dependent establishment of superinfection resistance has also been documented for flaviviruses *in vitro* (see Introduction: Section 1.4 E). Analysis of DENV superinfection exclusion have demonstrated that resistance to a secondary infection can be established by 20h post-primary infection and can persist for up to 50 days (Dittmar *et al.,* 1982, Igarashi 1979, Pepin *et al.,* 2008). Recent studies by Zou *et al.,* (2009) using WNV indicated that flavivirus superinfection exclusion occurs via an RNA synthesis driven mechanism whereby replication of the secondary virus is blocked via over-competition for cellular host factors involved in viral replication (Zou *et al.,* 2009). To determine if a similar phenomenon could be observed for YFV, Vero and $C_7$10 cells were superinfected with YFV 17D following primary infection with YFV Asibi. While $C_7$10 cells appear to become completely refractory to 17D infection/replication following primary infection with Asibi, superinfected Vero cells appear to be fairly permissive for 17D replication. It was noted by Zou *et al.,* (2009) that WNV superinfection resistance could be overcome by selection for viruses containing mutations which increased receptor binding affinity and RNA replication kinetics (Zou *et al.,* 2009). Indeed YFV 17D is a highly vertebrate cell culture adapted virus and as such may possess some, as of yet, unidentified mutations which allow for escape from superinfection resistance in vertebrate cells. The ability of CHIKV to replicate to similar titers and at similar kinetics in both Vero and $C_7$10 cells harboring a primary infection with YFV Asibi suggest that primary YFV infection does not induce a non-specific antiviral state in these cell types.

Taken together my observations as reported in this dissertation and those published by others strongly argue that: 1) if a recombinant flavivirus does arise

following acute dual infection in a vertebrate host then it would likely be attenuated and as such it would likely be selected against, and 2) should a arthropod vector become exposed to two distinct flaviviruses, the likelihood of sequential co-infection and/or recombination is extremely remote.

CHAPTER 4: *IN VITRO* AND *IN VIVO* ANALYSIS OF THEORETICAL "WORST CASE" FLAVIVIRUS WILD-TYPE/VACCINE CHIMERAS

4.1 INTRODUCTION

Dengue is the most important emerging arboviral disease (Gratz 1999, Gubler 1998). Due to increased travel to tropical locals and expansion of vector distribution, the incidence of dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) have increased dramatically in many regions of the world with an estimated 2.5 billion people at risk (Gubler 2006). Classical vector control has been the primary strategy to combat mosquito-borne diseases; however, population expansion of *Aedes aegypti*, the predominant peridomestic vector of the dengue viruses, serves to emphasize the urgent need for a dengue vaccine (Gubler 2004, Gubler 2006, Mackenzie *et al.*, 2004). DHF/DSS is commonly associated with secondary exposure of an individual with pre-existing antibody to a heterologous serotype. Therefore, since there are four serotypes of dengue viruses (DENV 1-4), an efficacious DENV vaccine must confer long-lasting tetravalent humoral and cellular immunity (Chaturvedi et al., 2005). Several approaches toward the development of a vaccine platform capable of inducing tetravalent DENV immunity are currently under investigation and in various phases of pre-clinical/clinical testing (Guy & Almond 2008, Webster et al., 2009). One particularly attractive strategy to achieve long-lived cellular and humoral immunity is through the use of vaccine formulations comprised of live attenuated viruses representing all four DENV serotypes (Guy & Almond 2008). However, the result of vaccination with such a cocktail is that four distinct but related viruses replicate in a single individual simultaneously. In many cases, flavivirus live attenuated vaccines (LAV) will be administered in wild-type (wt)

endemic regions further increasing the potential for a single host to be infected with multiple viruses. Since recombination has been identified as a major cause of post-vaccination reversion when RNA virus vaccines have been administered as multivalent formulations and/or in endemic regions (oral polio vaccine) (Furione et al., 1993, Georgescu et al., 1994, Kew et al., 1981, Minor et al., 1986), caution has been recommended with regard to the use of live flavivirus vaccines (Holmes *et al.,* 1999, Seligman & Gould 2004, Worobey *et al.,* 1999).

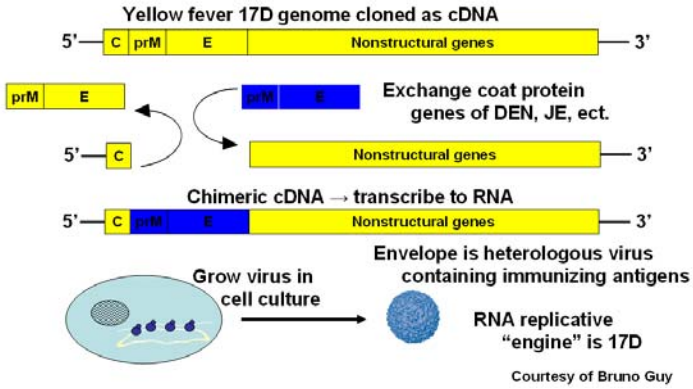


FIGURE 4.1 SCHEMATIC REPRESENTATION OF 17D BASED CHIMERIC VACCINE TECHNOLOGY: CHIMERIVAX™.

The yellow fever (YF) 17D LAV has been regarded as one of the safest and most efficacious vaccines (Marianneau *et al.,* 2001). The success of the 17D LAV has been coupled with reverse genetic technologies to generate the so called ChimeriVax™ platform, originally developed by Acambis Inc. The ChimeriVax platform attempts to provide safe and efficacious flaviviral immunity via delivery of chimeric LAV viruses, in which the genetic sequences for the structural genes of the YF 17D vaccine (premembrane (prM) and envelope (E)) have been replaced with the corresponding sequences derived from heterologous flaviviruses (Guirakhoo *et al.,* 2000, Guy *et al.,* 2009, Monath 2001, Monath *et al.,* 1999) (**Figure 4.1**). This technology has been used to develop candidate vaccines for flaviviruses such as St. Louis encephalitis, West Nile virus (WNV), Japanese encephalitis virus (JEV), and DENV 1-4. The attenuation and immunogenecity of these chimeric viruses has been extensively characterized in vertebrate and insect systems both *in vitro*

and *in vivo* (Brandler *et al.,* 2005, Guirakhoo *et al.,* 2001, Guirakhoo *et al.,* 2006, Guirakhoo *et al.,* 2002, Guirakhoo *et al.,* 2004, Guirakhoo *et al.,* 2000, Guirakhoo *et al.,* 1999, Guy *et al.,* 2009, Guy *et al.,* 2008, Monath *et al.,* 2003, Monath *et al.,* 2005, Monath *et al.,* 2000, Monath *et al.,* 2006, Monath *et al.,* 2002, Monath *et al.,* 2005, Monath *et al.,* 1999, Pugachev *et al.,* 2005, Pugachev *et al.,* 2003, Wills *et al.,* 1985). Additionally, experimental observations of ChimeriVax vaccine candidate virus-vector interactions have identified attenuations for mosquito infectivity similar to the parental 17D vaccine (Bhatt *et al.,* 2000, Higgs *et al.,* 2006, Johnson *et al.,* 2002, Johnson *et al.,* 2004) suggesting that even if a vaccinee were to mount significant viremia these viruses would not establish mosquito-borne transmission cycles.

To date over 500 million doses of YFV 17D have been administered worldwide, frequently in situations when vaccinees may be infected with wt YFV, and neither experimental nor field data exists that would suggest that recombination between vaccine and wt flaviviruses has ever occurred (Barrett *et al.,* 2007, Monath *et al.,* 2005). As such, the incidence of, and potential for, such an event may have been overestimated (de Silva & Messer 2004, Hornbach 2004, Monath 2004, Murphy *et al.,* 2004). A total of 33 amino acid differences exist between the 17D vaccine and Asibi wt strains of YFV (Hahn *et al.,* 1987, McElroy *et al.,* 2005). We are beginning to understand how these sequence differences may influence vector infection and vertebrate virulence (Engle 2006, McElroy *et al.,* 2006, McElroy *et al.,* 2006), but it is unknown if any phenotypic effects would result from a recombination/reversion event. Attenuating mutations appear to be scattered throughout the YFV 17D genome and in general substitution of virulent Asibi sequences into the 17D backbone does not restore an "Asibi-like" like phenotype with respect to vertebrate virulence or mosquito infectivity (Engle 2006, McElroy *et al.,* 2006, McElroy *et al.,* 2006). It has also been documented that chimerization typically

compromises replication competence (Blaney *et al.,* 2004, Blaney *et al.,* 2002) further underscoring the low probability that a vaccine/wt recombinant would possess a virulent phenotype. As such, the possibility for a recombination event or multiple mutational events that change the attenuated phenotype of a vaccine virus to one of virulence and simultaneously enhance the capacity for the virus to replicate, disseminate and be transmitted by the mosquito, seems highly unlikely.

For recombination to occur, a mosquito vector or vertebrate host would first have to become infected with both a wt and vaccine virus either simultaneously or sequentially. Following initial co-infection, a recombinant genome would have to be generated, selected for, and amplified. If such a change in a flavivirus vaccine platform did occur, the recombinant virus would have to replicate to sufficient titer in the vertebrate host to infect subsequently feeding mosquitoes and thus establish vector-borne transmission cycles. Although recombination following vaccination with a flavivirus LAV is theoretically unlikely, especially considering my inability to quantify 17D homotypic recombination despite achieving artificially high levels of co-infection (Chapter 3), it is important to test the consequences of recombination in a laboratory setting. The parental YFV 17D and derivative vaccine ChimeriVax DEN 4 were selected for these experiments. ChimeriVax DEN 4 was chosen since although highly attenuated, it may be slightly more infectious than the other ChimeriVax DEN vaccine candidates for *Aedes* mosquitoes (Higgs et al., 2006). Additionally, ChimeriVax DEN 4 may produce a slightly higher and longer viremia in humans and non-human primates than YFV 17D and other ChimeriVax DEN vaccine candidates (average peak titers: 2.8 $\log_{10}$ pfu/mL, average duration: 4.4 days) when administered as a tetravalent formulation (Guirakhoo *et al.,* 2004, Higgs *et al.,* 2006, kanesa-Thasan 2005). A "worst-case scenario" recombinant is likely represented by a situation where two distinct and pathogenic flaviviruses

recombine exchanging their prM and envelope E genes, while preserving the functions of the nonstructural and untranslated regions (UTR) (Pugachev et al., 2007). Therefore "worst-case scenario" chimeras were constructed, in which the backbone of the well-characterized attenuated vaccines YFV 17D and ChimeriVax DEN 4, were wholly replaced with corresponding sequences of the virulent YFV Asibi strain. The phenotypes of the resulting recombinants were then evaluated with respect to mosquito infectivity and pathogenicity in non-human primates.

4.2 AIMS AND HYPOTHESES

The **aim of these studies** was to generate and evaluate the mosquito infectivity and vertebrate pathogenicity phenotype of flavivirus wt/LAV experimental recombinant viruses using YFV 17D, ChimeriVax DEN 4, and YFV Asibi. The hypotheses of these studies were as follows:

A. *Replacement of 17D vaccine sequences with corresponding YFV Asibi sequences will result in the generation of recombinant chimeric flaviviruses that are competent for replication in cell culture.*
B. *Rationally designed "worst-case" recombinant wt/vaccine flaviviruses will have significantly decreased mosquito infectivity as compared to their respective wt parental viruses.*
C. *"Worst-case" chimeric wt/vaccine recombinants will be fully infectious for mosquitoes following intra-thoracic injection.*
D. *"Worst-case" recombinant wt/vaccine flaviviruses will not have a YFV "Asibi-like" phenotype with respect to vertebrate pathogenicity in non-human primates.*

Data from these studies have been previously reported and were reproduced here with permission (McGee *et al.,* 2008, McGee *et al.,* 2008):

Charles E. McGee, K. Tsetsarkin, D.L. Vanlandingham, K.L. McElroy, J. Lang, B. Guy, T. Decelle, and S. Higgs. Substitution of Wild-Type Yellow Fever Asibi Sequences for 17D Vaccine Sequences in ChimeriVax™-Dengue 4 Does Not Enhance Infection of *Aedes aegypti* Mosquitoes. Journal of Infectious Diseases. 2008a. 197:686–692.

Charles E. McGee, M.G. Lewis, M. St. Claire, W. Wagner, J. Lang, B. Guy, K. Tsetsarkin, S. Higgs, and T. Decelle. Recombinant Chimeric Virus with Wild-Type Dengue 4 prM-E and Virulent Yellow Fever Asibi Backbone Sequences is Dramatically Attenuated in Non-human Primates. Journal of Infectious Diseases. 2008b. 197:693–697.

4.3 MATERIALS AND METHODS

A. Cells and viruses

African green monkey kidney (Vero), baby hamster kidney (BHK-21), and *Ae. albopicuts* (C6/36) cells were gown in Liebovitz L-15 media supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin, and 1% L-glutamine (cellgro® Mediatech, Inc., Herndon, VA) with vertebrate and invertebrate cell lines being maintained at 37ºC and 28ºC, respectively. ChimeriVax DEN 4 virus was obtained as frozen stocks from Acambis Inc., thawed once to prepare aliquots and then stored at -80ºC. The wt parent virus used for construction of ChimeriVax DEN 4 virus was DEN 4 (strain 1288). Infectious Asibi, 17D, Asibi/17D M-E, 17D/Asibi M-E, and Asibi/DEN 4 M-E (**Figure 4.2**), viral stocks were generated from respective infectious clones (i.c.) as previously described (Chapter 2: Materials and methods: Section C). Briefly, plasmids were linearized, purified using phenol/chloroform, and *in vitro* transcribed using the mMESSAGE mMACHINE kit in accordance with manufacturer's protocols. RNA was electroporated into BHK-21 cells and cell culture supernatant aliquots containing virus were harvested three to five days post-electroporation based on visualization of cytopathic effect (CPE) then stored at -80ºC. Stock titers were determined by virus titration (Higgs 1997).

B. Plasmid Constructs

All molecular cloning methodologies involved in the generation and evaluation of all full length flavivirus reverse genetics systems or infectious clones were as described (Chapter 2: Materials and Methods: Sections B-D). Oligonucleotide primers used for PCR amplification of cloning fragments are listed in **Table 4.1**.

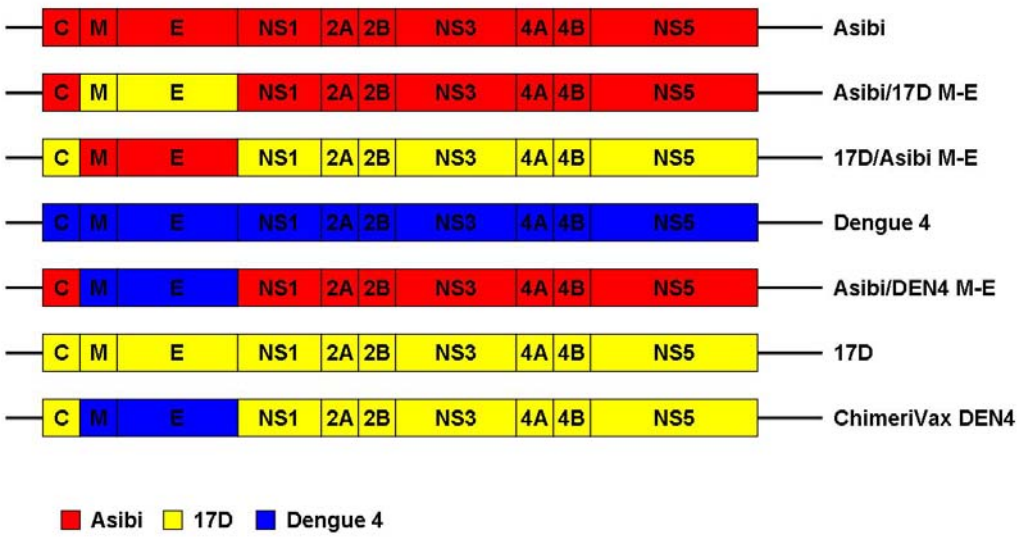


FIGURE 4.2 SCHEMATIC REPRESENTATION OF THE WILD-TYPE, VACCINE, AND CHIMERIC FLAVIVIRUSES USED IN THESE STUDIES.

Construction of the pYFV-Asibi i.c. and structural YFVAsibi-17D chimeric variant clones pAsibi/17D M-E and p17D/Asibi M-E (backbone/structural insertion), were previously described (McElroy et al., 2005, McElroy et al., 2006, McElroy et al., 2006). The YFV variant infectious clone expressing the DEN 4 (strain 1228) structural proteins prM and E in the virulent YFV Asibi backbone pYF-Asibi-DEN 4 M-E was constructed via the generation of an intermediate construct designated p69.2 in a two step cloning process. Plasmid 69.2 was made by three fragment ligation of vector (V) pAsibi 5 i.c. (p62.6 K. Tsetsarkin) digested with Not*I*, Mlu*I*, and calf intestinal phosphatase

(CIP), insert $(I)_1$ a fusion PCR amplicon templates p62.6 and pYF5'3'DEN 4 (Acambis Inc.) using primers (pr.) 17D-F1/17D-R8 and 17D-F8/Den-Bst-R digested with Not*I* and BstB*I*, and I_2 pYFM5,2DEN 4 (Acambis Inc.) digested with BstB*I* and Mlu*I*. Infectious clone pYF-Asibi-DEN 4 M-E (p70) was then produced via replacement of heterologous p69.2 sequences with PCR amplified YFV Asibi sequences by two fragment ligation of V p69.2 digested with Sfo*I*, Mlu*I* and CIP and I_1 a PCR amplicon of p62.6 using pr. YF-Nar-F/17D-R3 digested with Sfo*I* and Mlu*I*.

C. Analysis of viral replication kinetics

To elucidate the appropriate conditions to obtain maximum bloodmeal titers for infectious feeds (i.e. cell type which would produce maximal titers), ten day growth curves for all viruses were generated in duplicate. Vero ($1x10^6$ cells per well) and C6/36 ($5x10^6$) cells were seeded into six-well plates and infected 24h later at a multiplicity of infection (moi) of 0.001 for 1h at room temperature (rt) with agitation. The inoculum was then removed, monolayers washed three times with 1X phosphate buffered saline (PBS), and 2.0mL of the corresponding maintenance media added. Fifty microliters of cell culture supernatant was then harvested and replaced every 24h for 10 days post-infection (dpi). Samples were stored at -80ºC and virus quantified by titration.

TABLE 4.1 SPECIFIC PRIMERS INVOLVED IN THE CONSTRUCTION AND SEQUENCING OF FLAVIVIRUS PLASMID CONSTRUCTS IN THESE STUDIES.

Name	Sequence
17D-F1	5' AAGTGCCACCTGACGTGTCG
17D-F8	5`-ACGCCGTTCCCATGATGTTC
17D-R8	5`- ATTAGGAATTGCACAGTCA
17D-Mlu F	5' GAAAGTCCAGGAAAGAATGCC
17D-R3	5' AAGACTGCGTCCATGTACAC
17D-F3	5' CCTCGCTGGCCTTGGTTG
17D-Sap R	5' ACATCCCCACTTCTCCTAGC
17D-F7	5`-GCTTGCGCACTCTTGTGTTG
17D-NgoM F	5' TGTCTCCCAAAGGCATCAGT
17D-R5	5'CTTCTATGGCCTCTTTGTCC
17D-ClaI R	5' CATGTTGTGCGTCCTTGTG
17D-Vec-R	5`-GCCACGTTGTGTCTCAAAATC
YF-Nar-F	5'-GAGTTGGCGCCGATCAAGGATGCGCCATCA
Den-Bst-R	5'-GGCTCCAGACACGTAGTA

D. Mosquito infection and maintenance

Two strains of *Ae. aegypti* mosquitoes were used for infectious feeds to allow for investigation and comparison of the infection and dissemination of YFV variants in colonized and wild-caught potential vectors. The *Ae. aegypti* Rex-D white-eye (WE) Higgs strain was derived from a spontaneous mutation in the Rex-D strain originally isolated from Rexville, Puerto Rico (Wendell et al., 2000) with mosquitoes used for infectious feeds being >30F. Wild-caught *Ae. aegypti* eggs were collected in the spring of 2004 from Mae Sot Province, Thailand (16º 45'N, 98º 33'E), reared for several generations, and F5 eggs were collected and stored for use in these experiments.

Fresh virus for artificial bloodmeals was generated by propagation of virus stocks in cell culture or directly harvesting from electroporation. Virus inocula prepared in Vero cell culture included; 17D/Asibi M-E and ChimeriVax DEN 4. Those propagated in C6/36 cells included; Asibi/17D M-E and Asibi/DEN 4 M-E. YFV Asibi was harvested directly from electroporated BHK-21 cells. Virus, cells, and supernatant were harvested in accordance with predicted maximal titer post-infection and combined 1:1 with defibrinated sheep blood. Adenosine triphosphate was added as a phagostimulant, to a final concentration of 2-3 mM. Oral infection of *Ae. aegypti* mosquitoes was as previously described (Chapter 2: Materials and methods: Section F). A 1.0mL aliquot of infectious bloodmeal was stored at -80ºC for virus titration. Positive control mosquitoes for all viruses were generated via intra-thoracic (IT) inoculation of virus stocks diluted 1:3 in Liebovitz L-15 media.

E. Determination of virus titer

Mosquitoes were harvested at zero and 14 dpi and assayed for virus by titration as previously described (Higgs 1997, Higgs *et al.,* 2006). Whole individual mosquitoes (zero dpi) were triturated in 1mL of L-15 media (10% FBS + 10% tryptose phosphate

broth + 100 units/mL Penicillin + 100 µg/mL Streptomycin + 1 µg/mL Fungizone) and titrated as ten-fold serial dilutions on Vero cell culture. At 14 dpi, mosquito bodies and heads were assayed for infectious virus separately to determine infection and dissemination rates respectively. Mosquito bodies were triturated in 1mL and heads in 300µL of L-15 media (supplemented as above). 100µL of each body homogenate was loaded in duplicate then titrated in serial ten-fold dilutions in with the first eight wells of a 96-well plate. Head homogenates were titrated in the last four wells of the same rows as corresponding bodies. Titration plates were incubated at 37ºC for 10 days, then fixed with 1:1 acetone-methanol and stored at -20ºC for at least 30m, dried and analyzed by immunohistochemistry (IHC).

F. Immunohistochemical assay

Viruses varied in their capacity to produce CPE in Vero cells, therefore all titration plates were assayed for virus by IHC to determine the highest dilution at which antigen was present. Selection of the antibody and optimal dilution was based on visual evaluation of signal intensity using Vero cells infected with each of the viruses, grown on glass coverslips (Gould et al., 1985). For all YFV variant viruses it was determined that MA93, a hyperimmune mouse serum raised against YFV Asibi and 17D (produced by S. Higgs in 1993), at a concentration of 1:500 provided optimal detection.

Plates were dried and then incubated for 10m at rt in PBS supplemented with 1% normal horse serum (Sigma-Aldrich, St. Louis, MO), before the application of primary antibody (MA93) for 30m. Plates were then rinsed twice in 1X PBS followed by application of secondary antibody; an anti-mouse IgG horseradish peroxidase conjugate raised in goats (Southern Biotech, Birmingham, AL) at 1:500 for 30m. Plates were subsequently rinsed twice in 1X PBS followed by visualization using the Vector® VIP peroxidase substrate kit (Vector Laboratories Inc., Burlingame, CA) for 7.5 min, and

reactions were quenched in distilled water. Growth curve samples, bloodmeal and mosquito titers were calculated as $\log_{10}$ tissue culture infectious dose 50 ($\log_{10}$ $TCID_{50}$) per mL or mosquito respectively.

G. Animal manipulations

Forty-eight Cynomolgus macaques (*Macaca fascicularis*), three males/three females per group were immunized using a subcutaneous injection ~10^4 $TCID_{50}$ of a single flavivirus, either a wt (Asibi and DEN 4 1228), vaccine (17D (lot #UE813AA) and ChimeriVax DEN 4), experimental recombinant (17D/Asibi M-E, Asibi/17D M-E and Asibi/DEN 4 M-E) (backbone/structural insertion) or PBS control. This dose was chosen based on previous analyses of Asibi, 17D, and DEN 4 in non-human primates (T. Decelle unpublished data).

Animals were purpose-bred by Siconbrec Inc., (Philippines), housed and manipulated at Bioqual, Inc., (Rockville, MD) and prescreened for flavivirus antibodies using an IgG antibody ELISA assay (Panbio Inc, Columbia, MD). All animal manipulations were conducted in accordance with federal laws, regulations, and NIH guidelines and in compliance with AAALAC standards.

Animals were observed twice daily and weighed prior to each sampling. Blood samples, 3.0mL EDTA treated and 3.0mL whole blood, were collected at zero, two, four, six, eight, ten, and fourteen dpi representing a maximal draw of seven samples of six milliliters. Percutaneous fine needle liver biopsies were taken at two, four, and eight dpi from animals euthanized at six, ten, and fourteen dpi respectively using an 18 gauge biopsy needle. Representative animals from each group were euthanized and necropsied at six/seven, ten, and fourteen dpi. A board certified veterinary pathologist examined these tissues specimens and the collections were all of excellent quality. Larger liver samples were taken at necropsy and sections of grossly observed lesions examined.

H. Hematology

Complete blood counts and coagulation times (prothrombin time (PT) and activated partial thromboplastin time (APTT)) were analyzed using a SCA2000 veterinary coagulation analyzer (Synbiotics, San Diego, CA).

I. Serum chemistry

A standard panel of liver function serum chemistry assays was performed (Antech Diagnostics, Lake Success, NY).

J. Immunologic assessment

Seroconversion of animals that survived beyond or were not euthanized prior to six dpi was confirmed using a DENV IgG ELISA assay (PanBio Inc.).

K. Cytokine modulation

Serum cytokine levels were assessed using commercially available assays (IFN (interferon) α, IL (interleukin)-10, and IL-12- p70 & p40 Invitrogen, Camarillo, CA; IL-1α R&D Systems, Minneapolis, MN; and IFNγ, IL-1β, IL-2, IL-5, IL-6, IL-8, and TNF (tumor necrosis factor) α BD Biosciences, San Jose, CA) in accordance with manufacture's protocols.

L. Viral genome quantification

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed on plasma samples collected on days zero, two, four, six, eight, ten, twelve, and fourteen and on all biopsy samples and selected tissues collected at necropsy. RNA was isolated from 1.0mL of plasma. Samples were centrifuged at 12,000g for 1h at 4^{o}C. The resulting pellet was treated with 1.0mL of RNASTAT-60 (Tel-Test, Friendswood, TX) and vortexed. Chloroform, 250μL, was then added and the mixture vortexed. The samples were then centifuged again as above. The aqueous layer containing the RNA was

added to 500µL of isopropanol with 10µL of 10µg/mL of tRNA (Sigma-Aldrich, St. Louis, MO). The mixture was precipitated over night at -20^{o} C, and then centrifuged 1h. The RNA was washed with 75% ethanol and resuspended in 50µL nuclease free water. Homogenized tissues samples were processed the same as above, using 100mg of the collected samples. Viral genomes were then quantified using qRT-PCR kit from Applied Biosystems (Foster City, CA), by combining a 20µL master mix, comprised of 3.125µL of water, 12.5µL of buffer 2.5µL l.0µL each of primers (sequences listed below, 10µM. each), 1.25µL of probe (4µM) and 0.625µL of enzymes, with 20µL of RNA. Specific primer and probe sequences were designed using genome sequences of YFV: nt 9595-9677 and DENV4: nt 9950-10109 and are depicted in **Table 4.2**. The reactions were placed in a 7700 sequence detector Applied Biosystems, and run using the following thermo-cycling conditions: 30m at 50^{o} C, 15m at 95^{o} C, 45 cycles of 95^{o}C for 15s and 1m at 60^{o} C for YFV (58^{o} C for DENV).

TABLE 4.2 YELLOW FEVER VIRUS AND DENGUE 4 VIRUS SPECIFIC OLIGONUCLEOTIDE PRIMERS AND PROBES FOR QRT-PCR ANALYSES.

Virus	Name	Sequence
Yellow fever virus	YF-Fwd	5'-GCACGGATGTAACAGACTGAAGA
	YF-Rev	5'-CCAGGCCGAACCTGTCAT
	YF-Probe	5'-FAM-CGACTGTGTGGTCCGGCCCATC-TAMR
Dengue 4 virus	D4-Fwd	5'-GTCAATCCACGCTCATCATC
	D4-Rev	5'-TCCACACCACAAATCCTCTC
	D4-probe	5'-FAM-CAAAGTGTGGAACAGAGTG-MGB

FAM-fluorophore, Fwd-forward, MGB-quencher, Rev-reverse, TAMR-quencher.

M. Histopathology

At necropsy, relevant tissue samples were fixed in formaldehyde, trimmed, paraffin embedded, sectioned (~5µm), and then stained with hematoxylin and eosin. Stained sections were microscopically examined for pathologic morphologies and graded by a board certified veterinary pathologist.

4.4 RESULTS

A. *In vitro* analysis

The replication kinetics of all flaviviruses (wt, vaccine, and chimeric recombinant) were evaluated in both Vero and C6/36 cells (moi 0.001: **Figure 4.3 A-H**). All chimeric recombinants were competent for replication in both vertebrate (Vero) and arthropod (C6/36) cells. In general, growth kinetics were consistent with peak titers being observed by five dpi. Insertion of the Asibi structural genes (prM-E) into the 17D backbone (Asibi/17D M-E) resulted in the generation of a virus with similar growth kinetics to YFV Asibi. In fact, 17D/Asibi M-E was observed to grow more rapidly and to higher titers in Vero cells than either YFV Asibi or the highly vertebrate cell line adapted 17D vaccine (**Figure 4.3 B**). The reverse chimera 17D/Asibi M-E was also observed to have growth kinetics similar to the YFV Asibi at least in C6/36 cells (**Figure 4.3 G**). ChimeriVax DEN 4 replicated either more rapidly or to higher titer than both YFV 17D and DENV 4 viruses in both Vero and C6/36 cells (**Figure 4.3 D,H**). Interestingly, the heterotypic recombinant Asibi/DEN 4 M-E was replicated to higher titer than the parental DENV 4 in both cell lines (**Figure 4.3 A,E**).

B. Analysis of chimeric recombinant vector infectivity in *Ae. aegypti* mosquitoes

Whole body titers of mosquito triturates were measured on zero and 14 dpi for all YFV/DENV variant viruses. Mean titers and standard deviation were calculated for each group using those mosquitoes, from which infectious virus was recovered (**Table 4.3**). Day zero whole body titers confirmed the ingestion of infectious virions during blood feeding for all groups Infection and dissemination were measured by individual titration of mosquito bodies and heads (Higgs *et al.,* 2006) because previous studies have indicated that detection of DENV in mosquito head tissue is indicative of a disseminated

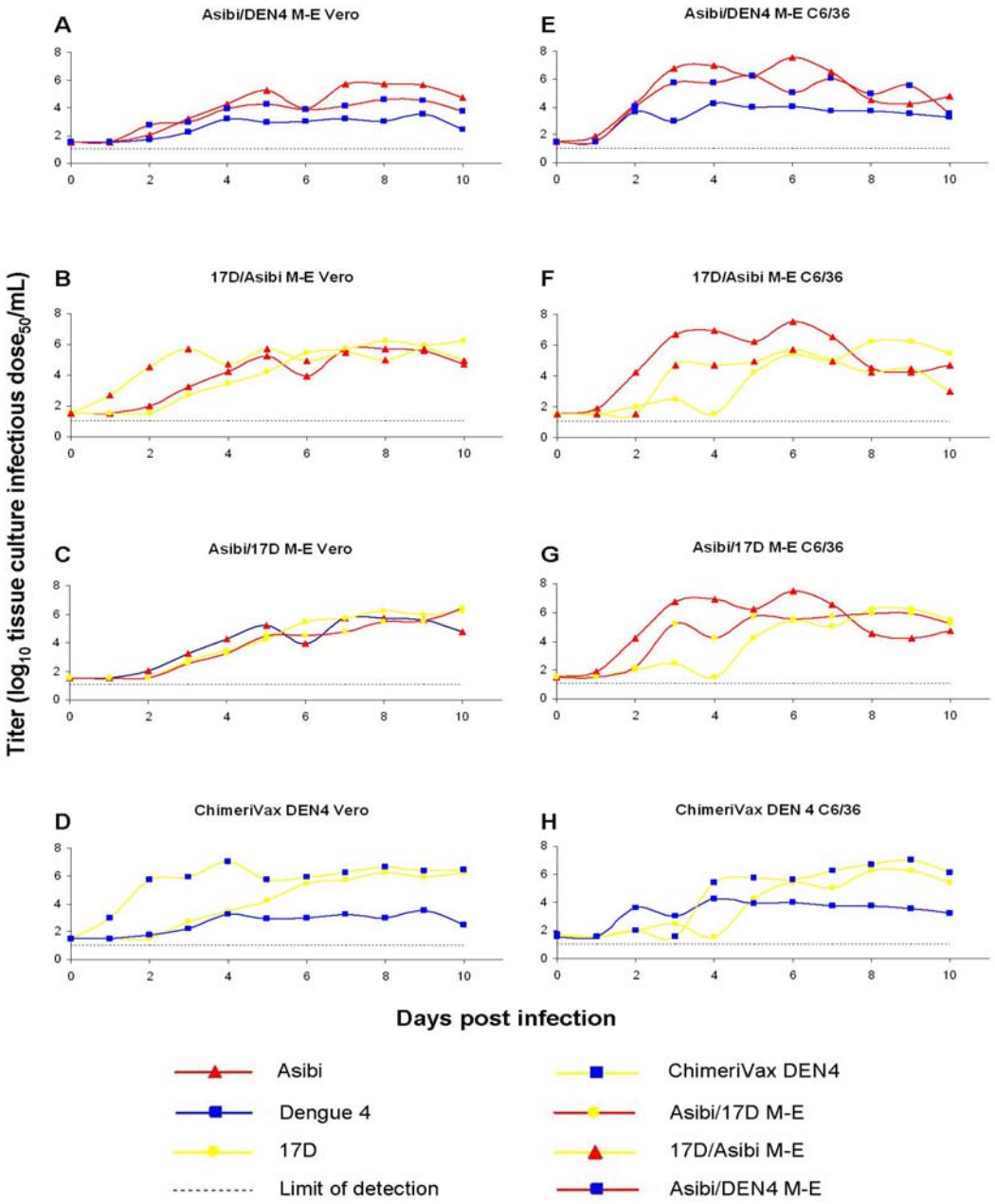


FIGURE 4.3 IN VITRO ANALYSIS OF THE GROWTH KINETICS OF CHIMERIC AND PARENTAL DENGUE AND YELLOW FEVER VIRUSES.
All growth curves represent the average of two replicates, error bars have been removed for clarity. Chimeric flavivirus growth curves are shown in comparison with parental viruses grown in the respective cell type as indicated. **A,E** Asibi/DEN 4 M-E; **B,F** 17D/Asibi M-E; **C,G** Asibi/17D M-E; **D,H** ChimeriVax DEN 4.

infection of salivary gland tissue (Olson *et al.,* 1996). Infection and dissemination rates were calculated as the percentage of positive mosquitoes from the total number tested. Positive and negative control *Ae. aegypti* were included in all virus titration assays.

TABLE 4.3 COMPARISON OF DAYS ZERO AND FOURTEEN POST INFECTION VIRAL TITERS ANALYZED BY TITRATION OF THE BODIES OF AE. AEGYPTI MOSQUITOES.

	Ae. aegypti RexD WE body titer[a]			*Ae. aegypti* Thai F5 body titer[a]		
Virus	Meal titer[b]	day 0 p.i.	day 14 p.i.	Meal titer[b]	day 0 p.i.	day 14 p.i.
Asibi	3.95	1.69 ± .72	3.74 ± 2.13	3.95	1.29 ± .33	3.82 ± 1.61
17D/Asibi M-E	5.52	3.33 ± .33‡	2.84 ± 1.00	5.52	3.28 ± .58	2.38 ± 1.16
Asibi/17D M-E	4.95	2.33 ± .73	1.52	4.95	3.33 ± .33	1.06
Asibi/DEN4 M-E	4.52	3.00 ± .50‡	Negative	4.95	2.81 ± .25†	3.52
ChimeriVax DEN4	3.95	1.58 ± .34‡	Negative	4.52	1.06†	Negative

[a] titers of mosquito bodies are reported as $\log_{10}TCID_{50}$/mosquito
[b]bloodmeal titers are reported as $\log_{10}TCID_{50}$/mL
‡ $p \leq .02$; † $p = .003$
limit of detection = 1.06 $\log_{10}TCID_{50}$/mosquito or mL respectively

Ae. aegypti RexD WE

High levels of infection (7/8) and dissemination (7/8) (**Figure 4.4)** were observed in those mosquitoes infected with virus derived from our Asibi i.c. (prototypical wt YFV) despite relatively low titers noted in the infectious bloodmeals and day zero mosquitoes (3.95 $\log_{10}$ $TCID_{50}$/mL and 1.69 ± .72 $\log_{10}$ $TCID_{50}$/mosquito respectively). Substitution of the YFV 17D structural genes into the YFV Asibi back bone resulted in a significant decrease in mosquito infection (1/8, $p = 0.01$) and prevented viral dissemination (0/8). The reverse substitution, YFV Asibi M-E in the 17D backbone, was associated with an intermediate infection rate (4/8). Substitution of the DEN 4 structural genes into the virulent YFV Asibi backbone, Asibi/DEN 4 M-E (**Figure 4.4)** also resulted in a significant decrease in mosquito infection (0/8, $p \leq 0.01$) as compared to the YFV Asibi wt (7/8). In fact Asibi/DEN 4 M-E displayed comparable mosquito infectivity as

compared to ChimeriVax DEN 4 which was not observed to infect (0/8) or disseminate (0/8) in RexD WE *Ae. aegypti*.

Ae. aegypti Thailand F5

Generally, observations of infection and dissemination kinetics, of YFV variants, were similar in both strains of *Ae. aegypti* (RexD WE and Thailand F5) with no significant differences in infection observed between the two strains for any of the viruses. YFV Asibi infected (6/8) and disseminated (5/8) at a high rate, while substitution of the DEN 4 or YFV 17D structural genes M-E resulted in significant decreases ($p < 0.05$) in mosquito infectivity (Asibi/DEN 4 M-E (1/8) and Asibi/17D M-E (1/8)) (**Figure 4.4)**. 17D/Asibi M-E again displayed an intermediate phenotype characterized by significantly increased ($p = 0.007$) mosquito infectivity (6/8) as compared to ChimeriVax DEN 4 (0/8), but decreased dissemination (1/8) compared to YFV Asibi (5/8) (**Figure 4.4**). Despite the significantly higher average titer observed in day zero mosquitoes ($p = .003$, **Table 4.3**) Asibi/DEN 4 M-E did not have significantly different mosquito infectivity (1/8) as compared to ChimeriVax DEN 4 which did not infect or disseminate in a near-wild Thai strain of *Ae. aegypti*.

Although no significant differences between the infection and dissemination rates of the hypothetical recombinant Asibi/DEN 4 M-E and parental vaccine ChimeriVax DEN 4 were observed. Asibi/DEN 4 M-E infected (1/8) and disseminated (1/8) in a single mosquito exposed to a relatively high titer infectious bloodmeal (4.95 $\log_{10}$ $TCID_{50}$/mL). A significantly lower infection rate (5/8, $p < .05$) than was observed for mosquitoes exposed to a lower titer infectious bloodmeal (3.95 $\log_{10}$ $TCID_{50}$/mL) of YFV Asibi. Since Asibi/DEN 4 M-E is a chimera of sequences derived from two viruses with high mosquito infectivity phenotypes (YFV Asibi (McElroy *et al.,* 2005, McElroy *et al.,* 2006, McElroy *et al.,* 2006, McGee *et al.,* 2008) and DEN 4 1228 (Higgs *et al.,* 2006) see

materials and methods), this result was not entirely unexpected. However, when we consider the low levels of infectious viremia and dramatically attenuated phenotype in non-human primates infected with Asibi/DEN 4 M-E (see below) as compared to the generation of maximal bloodmeal titers using cell culture systems we must conclude that it is extremely unlikely that a mosquito would become infected while feeding on an animal infected with this recombinant.

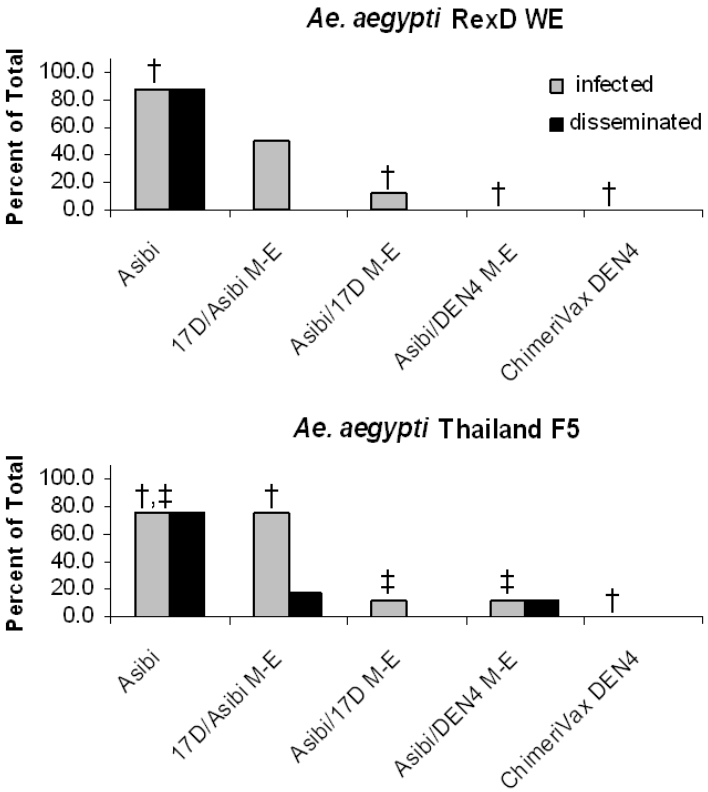


FIGURE 4.4 INFECTION AND DISSEMINATION RATES FOR WILD-TYPE, VACCINE, AND CHIMERIC RECOMBINANT FLAVIVIRUSES IN TWO STRAINS OF *AE. AEGYPTI* MOSQUITOES.
$^{‡}p \leq .05$; $^{†}p \leq .01$

C. Analysis of chimeric recombinant vertebrate pathogenecity in non-human primages (Cynomolgus macaques)

Clinical symptoms and blood parameters

In general, animals in groups I-VI and VIII (PBS, 17D, ChimeriVax DEN 4, DEN 4 1228, 17D/Asibi M-E, Asibi/17D M-E, and Asibi/DEN 4 M-E respectively) displayed no significant reactions to immunizations (noteworthy observations summarized in **Table 4.4**). Clinical parameters for these groups (behavior, weight, food intake, and activity) were within normal limits throughout the study and although anemia, leucopenia,

leucocytosis and increased granulocyte and eosinophil counts were noted, most of these changes could be attributed to the intensity of bleeding schedule (M. Lewis unpublished observations). Three animals (3/4) in group VI (Asibi/17D M-E) that were euthanized at 10 and 14 dpi, displayed minimal to mild liver inflammation, with mononuclear cell infiltrates. One group VIII (Asibi/DEN 4 M-E) animal (1/6) developed peritonitis, bile duct fibrosis, and slightly increased alkaline phosphatase (AP) levels (2306 units/liter (U/L); Reference range 100-277U/L), but this was likely due to experimental manipulations because viral RNA was not detected in liver samples from that animal. PT and APTT were not significantly affected by any of these viruses.

TABLE 4.4 CLINICAL OBSERVATIONS OF NOTE.

Group	Immunization	Clinical Observations	Histopathology	Hematology[b]	Serum Chemistry	Cytokines Modulated	Antibody
I	PBS	All parameters observed to be within normal limits					---
II	17D vaccine	All parameters observed to be within normal limits					+
III	ChimeriVax DEN4	All parameters observed to be within normal limits					+
IV	Dengue 4	All parameters observed to be within normal limits					+
V	17D/Asibi M-E	---	---	---	---	---	+
VI	Asibi/17D M-E	Liver lesion[a] Abdominal bruise[a]	Liver MCI	Abnormal APTT	---	IL-1β, IL-6,	+
VII	Asibi	Liver swelling Jaundice Fatal disease	Hepatocellular DN Acute inflammation Eosinophilic inclusions Spleen, lymph node, kidney DN	↑ PT[c]	↑ ALT ↑ AST ↑ AP ↑ bilirubin Ca:Phos Imbalance	INFα, IIL-6, IL-1β	+
VIII	Asibi/DEN4 M-E	Peritonitis and bile duct fibrosis[a]	---	---	Slight ↑ AP[a]	---	+

ALT alanine aminotransferase; AST aspartate aminotransferase; APTT activated partial thromboplastin time;AP alkaline phosphatase; Ca:Phos calcium phosphate ratio; DDN degeneration, and/or necrosis; MCI mononuclear cell infiltrates; PBS phosphate buffered saline; PT prothrombin time; ↑ increased

[a] – observation occurred in a single animal (1/6) and was likely associated with physical manipulations/surgical procedures

[b] – day 6 and 8 post infection samples for animals in groups XI and XII were lost due to testing failure

[c] – $P \leq 0.1$ compared to PBS control

At six dpi two YFV Asibi (group VII) animals died and the remaining four became moribund and were humanely euthanized. Animals infected with YFV Asibi consistently displayed a constellation of lesions involving the liver, spleen, and lymph nodes that was clearly related to infection. Diffuse hepatocellular degeneration/necrosis with (5/6) or without (1/6) acute inflammation was present in all animals (6/6) and the nuclei of many hepatocytes contained eosinophilic inclusions characteristic of viral replication. Coincident with severe liver pathology in YFV Asibi infected animals were dramatic elevations in alanine aminotransferase (5/6) (3548.2 $\pm$ 49.4 U/L; reference range 0-82 U/L), aspartate aminotransferase (5/6) (5671.6 $\pm$ 66.3 U/L; reference range 14-30 U/L), and AP (6/6) (931.8 $\pm$ 24.7 U/L; reference range 100-277 U/L), calcium:phosphate ratio imbalances (6/6) (7.4 $\pm$ 0.9 : 9.3 $\pm$ 2.0 mg/dL; reference range 9.0-11.8 : 3.5-6.5), and large increases in bilirubin (1/6) (7.9 mg/dL; reference range 0-3.7 mg/dL). Depletion or degeneration/necrosis of the germinal centers of the spleen (6/6), mandibular and mesenteric lymph nodes (5/6), and renal tubules (3/6) were also noted in these animals.

Viremia and viral loads in tissues

Viral RNA was never detected in serum/plasma samples or terminal tissues samples collected from individuals in groups I-IV (limit of detection ($\leq$500 copies/mL and $\leq$50 copies/mg respectively)). Nevertheless, serological analyses of animals in these groups indicated they had been successfully infected with viable viruses (**Table 4.4**). However, this lack of detection did not prevent an extensive analysis of the virulence of the recombinant Asibi/DEN 4 M-E compared to the YFV Asibi i.c.derived virus.

Viral genomes were detected in the plasma of all group V-VIII animals (6/6 per group) by four dpi with average peak titers for 17D/Asibi M-E (5 x 10^6 copies/mL), Asibi/17D M-E (1 x 10^7 copies/mL), Asibi (3 x 10^9 copies/mL), and Asibi/DEN 4 M-E (2

x 10^5 copies/mL) observed between four and eight dpi (**Figure 4.5**). Significant differences in average viremia, F (3, 7.5) = 4.461, $p < .05$) were observed at six dpi (Analysis of Variance, Welch procedure, SPSS 14.0). Viral RNA was also detected at variable titers in various tissues collected at necropsy from most of the animals in Groups V-VIII (**Figure 4.6**), although one group V (17D/Asibi M-E) and two group VIII (Asibi/DEN 4 M-E) animals had no detectable virus in any tissue examined.

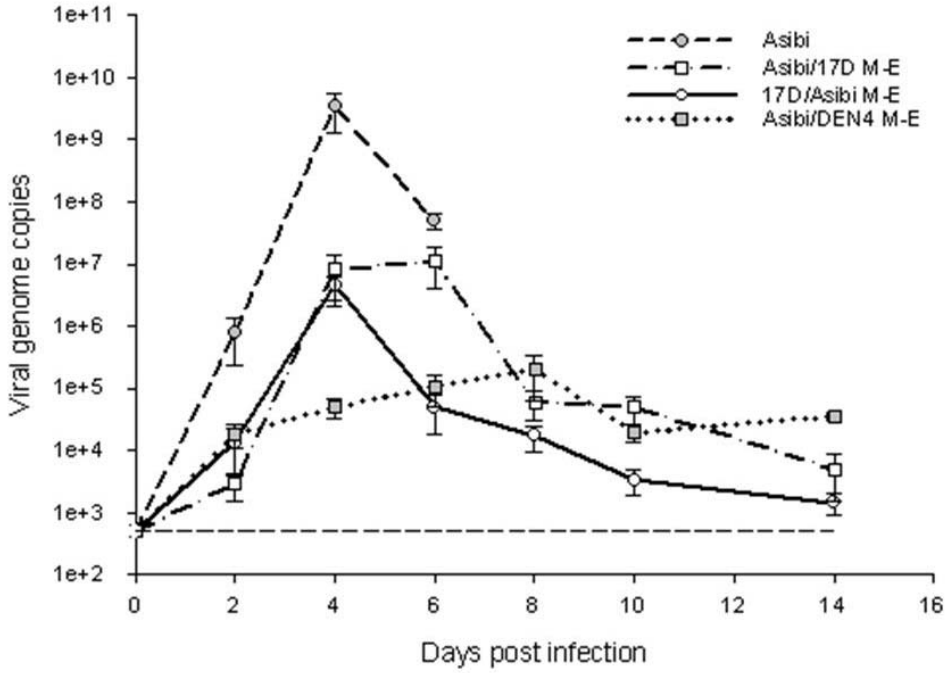


FIGURE 4.5 KINETIC QRT-PCR ANALYSIS OF VIRAL RNA IN PLASMA.
Asibi infected samples are absent on 10 and 14 days post infection as all animals in this group died or were euthanized by seven dpi. Dashed lines indicate lower limit of detection (error bars indicate standard error).

Cytokines

IFNγ levels were below the limit of detection in all groups except for two animals at one time point in groups, I and V. IL-2, TNFα and IL-10 were detected occasionally in most groups but values were within the same range as pre-immunization levels (200-1000 U/mL, 40-950 pg/mL, and 800-1000 pg/mL respectively). IL-6, IFNα, and IL-1β were

the only cytokines consistently induced by infection. Inconsistent or undetectable changes in IL-6, IFNα, and IL-1β were observed in groups I-V and VIII. However, IL-6 levels were increased in all group VI (Asibi/17D M-E) animals at least once between zero and 14 dpi (range 69-218 pg/mL, with one animal presenting values > 1500 pg/mL (upper limit of detection) at six dpi). A somewhat more robust response was observed in group VII (Asibi) animals with moderate (125-617 pg/mL) and high levels (100, 750, and >1500 pg/mL in two animals) of IL-6 detected at four and six dpi respectively. IFNα was increased in group VI animals (4/6) at four or six dpi (194-600 pg/mL), and high levels of IFNα were noted in all group VII animals at four (925-3195 pg/mL) and six dpi (86-860 pg/mL). Analyses performed using Dunn's non-parametric determined IL-6 and INFα were significantly increased by YFV Asibi infection ($p < 0.05$) at four and six dpi as compared to PBS control. Finally, levels of IL-1β were highly elevated in two group VI animals (512 and 1038 pg/mL) at two and four dpi respectively, and in group VII at six dpi (1058 pg/mL).

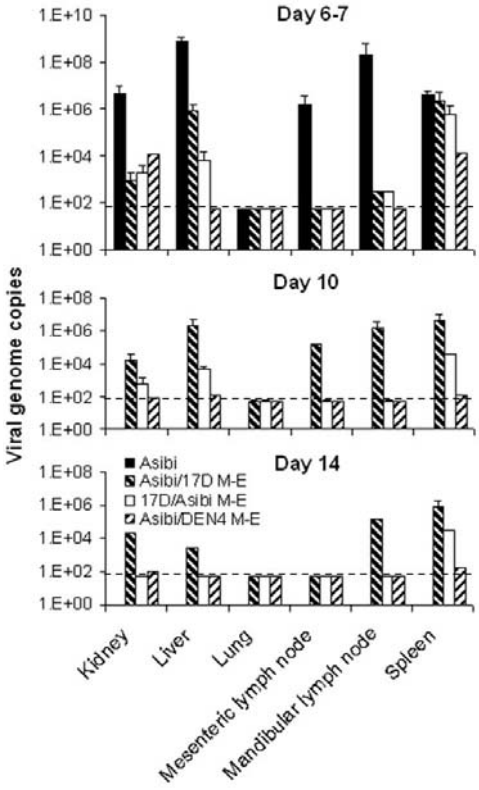


FIGURE 4.6 KINETIC QRT-PCR ANALYSIS OF VIRAL RNA IN SELECTED TISSUE SAMPLES. Asibi infected tissues are absent on 10 and 14 days post infection as all animals in this group died or were euthanized by seven dpi. Dashed lines indicate lower limit of detection (error bars indicate standard error).

Antibodies

All animals that survived beyond, or were not euthanized prior to, six dpi developed detectable levels of (YFV or DENV) binding antibodies, including those from groups developing no viremia (**Table 4.3**).

4.5 DISCUSSION

Recent experimental observations of YFV i.c, chimeras have identified some of the molecular determinants of infection and dissemination in mosquito vectors (McElroy et al., 2006, McElroy et al., 2006). Investigations of the relative contributions of structural and nonstructural genes to the mosquito infectivity phenotype of YFV Asibi have identified major determinants of dissemination in the structural genes specifically E, with the nonstructural (NS2A and NS4B) genes also contributing, albeit to a lesser extent (McElroy et al., 2006, McElroy et al., 2006). Substitution of the corresponding virulent YFV Asibi sequences into the 17D backbone does not necessarily restore high infectivity for *Ae. aegypti* mosquitoes (McElroy et al., 2006, McElroy et al., 2006). These observations support the conclusion that multiple attenuating mutations, distributed throughout the 17D backbone, contribute to decreased mosquito infectivity, therefore supporting its use as a safe platform for the development of heterologous flavivirus vaccines.

In vivo vector competence studies with ChimeriVax DEN vaccine candidates have consistently demonstrated decreased mosquito infectivity similar to the well known phenotype for the parental 17D vaccine virus (Higgs *et al.,* 2006, Johnson *et al.,* 2002, Johnson *et al.,* 2004, McElroy *et al.,* 2006, McElroy *et al.,* 2006, Miller & Adkins 1988, Whitman 1939). Analysis of ChimeriVax DEN 2 in *Ae. aegypti* and *Ae. albopicuts* indicated that this vaccine candidate exhibited comparable mosquito infectivity to the approved parental 17D vaccine and identified no dissemination at 14 dpi despite exposure

to high blood meal titers of 6.3 – 7.7 $\log_{10}$ plaque forming units (pfu)/mL (Johnson et al., 2004). Additionally, significant decreases in ChimeriVax DEN 2 mosquito infection, associated with a loss of dissemination, were observed in a DENV 2 susceptible wild isolate of *Ae. aegypti* collected in Puerto Rico (Johnson *et al.,* 2002). qRT-PCR analysis of ChimeriVax DEN 1, 2, 3, 4 in *Ae. aegypti* also identified attenuated oral infectivity (Johnson et al., 2004). Significant decreases have been identified in the infection and dissemination rates of ChimeriVax DENV when fed to near-wild strains of *Ae. aegypti* and *Ae. albopicuts,* isolated from Thailand, both individually and as a tetravalent mixture (Higgs et al., 2006).

Ae. aegypti mosquitoes experimentally infected with YFV Asibi have been observed to fatally infect monkeys for up to 168 dpi (Philip 1962). Vector competence studies are therefore a critical component of the evaluation of potential LAV candidates, even though, in regions of extreme and continuous YFV activity, as discussed below there are many reasons why productive recombination in the mosquito vector is highly unlikely. Viremias of YFV 17D produced in vaccinees are relatively low and of short duration, and this attenuated strain of virus is poorly infectious to mosquitoes (Barrett & Higgs 2007, McElroy *et al.,* 2006, McElroy *et al.,* 2006, Whitman 1939). Since YFV 17D does not disseminate from the midgut (McElroy *et al.,* 2006, McElroy *et al.,* 2006, Whitman 1939) and its derivative chimeric vaccines exhibit similar losses of dissemination, then if recombination were to occur within a vector it would require co-infection of midgut epithelial cells with both vaccine and wt viruses. Although the mechanism of superinfection resistance (the inability of cells to be sequentially infected by antigenically related viruses) is not fully understood and indeed may be different for different genera of viruses, it has been documented for mosquito-borne viruses (see Chapter 1: Sections 1.3 E & 1.4 E and Chapter 3: Section 3.5) and may further restrict the

potential for recombination. The tendency for *Ae. aegypti* to breed in close proximity to humans, coupled with the high propensity for human feeding (Scott *et al.,* 1993) may also restrict opportunities to feed on multiple different viremic hosts and therefore further reduce the likelihood of dual infection. For these multiple potential hurdles to recombination to be overcome in a vector that has a relatively short average life span of perhaps a few weeks (Christophers 1960) (W. Reisen personal communication), seems highly unlikely.

As observed for 17D, using an artificial IT inoculation method, *Ae. aegypti* became infected with all chimeric viruses. As such, it appears that the block to vector-borne transmission, inherited from the 17D backbone, occurs at the level of the midgut infection/midgut escape (see Chapter 1 Section 1.2 for a comprehensive explanation of potential mechanism(s)). No significant differences were observed in the infection and dissemination profiles of the two strains of *Ae. aegypti* although the near-wild Thailand mosquitoes appear to be slightly more susceptible to infection. Substitution of the wt virulent YFV Asibi sequences into either vaccine backbones (17D or ChimeriVax DEN 4) did not produce a virus with an "Asibi-like" phenotype with respect to mosquito infectivity. Additionally, the mosquito infectivity of Asibi/DEN 4 M-E was similar to previous reports of the infectivity of ChimeriVax DENV 4 for *Aedes* mosquitoes collected from Thailand (Higgs *et al.,* 2006). Although Asibi/DEN 4 M-E did disseminate in *Ae. aegypti* Thai F5, it occurred at a low rate (1/8) despite exposure to relatively high viral titer in the bloodmeals (4.95 $\log_{10}TCID_{50}$/mL), especially when we consider the significantly higher infection rates observed for those mosquitoes exposed to a lower titer infectious bloodmeal of YFV Asibi. Considering: 1) the short duration of viremia (0.4$\pm$1.6 days: 17D and 1.2 $\pm$ 1.42 to 1.9 $\pm$ 1.23 days; ChimeriVax DEN 2); 2) the low circulating titers of vaccine virus expected in vaccinees (44$\pm$116 pfu/mL; 17D

and 20±33 to 50±68 pfu/mL; ChimeriVax DEN 2) (Guirakhoo *et al.,* 2006); 3) the relative refractoriness of *Aedes* mosquitoes to oral infection with ChimeriVax DENV (Higgs *et al.,* 2006); 4) the low mosquito infectivity of 17D/Asibi M-E, Asibi/17D M-E, and Asibi/DEN 4 M-E; and 5) the dramatically attenuated phenotypes of 17D/Asibi M-E, Asibi/17D M-E, and Asibi/DEN 4 M-E in non-human primates (see below) it is concluded that even in the incredibly unlikely event of a complete "virulent" backbone reversion, the risk of mosquito transmission is minimal.

Importantly even if recombination did occur, the substitution of virulent YFV Asibi sequences for homologous vaccine sequences in YFV 17D and ChimeriVax DEN 4 does not result in reversion to vertebrate virulence in non-human primates. ChimeriVax DEN 4, 17D, and DEN 4 induced antibody production and were all well-tolerated with no untoward pathogenesis or replication. Consistent with previous reports for wt YFV Asibi (Hoskins 1935, Monath & Barrett 2003, Schlesinger *et al.,* 1986), virus derived from our YFV Asibi i.c. was uniformly lethal; inducing death or moribund state in all animals by six dpi and hepatic pathologies characteristic of wt YFV infection (Monath & Barrett 2003, Tsai 2000). Disease progression involved classical clinical signs including jaundice, discoloration or yellowing of the face and eyes, indicative of severe liver pathology. Indeed, viral replication in liver tissue induced pathological hepatocytic morphology, acute inflammation, swelling, and dramatic increases in serum liver enzyme levels.

Serum and tissue viral loads in YFV Asibi infected animals progressed rapidly reaching peak titers at four to six dpi with death rapidly following. Although viremia was induced by infections with 17D/Asibi M-E, Asibi/17D M-E, and Asibi/DEN 4 M-E it was dramatically reduced as compared to YFV Asibi and generally clinical and pathological signs were not associated with these infections. Cytokine results were in

agreement with symptomology and viremia intensity: with detectable to high levels of pro-inflammatory (IL-6) and antiviral (IFNα) cytokines observed only in Asibi/17D M-E and YFV Asibi infected animals, consistent with previous observations of severe/fatal yellow fever infections (ter Meulen et al., 2004)

In conclusion, these studies demonstrate that rationally designed "worst-case" recombinants such as Asibi/17D M-E and Asibi/DEN 4 M-E are highly attenuated in both invertebrate and vertebrate hosts. Additionally, given the low viremias in Asibi/DEN 4 M-E infected animals and the estimated ratio of YFV genomes to infectious units (between 1:1000 and 1:5000) (Bae et al., 2003) the likelyhood that an animal infected with a recombinant such as Asibi/17D M-E or Asibi/DEN 4 M-E could perpetuate a transmission cycle via infection of relatively refractory *Aedes* mosquitoes (see above) is extremely low. I therefore conclude that replacement of the 17D vaccine sequences with the homologous YFV Asibi sequences does not compromise the safety of the 17D vaccine or its ChimeriVax derivatives.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

There are distinct evolutionary advantages associated with the ability of RNA viruses to exchange and incorporate segments (modules) of genetic information, via recombination. Recombination is a potential mechanism for recovery from mutations incorporated via replicative error; the so called "Muller's Ratchet" phenomenon (Muller 1964, Worobey & Holmes 1999). Muller (1964) predicted that within an asexual or clonally evolving population there will be a non-recoverable accumulation of deleterious mutations. Considering the relatively high mutation rates of viral RNA dependent RNA polymerases (RdRp), ~10^{-4} per nucleotide per round of replication (deFilippis & Villarreal 2001, Drake *et al.,* 1998, Smith & Inglis 1987, Steinhauer & Holland 1987, Strauss & Strauss 1988), this process can proceed quite rapidly for RNA viruses.

Indeed, the ability of RNA viruses to restore lost or attenuated gene functions via a recombinant mechanism has been repeatedly demonstrated. Examples of these types of recombinant events include: 1) the recovery of proteolytic cleavage/maturation of the Influenza A haemagglutinin gene via incorporation of host ribosomal RNA sequence and the restoration of neuraminidase gene function by insertion of poly-adenylation signal sequences (Bergmann *et al.,* 1992, Khatchikian *et al.,* 1989); 2) restoration of function to non-replicative Sindbis virus (SINV) and poliovirus (Gmyl *et al.,* 1999, Hajjou *et al.,* 1996, Hill *et al.,* 1997, Raju *et al.,* 1995); 3) the recovery of a vertebrate virulent phenotype by oral polio vaccine viruses via vaccine/vaccine and vaccine/wild-type (wt) recombination (Furione *et al.,* 1993, Georgescu *et al.,* 1994, Kew *et al.,* 1981); and 4) recovery of deleted genes/sequences by various plant viruses (Bujarski & Kaesberg 1986, Greene & Allison 1994). Recombination can also allow for the incorporation of beneficial genes from heterologous viruses or of cellular origin that impart new and

diverse functions (Koonin & Dolja 1993, Strauss & Strauss 1988, Worobey & Holmes 1999). In this regard, recombinant driven incorporations of host cellular sequences into the bovine viral diarrhea virus (BVDV) genome has been repeatedly observed to result in changes in viral protein expression and processing associated with increased virulence *in vivo* (Kummerer *et al.,* 2000, Meyers *et al.,* 1991, Meyers *et al.,* 1989, Meyers *et al.,* 1991, Meyers & Thiel 1996).

The existence of highly conserved RdRp sequence motifs (Koonin 1991, Koonin & Dolja 1993, Poch *et al.,* 1989), a conserved catalytic mechanism of RNA polymerization (Butcher *et al.,* 2001, Steitz *et al.,* 1994, Steitz & Steitz 1993, van Dijk *et al.,* 2004), and functional protein elements (capsid, protease, and helicase) (Koonin & Dolja 1993) across all positive sense RNA viruses has led to the hypothesis that these viruses may have all evolved from a single common ancestor (Koonin & Dolja 1993, Strauss & Strauss 1988). Thus it has been postulated that the observed diversity among the currently recognized RNA viruses could be explained by recombination-driven insertion, deletion, duplication, and the rearrangement of exogenous viral and cellular genetic sequences (Koonin & Dolja 1993).

Because all RNA viruses encode a related RdRp it may seem logical to assume that template switching (recombination) would be a conserved molecular mechanism. However, unique evolutionary pressures that have given rise to specific virus families may affect the relative efficiencies of recombination by influencing polymerase fidelity and replicative complex stability. Indeed, picornavirus antiviral escape mutants have been observed to result from a specific mutation in the poliovirus RdRp (glycine-64-serine) which increased polymerase fidelity (Pfeiffer & Kirkegaard 2003, Vignuzzi *et al.,* 2005). This indicates that there is significant selectable plasticity in the viral polymerase. In these studies, it was hypothesized that the gly-64-ser mutation may have resulted in

increased hydrogen bonding or decreased flexibility in the polymerase finger domains, thereby stabilizing the RdRp-RNA template complex. However, the effects of this mutation on recombination efficiency were not investigated.

To date, all studies on RNA virus recombination fall into two categories: 1) comparative phylogenetic analysis of naturally occurring recombinant sequences, or 2) detection of recombinant progeny from experimental cell culture systems using high multiplicities of infection (moi). In both cases, these recombinant genomes observed have undergone extensive *in vivo/in vitro* replication and selection prior to sequence analysis. The nature of these RdRp template switches were then assessed retrospectively so that inferences could be made about the nature of the event which gave rise to the recombinant progeny. The vast majority of the evidence for mosquito-borne flavivirus recombination, generated in this way, has suggested that either genetic exchange occurs via homologous recombination or that these observed homologous recombination events were the result of the constraint on need to reproduce an intact and unmodified ORF. Simply put, if the genetic filter for genome viability requires homologous-like recombination, that is all that will be detected in nature. Homologous recombination of picornavirus oral polio vaccines is a well documented etiology of vaccine associated paralytic poliomyelitis (Furione *et al.,* 1993, Georgescu *et al.,* 1994, Kew *et al.,* 1981). As such, it has been hypothesized that homologous flavivirus vaccine/vaccine or vaccine/wt recombination must be considered as a potentially significant impediment to development of flavivirus live attenuated vaccines (Holmes *et al.,* 1999, Seligman & Gould 2004, Tolou *et al.,* 2001). However, picornaviruses and flaviviruses are separated by at least thousands or perhaps millions of years of evolution (Koonin & Dolja 1993, Strauss & Strauss 1988). Therefore, direct extrapolation of the conditions that favor picornavirus recombination and its consequences with respect to flavivirus vaccine safety

may be an overly simplistic view. Even across the three genera of the family Flaviviridae (*Flavivirus*, *Hepacivirus*, and *Pestivirus*) the hallmarks of recombination appear to be markedly different. Indeed several important differences, discussed below, exist at the molecular and epidemiological levels that may serve to dramatically alter recombination efficiency between picornaviruses and flaviviruses.

It is unarguable that the most important requirement for the generation of a recombinant viral genome is spatial and temporal association of the donor and acceptor templates to allow for polymerase template switching. Simply put, two genetically distinct viruses must be present in the same cell at the same time. A fundamental assumption of laboratory analyses of recombinant RNA viruses, arising from the efficient and reproducible generation of picornavirus recombinants, is that cross-over events occur within an acute, high level, co-infection. The emergence of specific recombinant virus is then driven by the selective advantages and relative viability of any given event (Banner *et al.,* 1990, Banner & Lai 1991, Copper *et al.,* 1974, Hirst 1962, Jarvis & Kirkegaard 1992, Kirkegaard & Baltimore 1986, Lai 1992, Ledinko & Hirst 1961). In cell culture these selective advantages are the result of specific "trait loci" responsible for *in vitro* growth characteristic such as temperature sensitivity (ts) (Cooper 1968, Cooper *et al.,* 1971, Hirst 1962, Kirkegaard & Baltimore 1986, Ledinko 1963, Ledinko & Hirst 1961) while virulent reversions are selected *in vivo* (Becher *et al.,* 1996, Furione *et al.,* 1993, Georgescu *et al.,* 1994, Kew *et al.,* 1981, Kummerer *et al.,* 2000, Meyers *et al.,* 1991, Meyers *et al.,* 1991, Meyers & Thiel 1996). However, similar experimental crosses, by others, using full length alphaviruses containing ts mutations did not result in the generation of detectable recombinants (Atkins *et al.,* 1974, Burge & Pfefferkorn 1966, Pfefferkorn 1971).

I hypothesized that alphavirus reverse genetic techniques could be used to engineer distinct sequence markers into the chikungunya virus (CHIKV) genome that would facilitate the detection of full length recombinant viruses. It was assumed, based on previous observations in our laboratory, that CHIKV replicon/defective helper systems were capable of recombination; at least with regard to the inter-genic sequence (K. Tsetsarkin unpublished observations). However, the specific conditions for cell culture purification of viable CHIKV recombinants had to be empirically derived. In order to duplicate some of the highly desirable characteristics observed in picornavirus recombinant crosses, I developed various replicon/defective helper systems to facilitate the detailed analysis of RNA arbovirus recombination.

The first of these recombinant crosses involved the engineering of 5' and 3' frame shift mutations into the CHIKV structural open reading frame. These replicon genomes, CHIK-LR-5'Δ (with and without GFP) Rep and CHIK-LR-3'Δ (with and without Cherry) Rep, were generated using the CHIKV LR2006 OPY1 reverse genetics systems previously described by Tsetsarkin *et al.,* (2006). Because these manipulations abrogated the ability of these genomes to express the CHIKV structural genes, viral genomes would only be released from infected cells if a recombination event has occurred. Therefore I hypothesized that co-infection of BHK-21 cells with these genomes would result in recombination between the homologous sequence (~1.0Kb) located between the two sequence deletions.

These genomes were transfected into BHK-21 cells via electroporation. Electroporation was chosen as the method to facilitate high moi co-infection because it avoids the potential problems associated with packaging the manipulated RNAs into virus like particles (VLP) such as; potentially low titers, recombination between replicons and defective helper genomes, and the potential need to agglutinate particles to achieve

efficient co-infection (Abel 1962, Brawner & Sagik 1971, Kirvaitis & Simon 1965). The genome organizations of my first generation alphavirus replicons, CHIK-LR-5'Δ Rep and CHIK-LR-3'Δ Rep, were also desirable because the generation of a viable full length recombinant would require an intra-open reading frame (ORF) cross-over event as would be the case for successful flavivirus recombination. As such, I hypothesized that optimization of the conditions for the detection of intra-genic alphavirus recombination would provide general insights that could be reasonably extrapolated in the design of an experimental flavivirus system.

The rationale that CHIKV would be capable of intra-genic recombination was based on the fact that western equine encephalitis virus (WEEV) is a naturally occurring intra-genic recombinant between "Sindbis-like" and "eastern equine encephalitis-like" viruses (Hahn et al., 1988). Therefore, the existence of a naturally occurring intra-genic alphavirus recombinant argued strongly that such events may influence alphavirus evolution in nature and should be able to be quantified in the laboratory. Preliminary experiments using co-electroporation of CHIK-LR-5'Δ Rep and CHIK-LR-3'Δ Rep failed to detect any viable full length recombinant virus. However, it is possible that recombinants may have been present in these experiments, but the frequency of the ability to generate an effective recombination event was lower in frequency than the loss of the gene of interest, a selective pressure which was avoided in later experiments via using only genomes which did not code for reporter genes. Interestingly, co-electroporation of either CHIK-LR-5'Δ GFP Rep or CHIK-LR-3'Δ Cherry Rep with an in-frame capsid (C) deleted defective helper (CHIK-LR-E2/E1-Help) genome produced self-limiting infectious foci which were originally thought to be representative of CHIKV recombination. However, detailed analysis of co-electroporation and cell culture passage of CHIKV replicons tagged with Cherry and defective helpers tagged with GFP provided

convincing evidence that these foci and the previous observations of K. Tsetsarkin (unpublished) were the result of co-packaging of segmented genomes and not true recombination.

The first convincing phenotypic evidence of recombination I observed, in the experiments described in this dissertation, involved the generation of bi-colored CHIKV infectious foci following co-electroporation of CHIK-LR-5'-GFP-2A-Help and CHIK-LR-Cherry-Rep2. In all previously reported instances of laboratory-generated alphavirus full length recombinants, the structural cassette was incorporated into the replicon genome along with its native subgenomic promoter (Pushko *et al.,* 1997, Schlesinger & Weiss 1994, Weiss & Schlesinger 1991). Therefore I hypothesized that if GFP were fused into the structural ORF under the control of a single subgenomic promoter, then it would be translocated with the subgenomic functional element upon recombination. If a defective helper genome with this gene topology were crossed with a replicon genome competent for the expression of a Cherry fluorescent protein, the recombinant progeny would be able to simultaneously express both fluorescent proteins from a single covalently linked genome. I hypothesized that if such a recombinant genome were generated all infected cells within any given foci would be positive for both fluorescent proteins. This would enable easy differentiation between foci generated by recombination and foci generated by co-packaging; the latter having a characteristic "bulls-eye" appearance (**Figure 5.1 A**).

The "bulls-eye" phenotype is a result of the single cell at the center of the foci being infected with both the replicon (Cherry) and defective helper (GFP) genomes and releasing only replicon-containing VLPs which form a Cherry only halo. Indeed, a population of full length recombinant CHIKVs that expressed both GFP and Cherry were clonally isolated following co-electroporation and just a single amplification in C6/36

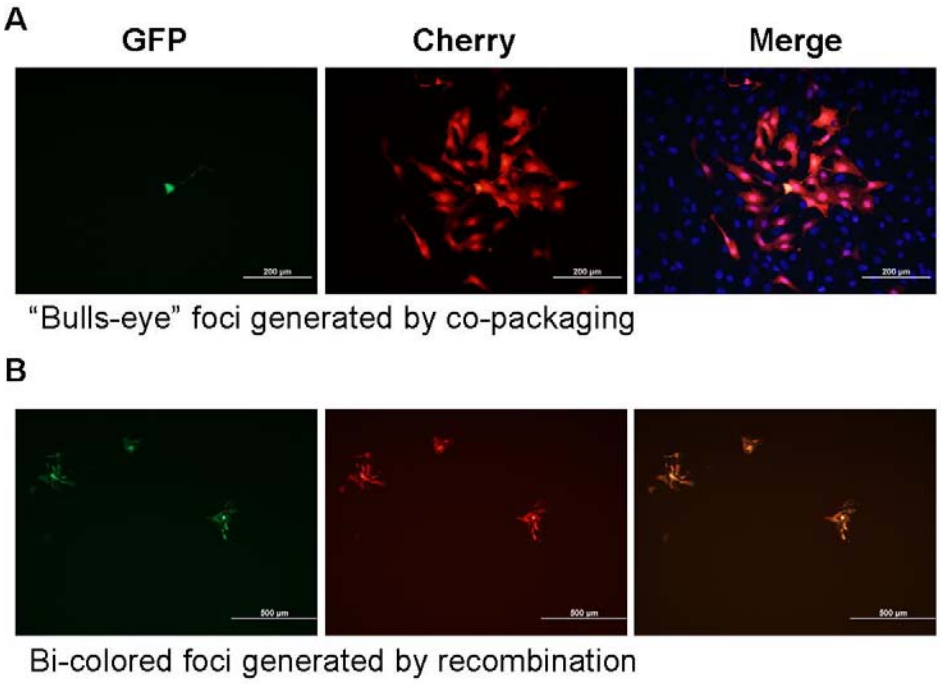


FIGURE 5.1 COMPARISON OF FOCI PHENOTYPES GENERATED BY REPLICON/DEFECTIVE HELPER CO-PACKAGING AND BI-COLORED RECOMBINANT CHIKUNGUNYA VIRUSES

A, Infectious replicon foci generated by co-infection of the center "seed-cell" with both Cherry replicon and GFP defective helper genomes. Note that only the center cell is positive for both fluorescent proteins. B, Three foci generated by a bi-colored CHIKV recombinant genome. Note how all cells in these foci are positive for both fluorescent proteins.

cells. These Cherry-GFP recombinants were characterized by having a full-length-like size genome by Northern blot analysis and were infectious to *Ae. albopictus* mosquitoes. The exogenous gene sequences in these recombinants were highly unstable with loss-of-reporter mutants being detected following passage in cell culture. This instability precluded sequence analysis of putative cross-over regions nevertheless, this system allowed me to establish optimized protocols for the purification and characterization of putative recombinants for all future crosses.

I next conducted a comparative analysis of inter vs. intra-genic recombination, using CHIKV. Data generated from these studies indicated that when the constraints of

coding frame maintenance and functional protein expression exist, then the efficiency of generation of viable, detectable, recombinants decreases ~100-fold. This was evidenced by the fact that inter-genic recombinant CHIKV could be isolated when as few as 10^3 cells were co-infected, while a co-infection level of 10^5 was required to generate recoverable intra-genic recombinants. Intra-genic recombination was characterized by aberrantly homologous recombination which resulted in sequence duplications of the C and E3 coding region despite 100% sequence identity between the two parental genomes. In fact, homologous recombination was not observed in any of the alphavirus experimental crosses I performed. One Sindbis virus (SINV) inter-genic recombinant isolate did exhibit "near homologous" recombination characterized by a duplication of only 26nt of inter-sequence. However, I cannot exclude the possibility that this organization arose by post-recombination evolution from the double subgenomic recombinant topology. It should be noted that double subgenomic SINV recombinants were also isolated from this sample. Indeed, recombinant CHIKV isolated from a replicon/defective helper cross containing intra-genic in-frame deletions was highly unstable, with evidence of reductive evolution (loss of duplicated sequences) immediately following clonal isolation. These in-frame deletion recombinants were observed to completely revert to the native CHIKV wt sequence following five serial passages in Vero cells. Although I observed homotypic recombination under all of the experimental conditions evaluated (with the exception of the dual helper negative control system), the selection of release and re-initiation sites appeared to be random and could not be consistently correlated with any predictable secondary structural elements or sequence homologies.

Furthermore, I observed heterotypic alphavirus recombination (i.e. between different alphavirus species) to reproducibly result in the generation of viable and stable

CHIKV/SINV and SINV/CHIKV (nonstructural genes/structural genes). These recombinants were characterized by cross-over events occurring within the inter-geneic sequences. However, it should be noted that CHIKV and SINV did not display reciprocal abilities to utilize each others regulatory sequences. As such, it appears that only certain recombinant genome topologies are likely to be viable and produce infectious virus. Therefore, these types of heterotypic compatibilities must be evaluated on a case-by-case basis. Thus it appears that under the specific experimental conditions I describe in this dissertation, homotypic and heterotypic recombination of alphaviruses was a highly favored and promiscuous process whereby viable non-homologous recombinants were readily generated following acute infections of two compatible genomes.

Using these data as a model, I designed similar experiments to evaluate the potential for the yellow fever virus (YFV) 17D vaccine to undergo homotypic recombination. 17D replicon genomes containing complementary in-frame deletions in the envelope (E) gene coding sequence (17D 5'Δ E and 17D 3'ΔE) were designed and constructed. I hypothesized that only a recombination event occurring within the homologous overlap sequence located between these two deletions would generate a functional E protein and result in the release of infectious recombinant progeny virus. I was unable to detect recombinant 17D following co-electroporation of these replicon genomes, despite relatively high levels of co-infection (~10^6 BHK-21 cells and ~10^5 $C_7$10 cells) and a lower limit of detection of ~5 primary infections. A similarly designed study, described by Taucher *et al.,* (2009), also failed to detect recombinant tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) (Taucher *et al.,* 2009). Furthermore, recent analyses of YFV 17D *trans*-complementing pseudoinfectious genomes also failed to detect the generation of any viable recombinant full length virus despite sustained serial passage (Shustov & Frolov 2010, Shustov *et al.,* 2007). It should

be noted, however, that specific sequence manipulations were engineered into the YFV 17D genomes used by Shustov *et al.,* (2007; 2010) to decrease sequence homology specifically with the goal of prevention of recombination. Although the study by Taucher *et al.,* (2009), did not result in TBEV or WNV recombination, it did provide the first experimental evidence of the generation of viable flavivirus recombinants under laboratory conditions using a Japanese encephalitis virus (JEV) replicon/replicon "recombination trap" (Taucher *et al.,* 2009). In these studies, persistent high level co-infection, via serial passage in BHK cells, of *trans*-complementing JEV genomes was required to yield full length virus (Taucher *et al.,* 2009). JEV recombinants isolated from this system were aberrantly homologous and contained sequence duplications. Cross-overs were observed to occur within the NS1 gene resulting in duplications of NS1 and both structural cassettes including their in-frame structural deletions (**Figure 5.2 B**). Analysis of the attenuated *in vitro* growth characteristics of these recombinants as compared to wt suggested that under a natural co-infection these recombinants would not be fit for selection relative to the parental viruses.

The lack of recombination of YFV using 17D in my studies as compared to JEV may be due to differences between the topologies of our replicons and those used by Taucher and coworkers (**Figure 5.1 A**). The 17D replicons described in this dissertation contained specific internal deletions of E coding sequences while JEV replicons described by Taucher *et al.,* (2009) contained major deletions of different proteins, C and E. Thus, while my system required recombination to occur within the coding sequence of a single protein, the JEV system employed by Taucher *et al.,* (2009) allowed for expression of both truncated and full length C and E from a single covalently linked genome (**Figure 5.2 B**). As such, it is possible that the efficiency of generating a viable recombinant within the 17D system was much less than that using the JEV system. A

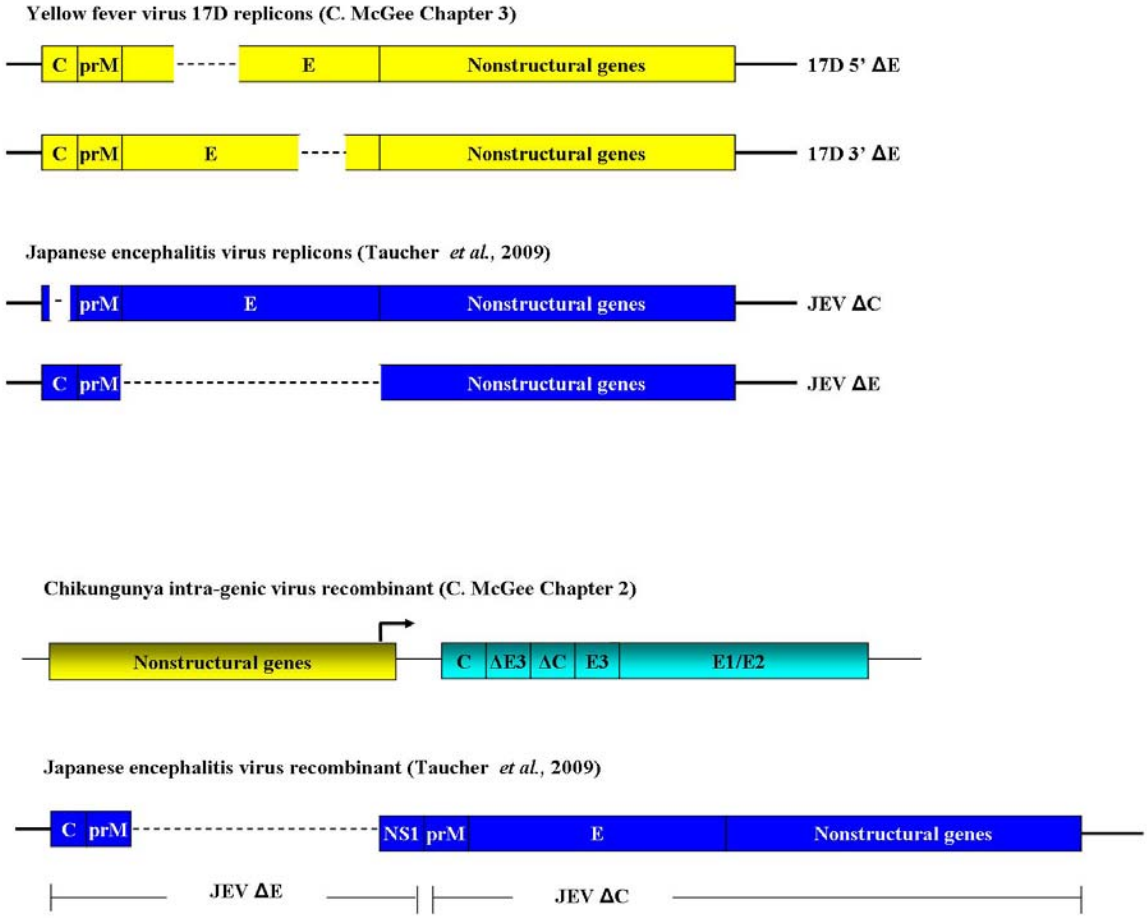


FIGURE 5.2 SCHEMATIC REPRESENTATION OF REPLICONS AND RECOMBINANT GENOME TOPOLOGIES.
A, Schematic representation of our yellow fever virus 17D recombinant cross and the Japanese encephalitis virus (JEV) recombinant cross used by Taucher *et al.*, 2009. B, Comparison of recombinant genome topologies of chikungunya intra-genic recombinants and JEV recombinants.

second possible explanation is that specific species of mosquito-borne flaviviruses may simply have different propensities to participate in recombination, indeed WNV and TBEV systems designed using similar topologies to the JEV recombinant cross did not generate viable recombinant virus. Furthermore, it is possible that the ability for different flavi/alpha viruses to undergo recombination and/or for recombinants to be detected may be highly influenced by the specific cell culture conditions employed. Interestingly, even

given its apparent propensity to recombine, the generation of JEV recombinants required three to five passages in cell culture. These data suggest that the efficiency of flavivirus recombination may be very low and in fact may require long-term sustained or persistent co-infection to allow for sporadic template switching to occur. Although such infections are known to occur for pestiviruses, via an immunotolerance mechanism, they appear to be unlikely for flaviviruses because of the acute nature of flavivirus infection in vertebrate hosts.

Distinct similarities in post-recombination genome topologies can be observed for CHIKV intra-genic recombinants reported in this dissertation with those of JEV recombinants as reported by Taucher *et al.,* (2009) (i.e. both contained aberrantly homologous duplications of truncated structural genes (**Figure 5.1 B**)). This supports my ability to extrapolate between alphavirus and flavivirus systems to draw general conclusions regarding RNA arbovirus recombination. Thus flavivirus recombination, like alphavirus recombination, when examined in the laboratory, appears to occur by non-homologus or aberrantly homologous template switching. This is perhaps not surprising giving that the likelihood of polymerase disassociation and re-initiation at specific homologous sites would require a high level of precision and perhaps facilitation by accessory proteins, none of which have been identified. However, reports of naturally occurring mosquito-borne flavivirus and alphavirus recombinants suggest that these viruses undergo homologus recombination in nature.

A critical analysis of the phylogenetic reports of DENV recombination suggests that not only is recombination occurring in nature, but that multiple completely homologous polymerase jumps are possible, and can result in the generation of mosaic DENV genomes (Chen et al., 2008, Perez-Ramirez et al., 2009, Tolou et al., 2001, Twiddy & Holmes 2003). In fact, one report by Chen et al., (2008) has identified a

DENV1 genome with three recombinant regions coincident with six homologous polymerase template switches. I propose here that the assertion that a flavivirus can undergo multiple homologous template switches and produce a recombinant progeny that is viable during a single co-infection represents a completely naïve view with respect to the nature of the molecular interactions involved. Working with CHIKV which seems highly amenable to recombination, or to a significantly disrupted gene order that allows for a much lower frequency recombination event to produce viable progeny, I have quantified the rate of intra-genic recombination to require at least 105 co-infected cells to facilitate a single template switching event. If we were to extrapolate the conditions required to achieve six polymerase jumps, even if the nature of those switches (homologous vs. nonhomologous) is disregarded, then ~1030 co-infections would be required. Given that the average human is only comprised of ~1013-1014 cells (Alberts et al., 2002) the possibility of even a double homologous template switch during acute replication is highly unlikely.

The central goal of the studies I described in this dissertation was to gain a more complete understanding of the potential for, and consequences of, flavivirus recombination under the auspices of live attenuated vaccine safety. Flavivirus recombination had not been previously quantified under laboratory conditions when these studies were initiated. I therefore designed a parallel set of studies using alphavirus reverse genetics, because of their documented propensity to undergo recombination (Hahn *et al.,* 1988, Pushko *et al.,* 1997, Schlesinger & Weiss 1994, Strauss & Strauss 1997, Vasilakis *et al.,* 2003, Weiss & Schlesinger 1991). Alphaviruses therefore appeared to be an ideal system to facilitate quantification of the parameters and sequences involved in positive sense single stranded RNA mosquito-borne virus recombination. I hypothesized that these alphavirus experiments would allow for the

optimization of cell culture techniques for the purification of putative recombinant viruses and allow for inferences to be made regarding the relative efficiency of various types of sequence to participate in recombination. The results of my experiments, coupled with analyses by others of naturally occurring instances of flavivirus co-infection and recombination and the phenotypes of rationally designed "worst-case" experimental recombinants, both described here and by others, allow inferences to be made about the natures of recombination in general and more specifically about flavivirus recombination.

In an attempt to reconcile the paradox observed between the nature of primary JEV recombination events, as observed by Taucher *et al.,* (2009), with the phylogenetically supported observation of homologus recombination in nature, criteria for the establishment of a co-infection circumstance capable of facilitating the generation, selection, and emergence of flavivirus recombinants are proposed. A critical analysis of the collective knowledge from genera: *Pestivirus*, *Hepacivirus*, and *Flavivirus* suggests that recombinants, within this family, arise from a multi-step mechanism characterized by sequential non-homologous cross-overs followed by post-recombination-selection and reductive evolution. This persistent co-infection can occur either within a single organism (as is the case for pestiviruses and hepaciviruses) or via sustained co-transmission as is proposed here for flaviviruses. This reductive evolution, loss of duplicated sequences, can be rapid, for example progeny of the CHIK-LR-Cap-Rep/CHIK-LR-E2/E1-Help recombinant cross reported in this dissertation were observed to completely revert to the CHIKV wt sequence after five passages in Vero cells. In fact, instability of these recombinants was suggested by Northern bolt analysis immediately following primary isolation.

Strong support for this persistence model to facilitate recombination has been provided by the pestiviruses and hepaciviruses. Viruses belonging to these genera are

known to cause persistent infection coincident with sustained replication in cattle and humans respectively. Interestingly, recombination-driven generation of cytopathic BVDV occurs only sporadically in persistently infected populations (Baker 1987). If the specific host gene incorporations that result in a cytopathic phenotype were driven by some specific sequence characteristics or accessory proteins, as would be expected for homologous recombination, then we would expect these genomes to be generated with some regular frequency. As this seems not to be the case, then it is much more likely that aberrant recombinants are frequently generated during the persistent replication of BVDV. However, only those rare organizations which are fit enough to compete against the parental genomes are detected.

Within the hepaciviruses recombination takes the more classic form of intra and inter-genotypic cross-over events. In this case the epidemiology of Hepatitis C virus (HCV) serves to set the stage for recombination. The lack of HCV cross protectivity to homologous and heterologous genotypes has been documented in non-human primates (Farci *et al.,* 1992, Prince *et al.,* 2005). Sero analysis of intra-venous drug users (IVDU) also indicated that ~10% demonstrated evidence of previous exposure with different serotypes/genotypes. Given the lack of protection to superinfection, and the probability for high risk groups such as IVDU to sustain multiple exposures, it is not difficult to imagine a scenario with humans persistently co-infected with multiple types. Under such conditions, recombination would likely be favored and the existence of sustained replication of the parental genomes could provide the viral nonstructural genes in *trans* to allow for replication and post-recombinant evolution of aberrant recombinants. Indeed phylogenetic analysis of the most well-characterized HCV recombinant, RF1_2k/1b, suggested this virus has been circulating in human populations for >50 years. As such, it is possible that the previously identified homologous hepacivirus recombinants are the

progeny of non- or aberrantly homologus cross-overs which when analyzed retrospectively appear to have resulted from homologous events.

Mosquito-borne flaviviruses on the other hand, typically have a life-cycle comprised of acute high titer replication in a vertebrate host, followed by death or immunity, and persistent low level non-cytopathic replication in the vector. Because vectors may not persist in nature for more than a few weeks (Christophers 1960) (W. Reisen personal communication) it is difficult to imagine how this short term persistence could contribute to recombination. Given the abundance of phylogenetic reports of DENV homologous recombination, the DENV will be used as an example of how sustained co-transmission following dual acute infections could establish persistent co-infection and allow for non-homologous recombination and evolution.

Although it has been demonstrated that mosquitoes may feed on multiple hosts during one gonadotropic cycle (Gould *et al.,* 1970, Molaei *et al.,* 2008) it is unlikely that a single mosquito which may feed on a few hosts would ingest two distinct viruses. Simply because, *Ae. aegypti*, the predominant peridomestic vector of the DENV, tends to remain within a single house (Gould *et al.,* 1970) where all infected humans would likely be infected with the same virus and even when multiple viruses have been documented to co-circulate the incidence of each virus has been relatively low ($\leq$60 confirmed cased per virus per month) (Adams *et al.,* 2006). However, given the potential human attack rates of 80-90% (Calisher 2005), the documented ability for multiple distinct DENV to co-circulate (Adams *et al.,* 2006, Twiddy *et al.,* 2002), and the potential for thousands of mosquitoes to feed on a single vertebrate host in a relatively short time period (Higgs *et al.,* 2005), the possibility for a human to become dually infected with distinct genotypes/serotypes during epidemic transmission seems more likely. Indeed cases of humans dually infected with distinct DENV serotypes have been repeatedly reported

(Araujo *et al.,* 2006, dos Santos *et al.,* 2003, Gubler *et al.,* 1985, Laille *et al.,* 1991, Wang *et al.,* 2003, Wenming *et al.,* 2005)

Although superinfection resistance was reported both in this dissertation using YFV Asibi and 17D variants encoding distinct reporter cassettes, and by others (Dittmar *et al.,* 1982, Igarashi 1979, Pepin *et al.,* 2008, Sabin 1952, Zou *et al.,* 2009), these studies are generally performed in cell culture where complete infection with the primary virus is established before the second is introduced. As such these results may not be directly translatable to the organismal level because: 1) It is unlikely that all the cells within a given individual would be infected by the primary virus and as such some cell population would exist that would be susceptible to secondary infection; and 2) co-infection in nature could theoretically occur during a very narrow time frame thus not allowing for establishment of a refractory state. Indeed, in many instances where superinfection has been reported a time dependent increase in exclusion has been noted (Beaty *et al.,* 1985, Borucki *et al.,* 1999, Davey *et al.,* 1979, Dittmar *et al.,* 1982, Peleg 1975, Pepin *et al.,* 2008). Under these circumstances it could be extrapolated that a single infected individual could mount sustained viremia of two distinct viruses sufficient to achieve dual infection of subsequently feeding mosquito vectors.

Support for the ability of two distinct DENV to achieve high titer replication was provided by a recent study of DENV 2/DENV 4 *in vitro* mixed infections. This study demonstrated that provided superinfection occurred within 6h both the primary and secondary virus were observed to replicate to titers >6.5 $\log_{10}$ pfu/mL (Pepin *et al.,* 2008). Based on analysis of DENV 1-4 infectivity in *Ae. aegypti* and *Ae. albopictus* (Higgs *et al.,* 2006), the titers observed by Pepin *et al.,* (2008) would have been sufficient to infect both species following oral exposure. This scenario allows for the establishment of persistent dual infection with distinct DENV via two potential mechanisms; 1)

sustained dual transmission between susceptible vertebrates and vectors or 2) persistent transovarial transmission within the vector population as has been observed to occur for DENV 3 (Joshi *et al.,* 2002).

One inherent assumption of the ability to establish persistent dual infection is that both viruses display high vertebrate and mosquito infectivities to facilitate sustained co-transmission. After persistent dual infection is established, then multiple non-homologous recombination events could theoretically occur to generate populations of recombinants that could be sustained by *trans*-complementation from their parental viruses. Various mechanisms of purifying selection, such as the need to replicate in two phylogenetically distinct hosts, and post-recombination reductive evolution may then function in concert to generate these phylogenetically identified mosaic recombinants. Support for persistent infection to generate apparently "homologous" DENV recombinants resulting from long term co-transmission has been provided by the repeated isolation of parental and recombinant variants from a single host (Aaskov *et al.,* 2007, Craig *et al.,* 2003, Perez-Ramirez *et al.,* 2009). A correlate of this model would be the potential for the use of defective genomes as "shuttles" for the evolution of genetic sequences and gene arrangements which could then be recombined into full length viral genomes. This could represent a potential mechanism by which flaviviruses may circumvent the problems associated with recombination such as lethal frame shifts and dysfunctional arrangements. A defective genome could tolerate an infinite number of events while being carried along in the population by *trans*-complementation.

An interesting correlate of flavivirus recombination via persistent dual transmission is how the introduction of live attenuated vaccines into regions of endemic/epidemic wt flavivirus transmission may effect the generation of naturally occurring recombinants. An important fact is that flavivirus candidate vaccines such as

YFV 17D and the ChimeriVax DEN viruses produce short duration viremias coincident with low circulating titers (Guirakhoo *et al.,* 2002, Guirakhoo *et al.,* 2004, Guirakhoo *et al.,* 2006). Furthermore, the attenuated phenotypes of these viruses in *Aedes* mosquito vectors (Higgs *et al.,* 2006, Johnson *et al.,* 2002, Johnson *et al.,* 2004) indicates that the potential for vector infection is very low, even after oral ingestion of artificially generated high titer bloodmeals. The potential for a person to be simultaneously infected with a wt virus and experience an adverse event post-vaccination allowing for both viruses to simultaneously replicate to high titers has been estimated at 1 per 300 million vaccinations (Monath *et al.,* 2005). Additionally, superinfection of YFV Asibi (virulent) infected non-human primates with an attenuated stain of YFV (neurotropic) protected these animals from lethal disease, suggesting that in the unlikely event that co-infection were to occur, then it would have an ameliorating effect on viral replication and disease.

Even if dual infection of wt and vaccine strains were to occur, and subsequently feeding vectors did become infected with a vaccine virus, it is extremely unlikely that such a cycle would be maintained long enough to allow for recombination and subsequent selection to occur. These conclusions are supported by the fact that no post-vaccination transmission cycles have ever been described for the YFV 17D vaccine. It is proposed here that if the "persistence" model of flavivirus recombination is true, then the responsible use of vaccines and mosquito control strategies should serve to limit the generation and emergence of naturally occurring flavivirus recombinants by interruption of natural co-circulation via establishment of herd immunity. Support for vaccine-dependent prevention of recombination is provided by the lack of evidence for YFV recombination. This is despite the fact that at least seven distinct genotypes of wt YFV have been identified (Barrett & Higgs 2007, Chang *et al.,* 1995, Mutebi *et al.,* 2001). The lack of epidemic spread of wt YFV, presumably due to the combined efforts of the

17D vaccine and vector control strategies, has kept genotypically distinct YFVs geographically isolated (Twiddy & Holmes 2003) thereby precluding the possibility for co-infection and recombination.

Regardless of the mathematical improbability, it may be theoretically possible that flavivirus wt/vaccine recombination could occur simply by virtue of the fact that both viruses are replication competent. We therefore designed experiments to examine the phenotypes of rationally designed "worst-case" theoretical recombinants (Chapter 4). All chimeric recombinants evaluated 17D/Asibi M-E, Asibi/17D M-E, and Asibi/DEN 4 M-E were replication competent in both vertebrate (Vero and BHK-21) and arthropod cells (C6/36). All recombinants replicated to $\geq$5 $\log_{10}TCID_{50}$/mL in at least one cell type, with some viruses replicating either more rapidly or to higher titer than their respective parental viruses *in vitro*. However, all of these viruses were observed to have significantly impaired mosquito infectivity and primate pathogenicity, despite exposure to relatively high titers of infectious inoculum. Thus I concluded that if a recombination-driven "virulent" reversion were to occur, then the most likely resultant organizations, based on the collected knowledge of flavivirus chimeric investigations, would not be sufficiently infections/virulent to allow for establishment of transmission cycles or human disease. This conclusion was further supported by similar study in which significant *in vitro* and *in vivo* attenuations were observed for a "worst-case" JEV (prM-E)/Kunjin recombinant (Pugachev *et al.,* 2007)

One major question remains: How do we explain the significant differences in the relative efficiencies and resultant genome organizations of picornavirus and flavivirus recombination? Given that these viruses have similar coding strategies, a single stranded positive sense genome comprised of a single ORF, it may seem logical to assume that they would have similar rates of recombination. However, a comparative analysis of the

RdRp and the relationships between replication and protein expression of these two distinct virus groups may provide significant insight into explaining the extreme permissiveness of picornavirus and relative refractivity of flavivirus recombination.

The overall structural conformation of the picornavirus and flavivirus RdRp resembles a right hand, comprised of palm, finger, and thumb domains (see Chapter 1: Section 1.1B). However, despite this conserved topology, significant differences exist that effect the specific mechanism of initiation of RNA replication for distinct virus groups. Picornavirus initiation of RNA synthesis is primer-dependent in that it requires a hydroxyl group that is provided by the (22-24 amino acid) VPg protein to allow for formation of a phosphodiester bond with the first nucleotide (Paul *et al.,* 1998, van Dijk *et al.,* 2004). To accommodate this protein, the picornavirus RdRp contains an open and unobstructed active site coincident with a relatively short thumb domain (**Figure 5.3 A**) (Ferrer-Orta *et al.,* 2006, Gruez *et al.,* 2008, Hansen *et al.,* 1997, Lescar & Canard 2009, Paul *et al.,* 1998, van Dijk *et al.,* 2004). In contrast the thumb domain of the flavivirus RdRp contains elongated motifs which interact with the finger domains and serve to restrict the template binding cleft (**Figure 5.2 B**) (Bressanelli *et al.,* 1999, Butcher *et al.,* 2001, Choi & Rossmann 2009, Lescar & Canard 2009, Malet *et al.,* 2007, van Dijk *et al.,* 2004, Yap *et al.,* 2007) resulting in a more condensed/restricted active site.

Comparative analysis of the HCV and poliovirus RdRp indicated that although poliovirus can initiate RNA internal elongation from double stranded (ds) complexes comprised of full length template hybridized to partial length primers HCV cannot (Hong *et al.,* 2001). The ability of HCV to recognize ds complexes and initiate internal elongation could be rescued by shortening of the thumb associated β-hairpin, presumably by removing steric hindrances and allowing for docking of a ds template. Unfortunately no crystal structure of the alphavirus RdRp exists, however, the observation of *de novo*

initiation, similar to flaviviruses, and the ability to internally initiate (to express subgenomic RNAs), similar to picornaviruses, suggests an intermediate configuration.

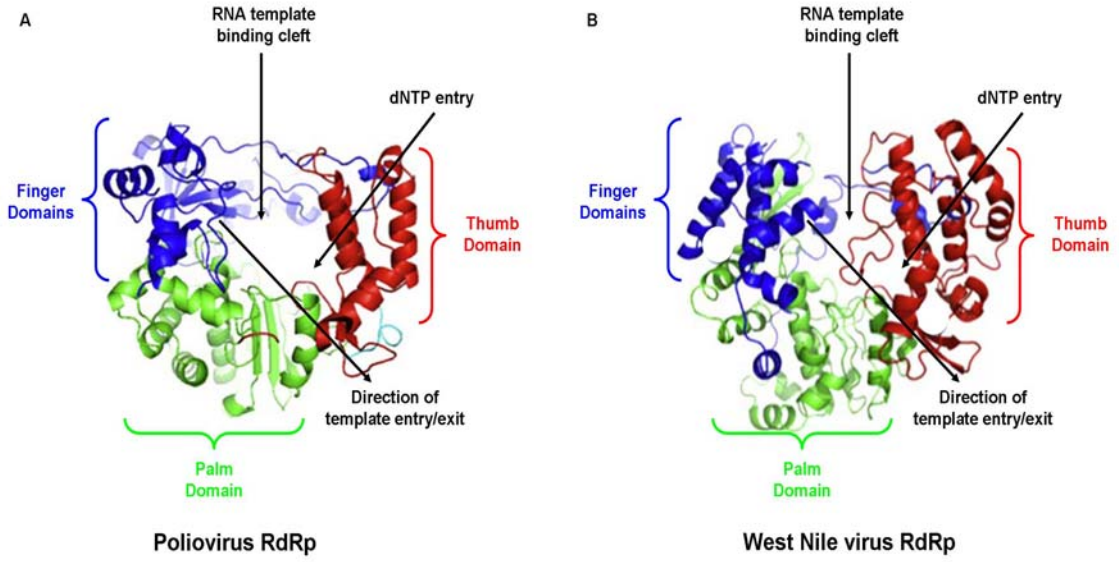


FIGURE 5.3 COMPARISON OF POLIOVIRUS AND WEST NILE VIRUS RNA DEPENDENT RNA POLYMERASES.
A, Poliovirus polymerase. B, West Nile virus polymerase. Please note specific differences in the RNA template binding cleft and thumb domain. (Adapted from Lescar & Canard 2009)

Specific characteristics of the function of the picornavirus RdRp/replicative complex may also serve to increase the spatial association of RNA templates and potential for cross-over events. The function of the picornavirus RdRp appears to require that two polymerases interact with one another along an interphase created by the thumb domain of one monomer and the palm domain of another monomer (Hansen *et al.,* 1997, Kok & McMinn 2009). As such, synthesis of two nascent RNAs occurs simultaneously but in opposite directions. It has also been observed, by electron microscopy, that up to eight nascent RNAs may be associated with a single picornavirus template RNA (Richards *et al.,* 1984). In contrast, the flavivirus replicative complex appears to remain

sequestered in viral induced cellular membrane compartments (Kopek *et al.,* 2007, Uchil & Satchidanandam 2003, Welsch *et al.,* 2009) and in general only a single nascent RNA is associated with any template (Westaway *et al.,* 1999). As such the potential for a flavivirus RdRp to interact with multiple distinct templates may be much less than for a picornavirus RdRp.

The accepted molecular mechanism of RNA virus recombination is "copy-choice" as originally proposed by Cooper *et al.,* (1974). In this mechanism the RdRp, and nascent RNA become destabilized from the parental template, reassociate with a secondary template, and RNA replication resumes. The selection of cross-over points is a matter of some controversy. With regards to the alphavirus recombinants reported in this dissertation, no consistent usage of primary or secondary sequence characteristics that would cause RdRp pausing, disassociation, or re-initiation could be identified. Indeed even with regards to picornaviruses, conflicting reports exist that support or refute the presence of recombination stem-loop "hot spots" (Jarvis & Kirkegaard 1992, King *et al.,* 1985, King *et al.,* 1982, Kirkegaard & Baltimore 1986, Romanova *et al.,* 1986, Tolskaya *et al.,* 1987). To reconcile these observations it has been proposed that recombination occurs at random sites followed by nascent RNA processing and/or selection resulting in the observed site specificity (Banner *et al.,* 1990, Banner & Lai 1991, Lai 1992).

If recombination can occur at random sites, then the question becomes what is the driving force for complex destabilization? Early in the RNA virus life-cycle the processes of replication and protein expression occur concurrently, and as such the possibility exists for a single template to be simultaneously utilized for protein production (5'→3') and negative strand synthesis (3'→5'). Analysis of poliovirus replication and translation suggest that the ribosomal complex may dislodge the replicative complex

before negative synthesis can be completed, while the converse is not true (Barton *et al.,* 1999). If such a collision event were to occur, it could do so randomly at any point along the RNA template and could result in disassociation of the RdRp nascent strand as a complex, or alternately as independent units (**Figure 5.4**).

It is not difficult to imagine that 3' truncated viral RNAs could be generated in this way. These aborted products could then hybridize with either heterotypic or homotypic full length templates. Since the picornavirus RdRp can re-initiate on these ds templates due to its open conformation (Arnold & Cameron 2000, Hong *et al.,* 2001) a recombinant genome can then be generated. A similar interaction would not be expected for flaviviruses due to the inability of the RdRp to accommodate ds templates (Hong *et al.,* 2001, Zhong *et al.,* 2000). If this were indeed a mechanism of template disassociation, then we would expect flavivirus recombination to be significantly less efficient than picornavirus recombination. Recombination in this way could result in a high probability of generating a homologous recombinant via extensive base-pairing between full length and aborted genomes. However, it could also potentially result in the generation of non-homologous recombinants. Such a mechanism was proposed to describe the insertion of short host ribosomal RNA sequences into poliovirus genomes via non-homologous recombination (Barton *et al.,* 1999). With the exception of this one report, recombination of picornaviruses has always been observed to be homologous. As previously suggested (see above) it is possible that these homologous picornavirus genomes result from rapid post-event reductive evolution and/or that non-homologus recombinants do occur but are simply not detected. Picornaviruses appear to require translation in *cis*, because analysis of polioviruses containing premature stop codons could not be replicated in *trans* (Novak & Kirkegaard 1994). As such, any cross-over events that generate a frame-shift picornavirus recombinant would not persist in the

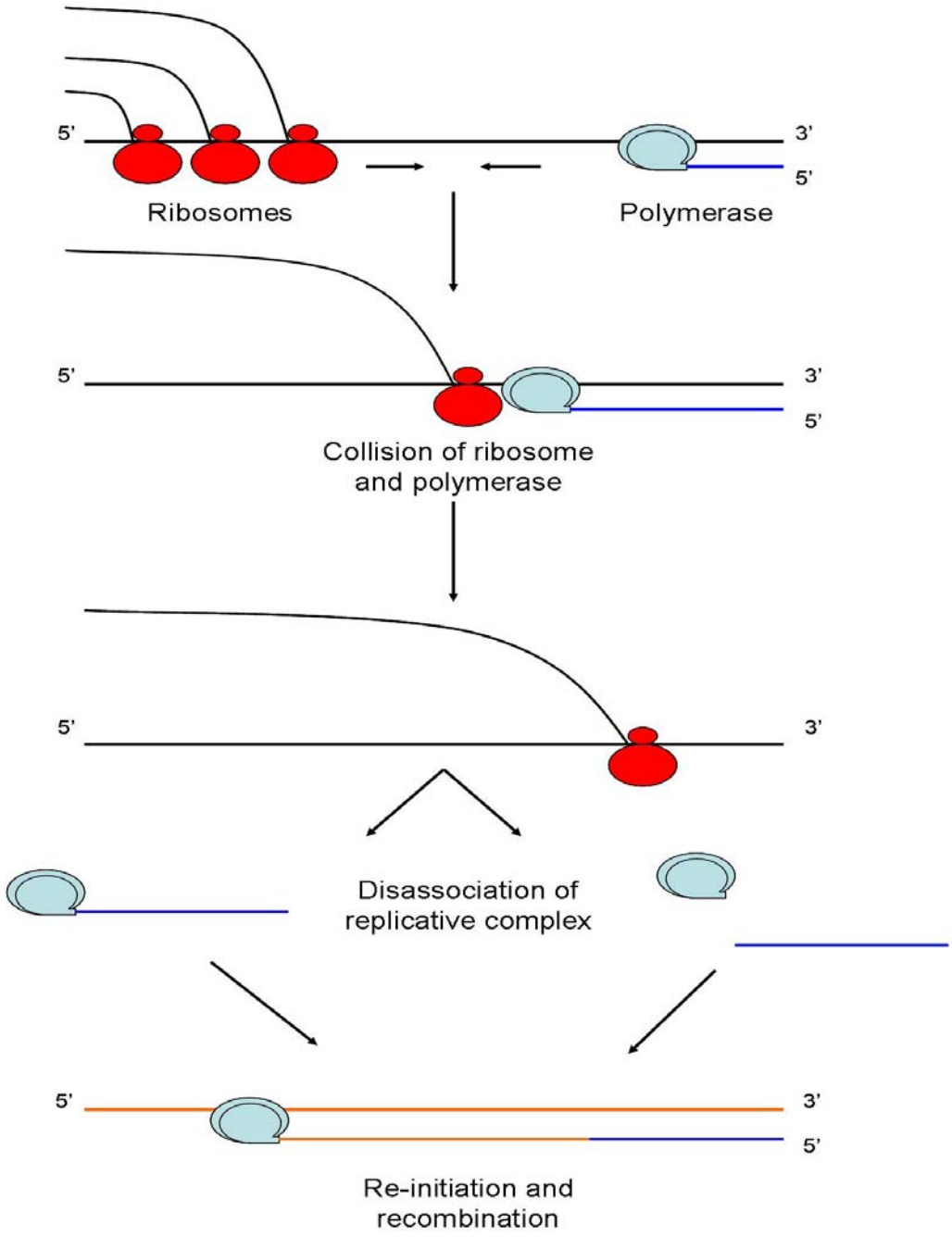


FIGURE 5.4 PROPOSED ALTERNATIVE MECHANISM OF REPLICATIVE COMPLEX TEMPLATE SWITCHING.
Compiled and extrapolated from Barton *et al.,* 1999 and Charini *et al.,* 1994

population. This does not appear to be the case for flaviviruses since repeated isolations of a DENV bearing an E stop codon suggest that at least this flavivirus can indeed replicate and allow for persistence of genomes containing ORF truncations (Aaskov *et al.,* 2006).

CONCLUSIONS

When taken together the data generated in this dissertation as well as by others strongly suggest the following:

- Alphavirus and flavivirus recombination occurs via a highly imprecise mechanism of genetic material exchange and that the apparently homologous events detected in nature are likely just a statistical result of the probability that a small fraction of non-homologous recombinant events happen in precisely the correct place to appear homologous.

- Investigation of the nature and phenotype of alphavirus homotypic and heterotypic recombinants must be performed on a case-by-case basis.

- The perceived nature of naturally occurring homologous events likely represents only those events that produced viable viral genome topologies.

- Recombinant viral genome topologies examined following replication *in vitro* or *in vivo* may be influenced by reductive evolution and as such must be interpreted very carefully.

- The unique molecular mechanisms of RNA replication and epidemiology of a virus strongly influence the potential for recombination and emergence of recombinants.

- The results of *in vitro* analyses of RNA virus recombination, evolution, and superinfection resistance must be interpreted in the larger context of organismal infection.

- Extrapolations between phylogenetically distinct systems such as picornaviruses, flaviviruses, and alphaviruses must not be based on superficial similarities but rather should reflect detailed comparative examinations at the molecular, phenotypic, and ecological levels.

FUTURE DIRECTIONS

It is clear from the observations reported in this dissertation, as well as by others, that although recombination may be a universal mechanism of RNA virus evolution the efficiency of recombination and resulting viable recombinant genome topology is highly virus specific. In terms of alphavirus recombination, my observations suggest that at least in the laboratory polymerase template switching is fairly promiscuous. However, it is difficult to determine if any of the heterotypic recombinant genomes generated would be selected for in nature, as was obviously the case for the naturally occurring recombinant WEEV. The CHIKV and SINV genomes contained in my heterotypic recombinants do not contain any known sequences that would impart selective advantages in a natural transmission cycle. Therefore future analyses of alphavirus recombination should employ viruses containing distinct phenotypic advantages (i.e.

increased vector infectivity or vertebrate pathogenicity) to determine if a laboratory generated alphavirus recombinant would be selected for in nature. Furthermore, given the documented co-circulation of New World alphaviruses, analyses of the potential for eastern, western, and Venezuelan equine encephalitis viruses to undergo homotypic recombination and the resulting phenotypes of these recombinants, in terms of their ability to cause human disease, should be evaluated. Given the non-reciprocal compatibilities of CHIKV and SINV replicon/defective helper systems reported in this dissertation it is likely the potential for alphavirus heterotypic recombination to result in the generation of viable chimeric genomes will have to be evaluated on a case-by-case basis.

Flavivirus recombination, on the other hand, appears to be much less efficient for reasons that are still unclear. In this dissertation I have proposed many factors which may contribute to the decreased efficiency of flavivirus recombination and/or the attenuated phenotype of flavivirus recombinants. The most important future direction in this regard would be detailed analysis of the flavivirus polymerase to re-initiate RNA synthesis on a double stranded intermediate template. This would required molecular manipulation of the polymerase to facilitate opening of the template binding cleft likely via shortening of the thumb domain as was previously achieved for HCV, followed by detailed analysis of effects of such manipulations on changes in recombination efficiency. Multiple reports exist to substantiate that dual flavivirus infections occur within mosquitoes and humans. Therefore, experiments should be designed to evaluate the potential for multiple distinct viruses to sustain simultaneous co-transmission. These studies should also include detailed sequence analyses of the viral quasi-species contained in dually infected arthropods and vertebrates. Furthermore, detailed analyses of the temporal onset, duration and mechanistic basis of superinfection resistance *in vivo*

are severely lacking and as such represent a major obstacle to our ability to understand and predict the consequences of naturally occurring co-infections.

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VITA

Charles Edwin McGee was born in Bridgeport, CT on February 10, 1980 to Charles McGee and Linda Lopez. Charles earned his B.S. in biology at Sacred Heart University in Fairfield, CT in 2002. Following completion of his undergraduate studies, Charles interned for three seasons at the Connecticut Agricultural Experiment Station, where he was involved in collection and identification of mosquitoes as part of the West Nile virus Surveillance Program. Charles was competitively recruited to The University of Texas Medical Branch, Galveston, TX in 2004 where he was supported from 2004-2007 by the Centers for Disease Control Fellowship Program in Vector-Borne Infectious Diseases. Additionally while a UTMB, Charles was also awarded a National Institutes of Health Emerging and Tropical Infectious Diseases Fellowship (2007-2008) and a Centers for Disease Control Public Health Dissertations Grant (2009-2010).

While at UTMB Charles was a graduate student representative to the UTMB Institutional Animal Care and Use Committee (2006-2008), Co-president of the Experimental Pathology Graduate Student Organization (2006-2008), and was the student representative to the American Society for Tropical Medicine and Hygiene's Education and Membership committees (2007-2008). Charles was also recognized with several prestigious awards while at UTMB including: the Robert Shope Ph.D. Endowed Scholarship, the Arthur V. Simmang Scholarship, the Edward S. Reynolds Scholarship, the Graduate School of Biomedical Sciences Associates Scholarship, and was named to *Who's Who of American Colleges* and Universities in 2006. Additionally, Charles received travel awards to attend both the American Society for Tropical Medicine and Hygiene annual meeting (2007) and the American Society for Virology annual meeting (2008). Charles was also selected to attend the prestigious 18th Biology of Disease Vectors course in Manaus, Amazonas-Brazil.

Charles has accepted a Post-Doctoral Fellowship at the University of North Carolina at Chapel Hill in the Carolina Vaccine Institute. Charles can be contacted through his mother Linda Lopez at 327 North Hoadley St. Naugatuck, CT 06770.

HONORS

2010	16th Annual Experimental Pathology Research Day Edward S. Reynolds Poster Presentation Award
2009	Robert Shope Ph.D. Endowed Scholarship Award
	Centers for Disease Control Grant for Public Health Research Dissertation (R36) PAR07-231

	15th Annual Experimental Pathology Research Day Edward S. Reynolds Poster Presentation Award
2008	Robert Shope Ph.D. Endowed Scholarship Award
	Arthur V. Simmang Academic Scholarship Award
	American Society for Microbiology Hurricane Ike Relief Award
	American Society for Virology Travel Award Recipient
2007	American Society of Tropical Medicine and Hygiene Travel Award Recipient
	UTMB Graduate School of Biomedical Science Associates Scholarship
	Edward S. Reynolds Scholarship
2007-2008	NIH T32 AI 07526 Training Grant in Emerging and Tropical Infectious Diseases
2006-2007	Who's Who Among American Colleges and Universities
2004-2007	Centers for Disease Control Fellowship Training Program in Vector-Borne Infectious Diseases T01/CCT622892
1998-2002	Sacred Heart University Trustee Scholarship

ABSTRACTS

Charles E. McGee, Konstantin A. Tsetsarkin, Dana L. Vanlandingham, and Stephen Higgs. Homotypic and Heterotypic Recombination of Chikungunya and Sindbis Viruses. Poster. 16th Annual Research Day. May 4, 2010. University of Texas Medical Branch, Galveston, TX. Abstract #11

Charles E. McGee. Homotypic and Heterotypic Recombination of Chikungunya and Sindbis Viruses. Invited Speaker. University of Pittsburgh. February 16, 2010.

Charles E. McGee. Homotypic and Heterotypic Recombination of Chikungunya and Sindbis Viruses. Invited Speaker. University of North Carolina at Chapel Hill. February 8, 2010.

Kanya C. Long, **Charles E. McGee**, Konstantin A. Tsetsarkin, Stephen Higgs, and Robert B. Tesh. Susceptibility of *Ae. aegypti* to oral infection with Mayaro virus.

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Charles E. McGee, Konstantin A. Tsetsarkin, Dana L. Vanlandingham, and Stephen Higgs. Developing Strategies to Detect Alphavirus Recombination. Poster. Department of Pathology 15th Annual Research Day. May 5, 2009. University of Texas Medical Branch, Galveston, TX. Abstract #11.

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Konstantin A. Tsetsarkin, Dana L. Vanlandingham, **Charles E. McGee**, and Stephen Higgs. Chikungunya Virus- Mechanism of Adaptation to *Aedes Albopictus* Mosquito. Poster. American Society of Tropical Medicine and Hygiene 57th Annual Meeting. December 7-11, 2008. New Orleans, LA. Abstract #1111.

Charles E. McGee, Konstantin Tsetsarkin, Jean Lang, Bruno Guy, Thierry Decelle, Mark G. Lewis, Marisa St. Claire, Wendeline Wagner, Dana L. Vanlandingham, Kate L. McElroy, and Stephen Higgs. Recombinant chimeric virus with wild type dengue 4 premembrane and envelope and wild type yellow fever Asibi backbone sequences is dramatically attenuated in Aedes aegypti mosquitoes and Cynomolgus macaques. Poster. American Society for Virology 27th annual meeting. July 12-16, 2008. Cornell University, Ithaca, NY. Abstract #22-9.

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Charles E. McGee, Konstantin Tsetsarkin, Jean Lang, Bruno Guy, Thierry Decelle, Mark G. Lewis, Marisa St. Claire, Wendeline Wagner, Dana L. Vanlandingham, Kate L. McElroy, and Stephen Higgs. Recombinant chimeric virus with wild type dengue 4 premembrane and envelope and wild type yellow fever Asibi backbone sequences is dramatically attenuated in *Aedes aegypti* mosquitoes and *Cynomolgus macaques*. Poster. Department of Pathology 14th Annual Research Day. May 6, 2008. Galveston TX. Abstract #7.

Konstantin Tsetsarkin, Dana L. Vanlandingham, **Charles E. McGee**, and Stephen Higgs. A single mutation in chikungunya virus affects vector specificity and epidemic potential. Poster. Department of Pathology 14th Annual Research Day. May 6, 2008. Galveston TX. Abstract #11.

Charles E. McGee, Konstantin Tsetsarkin, Jean Lang, Bruno Guy, Thierry Decelle, Mark G. Lewis, Marisa St. Claire, Wendeline Wagner, Dana L. Vanlandingham, Kate L. McElroy, and Stephen Higgs. Recombinant chimeric virus with wild type Dengue 4 prM-E and wild type yellow fever Asibi backbone sequences is dramatically attenuated in non-human primates and Aedes aegypti mosquitoes. Poster. Seventh McLaughlin Symposium in Infection and Immunity. November 15-17, 2007. Galveston, TX. Abstract #2.

Charles E. McGee, Alexandr V. Shustov, Konstantin Tsetsarkin, Ilya V. Frolov, Peter W. Mason, Dana L. Vanlandingham, and Stephen Higgs. Infection, Dissemination, and Transmission of a West Nile virus Green Fluorescent Protein Infectious Clone by *Culex pipiens quinquefasciatus* Mosquitoes. Oral Presentation American Society of Tropical Medicine and Hygiene 56th Annual Meeting. November 4-8, 2007. Philadelphia, PA. Abstract #658.

Charles E. McGee, Konstantin Tsetsarkin, Jean Lang, Bruno Guy, Thierry Decelle, Mark G. Lewis, Marisa St. Claire, Wendeline Wagner, Dana L. Vanlandingham, Kate L. McElroy, and Stephen Higgs. Recombinant chimeric virus with wild type Dengue 4 prM-E and wild type yellow fever Asibi backbone sequences is dramatically attenuated in *Aedes aegypti* mosquitoes and non-human primates. Poster. American Society of Tropical Medicine and Hygiene 56th Annual Meeting. November 4-8, 2007. Philadelphia, PA. Abstract #2696.

Konstantin Tsetsarkin, Dana L. Vanlandingham, **Charles E. McGee**, X. De Lamballerie, R.N. Charrel, and Stephen Higgs. Infectious Clones of Chikungunya Virus (La Reunion Isolate) for Vector Competence Studies. American Society of Tropical Medicine and Hygiene 56th Annual Meeting. Poster. American Society of Tropical Medicine and Hygiene 56th Annual Meeting. November 4-8, 2007. Philadelphia, PA. Abstract #613.

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Kate L. McElroy, Yvette A. Girard*, **Charles E. McGee***, Konstantin A. Tsetsarkin, Dana L. Vanlandingham, and Stephen Higgs. Distribution and tissue tropisms of three phenotypically distinct yellow fever virus strains in *Aedes aegypti*. Poster. American Society of Tropical Medicine and Hygiene 55th Annual Meeting. November 12-16, 2006. Atlanta, GA. Abstract #809

Yvette A. Girard, George F. Mayhew, Jeremy F. Fuchs, Bradley S. Schneider, **Charles E. McGee****, Thomas A. Rocheleau, Bruce M. Christensen, Stephen Higgs, and Lyric C. Bartholomay. Mosquito salivary gland gene expression during long-term cytopathological West Nile virus infection**.** Poster. American Society of Tropical Medicine and Hygiene 55th Annual Meeting. November 12-16, 2006. Atlanta, GA. Abstract #120

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Konstantin Tsetsarkin, Dana L. Vanlandingham, **Charles E. McGee**, Remi Charell, and Stephen Higgs. Infectious Clones of La Reunion Isolate of Chikungunya Virus for Vector Studies. Poster. Experimental Pathology BBSC Program Orientation Presentation and Poster Session. October 25, 2006. Galveston, TX.

Charles E. McGee, Bradley S. Schneider, Dana L. Vanlandingham, Kimberly A. Klingler, Ernest A. Gould, and Stephen Higgs. Nonviremic Transmission of West Nile Virus, Spatial & Temporal Aspects, Preliminary Data, and Experimental Design. Poster. Department of Pathology 12th Annual Research Day. May 3, 2006. Galveston, TX.

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Kate L. McElroy, Konstantin A. Tsetsarkin, Yvette A. Girard, **Charles E. McGee**, Dana L. Vanlandingham, Stephen Higgs. Genetic and Biological Determinants of Viral Dissemination from the Mosquito Midgut: Yellow fever virus in *Aedes aegypti*. Oral Presentation by Kate L. McElroy. American Society of Tropical Medicine and Hygiene 54th Annual Meeting. December 10-15, 2005. Washington, D.C. Abstract #1074.

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SUBMITTED:

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