DISSERTATION

MOLECULAR ANALYSIS OF LA CROSSE VIRUS TRANSCRIPTION AND REPLICATION IN HOST CELLS

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

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ABSTRACT OF DISSERTATION

MOLECULAR ANALYSIS OF LA CROSSE VIRUS TRANSCRIPTION AND REPLICATION IN HOST CELLS

La Crosse (LAC) virus is an arthropod-borne virus (family Bunyaviridae, genus *Orthobunyavirus*) transmitted by *Aedes triseriatus* mosquitoes and is an important cause of pediatric arboviral encephalitis in the United States. Like other arthropod-borne viruses, LAC virus can be very cytolytic in mammalian cells, but establishes a persistent, nonpathogenic infection in the mosquito following an infectious blood meal. Indeed, LAC virus is maintained in nature through transovarial transmission from an infected female mosquito to her progeny. The potential molecular basis of these long-term, persistent, nonpathogenic infections was investigated in this dissertation.

LAC virus primes transcription of its mRNA by using the scavenged 5' terminal cap and adjacent nucleotides from host mRNA. Previous studies demonstrated that certain 5' nonviral sequences predominated during LAC infection of mosquito cells. To characterize this further, the temporal specificity of LAC cap scavenging for transcription of S segment mRNA was analyzed for 42 days after infection of *Aedes albopictus* (C6/36) and *Aedes triseriatus* (MAT) cells and between 4 and 48 hours after infection of baby hamster kidney (BHK-21) cells. Two predominant 5' nonviral sequences [sequence A (5'-CAGTTACGTT-3') and sequence B (5'-TTGGTCGTCATCG-3')] were observed on LAC mRNA. Sequence A comprised 66.0% of sequences observed and sequence B comprised 5.2% of sequences

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observed. Also, 19.0% of sequences were unique and heterogeneous. The sequences of caps scavenged by snowshoe hare (SSH) and Tahyna (TAH) viruses and various LAC virus isolates in cell cultures and mosquito tissues were similar. Cap scavenging in cells dually infected with LAC virus and Sindbis (SIN) virus (family Togaviridae, genus *Alphavirus*) revealed interactions between unrelated viruses within the same cell, including altered growth kinetics of both viruses and differential targeting of host mRNAs. Scavenged 5' nonviral- and SIN mRNA sequences were shown to be present on LAC virus mRNA. Comparison of scavenged sequences with available databases indicated similarities with mitochondrial genes and ribosomal RNA genes.

Previous studies utilizing a reverse transcription-PCR (RT-PCR) based assay examined co-regulation of LAC virus RNA synthesis with host metabolic activity. A more sensitive real-time quantitative PCR (Q-PCR) based assay was developed during this study to detect and quantitate the LAC virus negative-sense small (S) segment RNA genome (vRNA), the full-length positive-sense complement (vcRNA), and the positive-sense subgenomic messenger (mRNA) transcript in mosquito tissues. Results showed that over a five-week period following virus infection of the midgut there was a slight reduction in mRNA and vcRNA quantities, and stable vRNA quantities. Following a second blood meal or a sugar meal at day 30, quantities of the LAC RNAs decreased in midguts. In ovaries, quantities of the LAC RNAs increased following infection, and as the ovaries shifted from biosynthetically active to quiescent metabolism after the initial infectious blood meal, the quantities remained stable. Within 24 hours following a subsequent blood meal, quantities of the LAC RNAs increased, suggesting co-regulation of LAC virus RNA synthesis with host metabolic activity. Interestingly, the same up-regulation was observed in ovaries from

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mosquitoes fed a sugar meal. Quantitative analysis of virus infection in mosquito and mammalian cell cultures was also performed. This approach quantitatively confirmed co-regulation of viral and host metabolic activity as a determinant of efficient transovarial transmission.

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DEDICATION

This work is dedicated to my wife, Emily Travanty; my parents, James and Mary Kempf; and my brother and sister, Michael Kempf and Jennifer Kempf. They are my family and without them I would not be here today. Their encouragement in rough times, praise in accomplishments, and love and support at all times have made this work possible.

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CHAPTER 1: LITERATURE REVIEW

A. Introduction

Arthropod-borne diseases are significant threats to human and animal health worldwide. Approximately 40% of the world's population is at risk of malaria, an arthropod-borne parasitic disease. Malaria is responsible for 300 million acute illness cases and over 1 million deaths annually (World Health Organization (WHO) http://www.who.int/). The dengue viruses (family Flaviviridae, genus *Flavivirus*) place at risk 40% of the world's population, or 2.5 billion people, and there are over 50 million reported cases of dengue infection annually (WHO http://www.who.int/). A more severe form of the disease, dengue hemorrhagic fever and dengue shock syndrome, affects 500,000 individuals annually. Yellow fever virus (family Flaviviridae, genus *Flavivirus*) causes 200,000 new cases of disease annually in Africa and South America with approximately 30,000 deaths (WHO, 1998; 2000; Monath, 2001). These numbers are believed to be greatly underreported.

Arthropod-borne viral diseases continue to emerge in new geographic areas with disastrous consequences. The introduction of West Nile virus (family Flaviviridae, genus *Flavivirus*) into New York in 1999 and its rapid subsequent spread throughout the United States is testimony to the potential for arboviral disease to traffic in this country and around the globe. Usutu virus (family Flaviviridae, genus *Flavivirus*) recently trafficked into Europe from Africa by migrating swallows (Weissenbock et al., 2002), and caused extensive mortality in bird populations in Europe. If this virus is able to establish a transmission cycle

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in Europe, avian populations may be at risk, and the potential effects to humans are unknown. The recent trafficking of Rift Valley fever virus (family Bunyaviridae, genus *Phlebovirus*) from the African continent to Saudi Arabia and Yemen with infections of humans and livestock demonstrates the possibility of the spread of a hemorrhagic fever disease to Europe and Asia (Ahmad, 2000). The recent outbreaks of La Crosse encephalitis in children in the southern United States and the putative role of *Aedes albopictus* mosquitoes is cause for concern and may result in increased number of human infections annually (Gerhardt et al., 2001).

B. Introduction to the Bunyaviridae

The family Bunyaviridae is the largest group of RNA viruses, consisting of more than 250 registered viruses (Karabatsos, 1985; Beaty and Calisher, 1991). The majority of bunyaviruses are arthropod-borne and some are pathogens for a wide variety of species, including humans, animals, and plants.

C. Taxonomy and Classification of the Bunyaviridae

1. Bunyaviridae. The family is divided into five genera based on biological, serological, and genetic relationships (Calisher and Karabatsos, 1988; Elliott 1990; Schmaljohn and Hooper, 2001). The five genera within the Bunyaviridae are *Orthobunyavirus, Nairovirus, Phlebovirus, Hantavirus*, (Calisher and Karabatsos, 1988; Elliott 1990; Schmaljohn and Hooper, 2001) and *Tospovirus* (deHaan et al., 1990; Schmaljohn and Hooper, 2001). Members of the first three genera are arthropod-borne and infect humans and other animals. The hantaviruses are not arthropod-borne; instead, they are transmitted to humans from persistently infected rodents through aerosolized excretions (Elliott, 1990). The tospoviruses are arthropod-borne plant viruses (deHaan et al., 1990).

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The viruses within a genus share complement-fixation antigens (Calisher and Karabatsos, 1988; Elliott 1990; Beaty and Calisher, 1991). Further separation into serogroups is accomplished by hemagglutination-inhibition and neutralization cross-reactions (Beaty and Calisher, 1991).

2. *Orthobunyavirus* genus. The largest genus is *Orthobunyavirus*, which includes more than 155 viruses in eighteen serogroups (Calisher and Karabatsos, 1988; Beaty and Calisher, 1991). The California (CAL) serogroup consists of fourteen viruses, of which eight cause human diseases, including La Crosse (LAC) virus (Calisher, 1983; 1994). The CAL serogroup is named for the prototype virus, California encephalitis (CE) virus, which was isolated in 1943 (Hammon and Reeves, 1945), and was the first virus of the CAL serogroup to be isolated. Other members of the CAL serogroup that cause human disease are snowshoe hare (SSH), Jamestown Canyon (JC), and trivitattus viruses in the United States (LeDuc, 1987; Calisher and Karabatsos, 1988; Tsai, 1991) and Tahyna virus in Europe (Casals, 1962).

D. Medical and Veterinary Importance of the Bunyaviridae.

Viruses in the family Bunyaviridae cause a wide variety of illnesses in both humans and other animals. The illnesses range from mild fevers to encephalitis to hemorrhagic diseases. Only a small proportion of viruses within the family are known to cause disease, but a number of them are significant in terms of morbidity and mortality in humans and other animals (Elliott, 1990; Gonzalez-Scarano and Nathanson, 1996; Nichol, 2001). These include Rift Valley fever, Crimean-Congo hemorrhagic fever, Korean hemorrhagic fever, Akabane, Oropuoche, and LAC viruses (Bishop, 1990; Nichol, 2001). Another bunyavirus, Cache Valley virus, also causes significant teratology and abortion in domesticated livestock and perhaps humans (Calisher, 1995). The rodent-borne Sin Nombre hantavirus emerged in

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1993 as a human pathogen, and it, as well as many other related viruses, cause adult respiratory distress or hantavirus pulmonary syndrome (HPS) in the United States and Central and South America (Elliott et al., 1994; Khan et al., 1996; Nichol, 2001). Hantaan virus, the prototype strain for the genus *Hantavirus*, causes severe hemorrhagic fever with renal syndrome in Asia and Europe (Nichol, 2001).

E. Characteristics of the Bunyaviridae

1. Virion structure. The bunyaviruses are spherical or pleomorphic in shape with a diameter of 80-110nm (Hewlett and Chiu, 1991). Pleomorphic virions were probably the result of preparation methods used in electron microscopy. Newer techniques have shown LAC virus to have spherical morphology (Talmon et al., 1987; Hewlett and Chiu, 1991). Virions are composed of three individual helical nucleocapsids surrounded by a host cell-derived lipid envelope (Obijeski et al., 1976b). The lipid envelope contains the two virus-encoded glycoproteins (Pringle, 1991), which are displayed on the virion surface (Hewlett and Chiu, 1991). The lipid bilayer of the envelope is obtained from the Golgi membranes of the host cell during virus maturation and budding (Kuismanen et al., 1982; 1985). The virions also contain the RNA-dependent RNA polymerase protein (von Bonsdorff et al., 1970; 1975; Patterson et al., 1984; Bishop 1990; Schmaljohn and Hooper, 2001).

The virus particles have four structural proteins, the two external glycoproteins, G1 and G2, and two internal proteins, the nucleocapsid N protein, and the viral polymerase L (Schmaljohn and Hooper, 2001). Each RNA segment is complexed with numerous N proteins to form the individual L, M, and S viral ribonucleoprotein (RNP) complexes (Schmaljohn and Hooper, 2001). Each RNP is associated with one or a few polymerase molecules (von Bonsdorff et al., 1970; 1975; Patterson et al., 1984; Bishop 1990;

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Schmaljohn and Hooper, 2001), and the RNPs are packaged in virion particles. In some instances, more than one RNP for each segment may be packaged and may be related to the size variability of the virion (Bishop and Shope, 1979; Talmon et al., 1987, Borucki et al., 1999).

2. Genome structure. The bunyavirus genome consists of three single-stranded RNA segments of negative or ambisense polarity. The genome segments are designated large (L), medium or middle (M), and small (S) (Obijeski et al., 1976a). The sizes and products of the encoded proteins are genus specific (Bishop, 1990; Elliott, 1990; Schmaljohn and Patterson, 1990). The three bunyavirus genome segments code for four structural proteins. The L segment codes for the L protein, the RNA-dependent RNA polymerase, the M segment codes for G1 and G2, the envelope glycoproteins, and the S segment codes for the N protein, the nucleocapsid protein (Schmaljohn and Hooper, 2001). Two non-structural proteins, NS_S and NS_M, are coded from the S and M RNA genome segments, respectively (Schmaljohn and Hooper, 2001). The 3'- and 5'-terminal nucleotide sequences of the genomic RNA segments are conserved within each genus and are complementary (Clerx-van Haaster et al., 1980; 1982; Obijeski et al., 1980, Akashi and Bishop, 1983; Bouloy et al., 1984, Cabradilla et al., 1983; Schmaljohn and Dalrymple, 1983; Elliott et al., 1991; Schmaljohn and Hooper, 2001). The conserved sequences are able to form panhandle structures (Obijeski et al., 1980; Patterson et al., 1983; Raju and Kolakofsky, 1989b), which may provide a recognition site for the viral polymerase or may act as a signal for encapsidation by the nucleocapsid or N protein (Raju and Kolakofsky, 1989b).

3. Evolutionary potential of bunyaviruses. In RNA viruses with segmented genomes, both genetic shift through segment reassortment and genetic drift through

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nucleotide insertions, deletions, and substitutions within the genome can lead potentially to the evolution of new and distinct viruses.

a. Genomic RNA segment reassortment. Viruses with segmented genomes have the potential for genome segment reassortment, which permits rapid evolution. This could yield viruses with new phenotypes including differences in host range and pathogenicity. Closely related bunyaviruses within a serogroup can undergo segment reassortment (Bishop, 1979; Pringle et al., 1984; Elliott et al., 1983; Bishop 1990). The ability to reassort genome segments may be one reason for the great number and diversity within the Bunyaviridae family.

Genome segment reassortment has been demonstrated in infected cell culture (Gentsch et al., 1977; 1979; Bishop, 1979; Schmaljohn and Patterson, 1990), in mosquitoes by intrathoracic inoculation or infection by blood feeding, and in transovarially-infected mosquitoes that are then infected with a second virus by blood feeding (Beaty et al., 1981b; 1985; Chandler et al., 1991; Borucki et al., 1999). The *Aedes* vector could become dually infected or superinfected when ingesting two bunyaviruses either simultaneously or through interrupted feeding (Beaty et al., 1981b). The infected mosquito can serve as the site for genome reassortment between homologous and heterologous strains of LAC and SSH viruses (Beaty et al., 1985; Beaty and Bishop, 1988; Chandler et al., 1990, Borucki et al., 1999) and LAC and Tahyna viruses (Chandler et al., 1990). The newly evolved reassortant viruses can be transmitted to vertebrates during blood feeding and to progeny mosquitoes by transovarial transmission (Chandler et al., 1990).

Several viruses within a serogroup may be sympatric in nature. The ranges of LAC, JC, and SSH viruses overlap in many areas in North America. The overlap may provide the

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opportunity for these viruses to dually infect mosquitoes after feeding on hosts infected with different viruses and allow for segment reassortment. Reassortant bunyaviruses have been isolated in nature and include LAC virus (El Said et al., 1979; Klimas et al., 1981), several members of the Patois serogroup bunyaviruses (Ushijima et al., 1981), and Group C bunyaviruses (Casals and Whitman, 1961; Shope and Causey, 1962). Genome segment reassortment has been observed in female mosquitoes transovarially-infected with LAC virus and superinfected with SSH virus (Borucki et al, 1999). Also, reassortant viruses have been demonstrated to be transovarially-transmitted to progeny (Chandler et al., 1990; Borucki et al., 1999), and subsequently to be transmitted to mice during feeding (Chandler et al., 1990). Because several reassortant bunyaviruses have been recovered in nature and viruses can reassort in arthropods, arthropod vectors may be important to the establishment of these new genome combinations (Klimas et al., 1981; Shope, 1988; Bishop, 1990). During an outbreak of Rift Valley fever virus in Africa in 1997 and 1998, a new reassortant bunyavirus associated with hemorrhagic disease was isolated. This virus, tentatively named Garissa virus, has L and S segments identical to Bunyamwera virus and an M segment from an unidentified source (Bowen, et al. 2001).

Reassortant bunyaviruses have been used in gene structure-biological function studies to determine the characteristics of the genome segments including coding assignment (Bishop et al., 1984; Bishop, 1990), virulence (Elliott et al. 1983; 1991; Bishop et al., 1984; 1987; Janssen et al., 1984; Gonzalez-Scarano et al., 1988; 1992; Griot et al., 1993), and virus-vector relationships (Beaty et al., 1981a; 1982; Beaty and Bishop, 1988; Beaty and Calisher, 1991). Biological characteristics of each genome segment are described in the sections describing each segment.

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b. Genomic RNA mutation. Genetic drift in RNA viruses results from poor fidelity and lack of proofreading mechanisms in the RNA-dependent RNA polymerase (Holland, et al., 1982). The ability for mutations to occur may be another reason for the great number and diversity within the Bunyaviridae family, which like the majority of arboviruses, contain RNA genomes. Genetic stability has been observed for many arboviruses, notably in the flaviviruses and alphaviruses (Monath, 1985; Duebel et al., 1986; Weaver, 1995; Zanotto et al., 1996; Beaty et al., 1997). The ability to replicate in the two distinct systems may require genome sequence conservation. Arbovirus evolution occurs ten-fold slower than in RNA viruses with a single host (Weaver, 1995).

An example of bunyavirus genome stability is given by RNA sequence determination of two LAC virus isolates from fatal human cases that occurred 18 years (1960 and 1978) and 50 miles apart in endemic regions of Wisconsin. Two nucleotide changes within a 668 nucleotide portion of the LAC S RNA were demonstrated between the two viruses, suggesting that in endemic areas, LAC virus is stable over long distance and extended periods of time (Chandler et al., 1998; Borucki et al., 2002).

Studies were conducted in collaboration with Dr. Monica Borucki, as part of this dissertation research, to determine the genetic consequences of oral and transovarial passage of different LAC virus isolates in mosquitoes. This work has been published and is provided in the appendix (Borucki et al., 2001; 2002). Genetic drift of LAC virus was observed following passage through *Aedes triseriatus* mosquitoes. The nucleotide sequences of small regions of the N, G1, and G2 open reading frames were examined in mosquitoes infected with either of two strains passed repeatedly in cell culture (WT or ORI) or a strain passed only once after isolation through suckling mouse brain (SM1-78). The N and G2 open

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reading frames were stable upon passage of any of the three viruses in mosquitoes (Borucki et al., 2001; 2002; Appendix). In contrast, after passage in mosquitoes, the G1 open reading frame of LAC WT and LAC ORI had numerous nucleotide changes, many of which resulted in amino acid changes. However, the G1 open reading frame from mosquitoes infected with LAC SM1-78 exhibited only two nucleotide changes, with one of these resulting in an amino acid change (Borucki et al., 2001; 2002; Appendix). The mutations observed in LAC WT and LAC ORI occurred in the midgut (75%) and the ovaries (21%) of the infected parental mosquito. One additional nucleotide change was observed in two of the transovarially-infected progeny. The mutations observed in the midguts were also observed in the salivary glands and 77% of them were also observed in the progeny mosquitoes, suggesting that selection for mutants occurred in the midgut (Borucki et al., 2001; 2002; Appendix). Mutations in the viral genome acquired during infection of the parental mosquito were detected in their progeny, suggesting that transovarial transmission can serve in amplification and maintenance of new virus genotypes in nature.

The population of viruses was also examined after passage in mosquito cell culture and mosquito tissues. The genetic variability in LAC virus S segment was determined during passage of the virus through a single mosquito following an infectious blood meal. Single strand conformation polymorphism analysis (SSCP) (Black et al., 1995) revealed that LAC virus quasispecies selected by growth in C6/36 cultured mosquito cells demonstrated less variability than viruses from an infected mosquito. Additionally, as virus infection disseminated from the midgut to ovaries and salivary glands, the genetic variability of the virus also decreased. It was observed that within the midgut of a single infected female mosquito, at least 13 different genotypes were present, 11 genotypes in the ovaries, 9

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genotypes in the salivary glands, compared to 4 genotypes in C6/36 cells. The predominant genotypes observed in each tissue and in cultured cells were identical and the same as the input virus. Sequence analysis of individual quasispecies genotypes revealed that in the ovary the different genotypes had between one and eight nucleotide changes from the input virus and between each other. This suggests that a greater variety of virus genotypes are able to replicate in the midgut and that as dissemination to other target tissue occurs the number of genotypes is reduced. In cell culture, only a small proportion of genotypes are able to replicate (Borucki et al., 2001; 2002; Appendix). Mutations in the viral genome may allow for efficient infection and dissemination in the mosquito as well as affect oral and transovarial transmission.

4. The S RNA genome segment and its products. The complete nucleotide sequences of the S RNA genome segments of several bunyaviruses, including LAC virus, have been published (Bishop et al., 1982; Akashi and Bishop, 1983; Cabradilla et al. 1983; Akashi et al., 1984; Elliott and McGregor, 1989; Elliott et al. 1991; Dunn et al., 1994; Bowen et al., 1995). Accession numbers for California serogroup viruses on GenBank include: LAC virus NC 004110, AF528167, K00610, and K00108; California encephalitis virus U12800 and U12797; Jamestown Canyon virus U12799 and U12796; Jerry Slough virus U12798; Keystone virus U12801; Melao virus U12802; Morro Bay virus U31989; San Angelo virus U47139; Serra do Navio virus U47140; snowshoe hare virus J02390; South River virus U47141; trivitattus virus U12803; Inkoo virus U47138, U47137, and Z68496; Tahyna virus U47142 and Z68497. The length of the S RNA genome segment ranges from 850 to 1,077 nucleotides, depending on the virus. The length of the S RNA segment of LAC virus is 984 nucleotides (Schmaljohn and Hooper, 2001).

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The S RNA genome segment of LAC virus encodes the nucleocapsid protein N and the non-structural protein NS_S in overlapping reading frames on the viral complementary or positive-sense RNA (Gentsch and Bishop, 1978; Cash et al., 1979; Bishop et al., 1982; Clerx-van Haaster et al. 1982; Fuller and Bishop, 1982; Fuller et al., 1983; Cabradilla et al., 1983; Elliott, 1985; Schmaljohn and Hooper, 2001). The mRNA coding for the two proteins is bicistronic (Bouloy, 1991) with the first open reading frame coding for the N protein and the second open reading frame, in +1 register, coding for the NS_S protein (Elliott and McGregor, 1989; Schmaljohn and Hooper, 2001). Two AUG initiation codons on the mRNA are used to initiate translation of the N and NS_S proteins. The first AUG codon is utilized preferentially by host ribosomes in translation of the N protein. The second AUG codon is used much less frequently in the translation of NS_S protein. As a result, a larger amount of N protein is synthesized than NS_S. To allow for initiation of translation from the second AUG codon the ribosomes must bypass the first AUG codon in a mechanism termed "leaky scanning" or "alternative initiation of translation" (Bishop et al., 1983; Kozak, 1986a; 1986b; 1991; Elliot and McGregor, 1989). Consensus sequences observed in many eukaryotic mRNAs and favored for initiation of translation in vertebrates (Kozak, 1987) are not present in the context of any initiation codons in LAC mRNA.

The size and amino acid sequence of N protein is highly conserved among members of the *Orthobunyavirus* genus. The molecular mass of the N protein is 19-26 kilodaltons (kDa). The N protein of LAC virus is 235 amino acids in length with a molecular mass of 26,586 daltons (Elliot, 1989b). The N protein acts as the complement-fixing antigen and is the primary group specific antigen (Bishop, 1990). The N protein interacts to encapsidate all three RNA genome segments, possibly initiating interaction at the terminal panhandle

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structures (Elliott, 1990). The N protein also encapsidates full-length virion complementary (vc) RNA.

The NS_S protein is 92 amino acids in length with a molecular mass of 10,467 daltons. It is not a component of virions but is detectable in infected cells (Elliott, 1990). Recent evidence suggests that the NS_S protein of some bunyaviruses inhibits the induction of interferon-mediated antiviral pathways in infected cells (Bouloy et al., 2001; Bridgen et al., 2001; Weber et al., 2001; 2002). Studies with Bunyamwera virus (genus *Orthobunyavirus*), the prototype virus for the family, and Rift Valley fever virus (genus *Phlebovirus*) suggest that NS_S blocks transcriptional activation of interferons alpha and beta (IFN-α/β) (Bouloy et al., 2001; Bridgen et al., 2001; Weber et al., 2001; 2002).

The nucleotide sequences of S RNA genome segments of CAL serogroup viruses exhibit between 80 and 98% nucleotide identity and 70 to 87% amino acid identity of both proteins. Between members of the CAL serogroup and other members of the genus *Orthobunyavirus*, there is 60 to 65% nucleotide identity and 40 to 45% amino acid identity (Akashi and Bishop, 1983; Bowen et al., 1995; Huang et al., 1996).

5. The M RNA genome segment and its products. The M RNA genome segments of nearly all CAL serogroup viruses have been sequenced (Eshita and Bishop, 1984; Lees et al., 1986; Grady et al., 1987; Pardigon et al., 1988; Gerbaud et al., 1992; Huang et al., 1995). Accession numbers for California serogroup viruses on GenBank include: LAC virus NC 004109, AF528166, U18980, U18979, U70205, U70206, U70207, U70208, D10370, and M87664; California encephalitis virus AF123483; Jamestown Canyon virus U88058; Jerry Slough virus AF123487; Keystone virus AF123489; Melao virus U88057; San Angelo virus AF123486; Serra do Navio virus AF123490; snowshoe hare virus M11783; South River

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virus AF123488; trivitattus virus AF12341; Inkoo virus U88060 and U88059; Tahyna virus AF229129 and AF123484. The size of the M RNA genome segment varies in length from 4,458 to 4,534 nucleotides. The M RNA genome segment of LAC virus is 4,527 nucleotides in length (Grady et al., 1987; Huang et al., 1995; Schmaljohn and Hooper, 2001).

The M RNA genome segment of LAC virus contains one long open reading frame of 4,326 nucleotides in the positive-sense sequence, coding for the two viral glycoproteins, G1 and G2, and the non-structural protein NS_M (Gentsch and Bishop, 1979; Fuller and Bishop, 1982; Eshita and Bishop, 1984; Elliott, 1985; 1990). The order of the gene products of bunyavirus M segment mRNA are genus specific (Schmaljohn and Patterson, 1990; Bouloy, 1991; Schmaljohn and Hooper, 2001). Based on the nucleotide and amino acid sequences, the gene order of the LAC virus M segment mRNA is 5'-G2- NS_M–G1-3' (Fazakerley et al., 1988; Bouloy, 1991). During virus replication a single polyprotein, with a proposed molecular weight of 162,365 daltons, is translated from the open reading frame, from which the mature proteins are cleaved to yield G1, G2 and NS_M (Fazakerley et al., 1988; Elliott et al., 1991). The polyprotein has never been isolated from infected cells, suggesting that the mature proteins are cleaved co-translationally (Pennington et al., 1977; Lees et al., 1986). For most of the viruses in the family, G1 and G2 have N-terminal signal sequences, suggesting that cleavage of the polyprotein precursor is most likely mediated by host signalase (Fazakerley and Ross, 1989).

The protein products of the M RNA segment are responsible for several of the biological characteristics of bunyaviruses. These characteristics include cell tropism and fusion, elicitation of neutralizing and hemagglutinating antibodies, and virus virulence (Gentsch et al., 1980; Gonzalez-Scarano et al., 1991). The M RNA segment confers the

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ability of LAC virus to infect and replicate in *Aedes (Ochlerotatus) triseriatus* mosquitoes (Beaty et al., 1981a; 1982). (Recently, the subgenus of *Aedes*, *Ochlerotatus,* was elevated to the genus level (Reinert, 2000), but the genus *Aedes* will be kept in this dissertation.) Studies using LAC and SSH reassortant viruses showed that virus containing the LAC M RNA segment exhibited more efficient infection and replication in *Ae. triseriatus* mosquitoes than did reassortants containing the SSH M RNA segment (Beaty et al., 1981a; 1982). The M RNA segment also co-segregates with neuroinvasiveness and neurovirulence in mice (Shope et al., 1981; Janssen et al., 1984; 1986; Gonzalez-Scarano et al., 1988; 1992; Endres et al., 1990). These characteristics are likely due to the ability of the virus to attach to cells within the central nervous system (Endres et al., 1990).

The G1 glycoprotein has a molecular mass of approximately 108-120 kDa; the G2 glycoprotein has a molecular mass of approximately 30-40 kDa; and the non-structural protein NS_M has a molecular mass of 15-18 kDa (Eshita et al., 1985; Elliott et al., 1991). G1 glycoprotein is the major neutralizing antigen (Kingsford et al., 1983; Kingsford, 1984; Kingsford and Bouquey, 1990; Pekosz et al., 1995). Recombinant vaccinia virus systems expressing either G1 or G1 and G2 showed that a single immunization with the virus expressing G1 alone was able to protect mice from challenge with a lethal dose of LAC virus (Pekosz et al., 1995). This confirmed that anti-G1 antibodies are sufficient in providing protection to LAC infection. G1 has also been shown to be responsible for virus attachment to vertebrate host cells and is necessary for infection both *in vitro* and *in vivo* (Hacker et al., 1995).

G1 glycoprotein is also responsible for the ability of LAC virus to orally infect *Ae. triseriatus* mosquitoes (Sundin et al., 1987). Using a monoclonal antibody-resistant mutant,

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LAC virus V22, the role of G1 in LAC virus infection of mosquitoes was studied. Of the mosquitoes ingesting the V22 mutant, only 15% became infected, whereas 74% of mosquitoes ingesting wild-type LAC virus became infected. When mosquitoes were intrathoracically injected with V22 or wild-type LAC virus, similar numbers of mosquitoes were found to be infected (Sundin et al., 1987). These results suggest that G1 glycoprotein is important in the ability of LAC virus to attach to cells in the *Ae. triseriatus* midgut.

The role of the G2 glycoprotein in LAC virus infection of *Ae. triseriatus* mosquitoes is not fully understood. In studies to determine the roles of G1 and G2 in attachment of LAC virus to vertebrate and invertebrate cells, G1 was shown to be necessary for LAC virus attachment to vertebrate cells while G2 was shown to be necessary for attachment to mosquito cells both *in vitro* and *in vivo* (Ludwig et al., 1989; 1991). To study the glycoprotein functions, mature LAC virions were treated with proteases to specifically cleave G1, leaving G2 intact on the virion surface. Treated viruses were able to more efficiently infect mosquito cells and midguts than non-treated viruses. This suggested that G2 was important as the attachment glycoprotein in mosquito midguts and that protease cleavage of the G1 to unmask G2 within the mosquito midgut was necessary for efficient infection (Ludwig et al., 1989). Affinity purified G2 glycoproteins were also shown to bind efficiently to receptors on mosquito midgut cells while G1 glycoproteins were shown to bind to receptors on vertebrate cells, thus supporting the previous results (Ludwig et al., 1991). In contrast, similar experiments performed using CE virus showed that G1 glycoprotein was necessary for both vertebrate and invertebrate cell infection (Hacker et al., 1995). Virions proteolytically processed using the same proteases as the previous study were shown to have

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greatly reduced infectivity as compared to untreated virus in mosquito and mammalian cell culture and in mosquitoes by the oral route (Hacker et al., 1995).

The non-structural protein NS_M is found in infected cells but its function is poorly understood (Fuller and Bishop, 1982; Elliott, 1985). Intracellular localization of NS_M of Bunyamwera virus has been studied using monoclonal antibodies. NS_M has been shown to localize at the Golgi complex of infected cells (Nakitare and Elliott, 1993). It has been hypothesized that NS_M may function in movement or transport of viral proteins through the cell during viral replication (Elliott, 1990). Indeed, the NS_M protein of tomato spotted wilt virus (genus *Tospovirus*) has been shown to function as a movement protein between plant cells (Kormelink et al., 1994; Storms et al., 1995; Soellick et al., 2000). Studies using recombinant vaccinia virus expressing Bunyamwera NS_M, G1 and G2 showed that NS_M was not necessary for transport of G1 to the Golgi (Lappin et al., 1994); rather G2 most likely contains the Golgi targeting signal and is required for G1 localization to the Golgi complex. Recent evidence from studies on Golgi localization of Hantaan virus glycoproteins demonstrated that separately, G1 and G2 localize to the endoplasmic reticulum but expressed together, they localize to the Golgi. It was further observed that G1 expressed with the signal sequence of G2 localized to the Golgi. Together these results suggest that the G1 cytoplasmic tail and the downstream signal peptide of G2 are required for Golgi localization and that the complex of G1 and G2 is more important to localization than actual amino acid sequence (Shi and Elliott, 2002). Sindbis virus (family Togaviridae, genus *Alphavirus*) replicon vectors expressing either LAC virus G1, truncated G1, G2, or G1 and G2, demonstrated that in mosquito and mammalian cell culture both proteins colocalized to the Golgi complex, while when G1 or truncated G1 were expressed alone, the protein was

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excreted. Expression of G2 sequestered G1 and truncated G1 to the Golgi complex (Kamrud et al., 1998).

The nucleotide sequences of M RNA genome segments of CAL serogroup viruses exhibit between 78 and 82% nucleotide identity between the viruses and 63 to 84% amino acid identity (Campbell and Huang, 1999). Between members of the CAL serogroup and other members of the genus *Orthobunyavirus*, there is 60 to 89% nucleotide identity and 29 to 39% amino acid identity (Grady et al., 1987; Elliott 1990; 1995; Wang et al., 2001).

6. The L RNA genome segment and its products. Sequence data are available for the L RNA genome segments of only Bunyamwera virus (Elliott, 1989a) and two LAC isolates (Roberts et al., 1995). The L RNA genome segment of LAC virus is 6,980 nucleotides in length with a single open reading frame in the positive-sense sequence of 6,792 nucleotides, coding for the L protein RNA-dependent RNA polymerase (GenBank accession numbers NC 004108, AF528165, and U12396). Based on RNA sequence information, the L protein of LAC virus is 2,264 amino acids long with a molecular mass of approximately 263 kDa (Elliott, 1989a; Endres et al., 1989; Roberts et al., 1995). When preparations of L protein are separated on denaturing polyacrylamide gels, the molecular mass is approximately 200 kDa (Endres et al., 1989). In addition to the L protein coding sequence in the vcRNA, two small open reading frames are present in the genomic L RNA sequence, but neither has been associated with any protein product (Roberts et al., 1995).

L protein was shown to function as the viral polymerase in reassortant studies with LAC and TAH viruses (Elliott, 1989a; Endres et al., 1989). The L protein also acts as a virulence determinant (Endres et al., 1991; Gonzalez-Scarano et al., 1992). Reassortant

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viruses with L RNA genome segments from neuroattenuated parental virus had a neuroattenuated phenotype (Janssen et al., 1986; Endres et al., 1991).

The L protein contains a methylated cap-dependent endonuclease function believed to be responsible for attaching to and cleaving the 5' 7-methyl guanosine cap and adjacent nucleotides from host mRNA to prime transcription of viral mRNA (Patterson et al., 1984; Patterson and Kolakofsky, 1984; Bouloy et al., 1990; Dobie et al., 1997). This phenomenon is known as "cap-scavenging" or "cap-snatching" and has been observed and studied in a variety of bunyaviruses (Bishop et al., 1983; Patterson et al., 1984; Patterson and Kolakofsky, 1984; Ihara et al., 1985; Bouloy et al., 1990; Simons and Pettersson, 1991; Kormelink et al., 1992; Jin et al., 1993; Garcin et al., 1995; van Poelwijk et al., 1996; Dobie et al., 1997; Duijsings et al., 1997; 2001), orthomyxoviruses (Krug, 1981; Plotch et al., 1981; Leahy et al., 1997; 1998), arenaviruses (Garcin et al., 1990; Raju et al., 1990; Meyer and Southern, 1994), and the tenuiviruses of plants (Huiet et al., 1993, Ramirez et al., 1995; Nguyen et al., 1997).

The L RNA genome segment and protein product of LAC virus have 86% nucleotide identity and 54% amino acid identity with Bunyamwera virus. The LAC L protein also contains amino acid sequences that are highly conserved in other RNA-dependent RNA and DNA polymerases (Poch et al., 1989).

F. Replication of bunyaviruses

1. Virus entry into the host cell. Bunyavirus entry into the host cell is most likely initiated by interaction and binding of the virus particle to host cell receptors. The early steps of bunyavirus replication have not been well studied and the exact receptors on host cells have not been determined (Elliott, 1990; Schmaljohn and Hooper, 2001). Attachment to

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vertebrate cellular receptors is initiated by the G1 glycoprotein (Ludwig et al., 1991; Pekosz et al., 1995), while the G2 glycoprotein has little to no involvement. The G2 glycoprotein is necessary for membrane fusion, but it is unclear if both G1 and G2 are necessary. Bunyaviruses are able to infect and replicate in a wide variety of cells, suggesting that a common cellular receptor or multiple cellular receptors are used for virus attachment.

Following virus attachment to the cell membrane, the virions are apparently endocytosed via coated vesicles, which then fuse with intracellular endosomes. The low pH or acidic environment of the endosome leads to fusion of the viral envelope with the endosomal membrane and release of the three RNP complexes (Gonzalez-Scarano et al., 1984; 1985; Hewlett and Chiu, 1991). As evidence of this, in cells treated with ammonium chloride to prevent endosomal acidification, infection with CE virus (Hacker and Hardy, 1997) and Uukuniemi virus was inhibited (Ronka et al., 1995). Replication and maturation of bunyaviruses occurs within the cytoplasm of the infected cell (Rossier et al., 1986).

2. Primary transcription. Replication of bunyaviruses begins with primary transcription of positive-sense viral mRNAs by the virion-associated viral polymerase from the negative-sense genomic RNA templates (Patterson et al., 1984). Initiation of mRNA synthesis is primed by the 5' 7-methyl guanosine cap plus adjacent oligonucleotide sequence 10-18 bases in length cleaved from cellular mRNA by the viral polymerase (Patterson et al., 1984; Hacker et al., 1990). As a result of cap scavenging, bunyavirus mRNAs have a heterogeneous, nonviral-coded sequence at their 5' ends (Bishop et al., 1983; Patterson et al., 1984; Patterson and Kolakofsky, 1984; Ihara et al., 1985; Bouloy et al., 1990; Simons and Pettersson, 1991; Kormelink et al., 1992; Jin et al., 1993; Garcin et al., 1995; van Poelwijk et al., 1996; Dobie et al., 1997; Duijsings et al., 1997; 2001). LAC virus replication is resistant

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to drugs that inhibit DNA-dependent RNA polymerases used in host cell transcription, which suggests that for cap scavenging to occur, a stable pool of host cell mRNAs and not newly synthesized transcripts provide primers for transcription (Rossier et al., 1986). Cap scavenging will be described in detail (Section I. Cap scavenging, page 26).

LAC virus mRNAs are not full-length copies of the genomic RNA segments. Transcription of the S and M segments is terminated approximately 60-100 bases prior to the end of the template genomic RNA. A transcription termination sequence on the genomic RNA may be responsible for premature transcription termination. A consensus 3'-GUUUUU-5' sequence present at bases 47-53 and 238-242 of the S and M genomic RNA segments, respectively, has been hypothesized to be the transcription termination signal (Patterson and Kolakofsky, 1984; Eshita et al., 1985; Bouloy et al., 1990; Collett, 1986; Eshita and Bishop, 1986; Kolakofsky and Hacker, 1991; Hutchinson et al., 1996), although the sequence has not been found in the genomes of all bunyaviruses that have been sequenced (Elliott, 1990). It is hypothesized that other purine-rich regions in the genome may function as the transcription termination signal (Collett, 1986; Raju and Kolakofsky, 1987; Hutchinson et al., 1996).

The 3' ends of bunyavirus mRNAs are not polyadenylated and do not bind to oligo dT columns (Ulmanen et al., 1981; Pattnaik and Abraham, 1983; Pettersson et al., 1985; Bouloy et al., 1990, Bouloy, 1991; Kolakofsky and Hacker, 1991). Sequence analysis has shown that polyadenylation signals are not present in genomic RNA sequences. Because the 3' ends of the viral RNAs are truncated, they are unable to form secondary structures via 5' to 3' base pairings as shown for genomic RNAs. This could function to keep the mRNAs translatable and prevent mRNAs from being encapsidated. This has not been proven, and

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there are reports of LAC virus mRNAs being encapsidated as a mechanism of translational control (Raju and Kolakofsky, 1987; Hacker et al., 1989).

3. Translation of viral proteins. Following mRNA transcription, translation of viral proteins begins in the cytoplasm. Within three to five hours after infection, new viral proteins are detectable in cells (Rossier et al., 1988). LAC virus L, G1 and G2 proteins were all detectable in pulse-labeling experiments by three hours after infection (Madoff and Lenard, 1982) and N encapsidated RNAs were detectable by 5 hours after infection (Raju and Kolakofsky, 1987). The L and S segment mRNAs are translated by free ribosomes and their gene products, L, N, and NS_S proteins, are not known to be modified after they are synthesized (Schmaljohn and Patterson, 1990). M segment mRNA is translated by membrane-bound ribosomes on the endoplasmic reticulum. The G1, G2, and NS_M proteins are translated as a polyprotein and cleaved co-translationally (Fazakerley et al., 1988; Matsuoka et al., 1991). G1 and G2 glycoproteins are modified by glycosylation in the endoplasmic reticulum and Golgi complex. Both G1 and G2 glycoproteins contain a region of hydrophobic amino acids near their N-termini that probably functions as a signal for translation and transport of the proteins through the endoplasmic reticulum and the Golgi complex. Sequence analysis has shown that a signal sequence in G1 is used for targeting of the proteins to the Golgi complex (Lappin et al., 1994; Matsuoka et al., 1994; Melin et al., 1995). Signal sequences from G2, as well as G1-G2 complexes, have been demonstrated to be necessary for targeting to the Golgi complex (Shi and Elliott, 2002). Expression of the G1 and G2 glycoproteins in mosquito cells using Sindbis virus expression vectors demonstrated co-localization of the glycoproteins in the Golgi following staining with wheat germ agglutinin, a Golgi marker (Kamrud et al., 1998). Studies of Uukuniemi virus (genus

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Phlebovirus) have shown that G1 and G2 are glycosylated both at the endoplasmic reticulum and the Golgi complex (Kuismanen et al, 1982; 1985; Ronnholm et al., 1992; Andersson and Pettersson, 1998).

4. Replication of the genome. Following transcription of mRNA and accumulation of viral proteins, the genome is replicated. Genome replication requires a switch from mRNA transcription to full-length virion complementary (vc) RNA synthesis. The mechanism through which the switch is mediated is poorly understood, although increased concentration of N protein not in RNP complexes may be a contributing factor, as occurs with nonsegmented negative strand RNA viruses (Kolakofsky and Hacker, 1991; Schmaljohn and Hooper, 2001). The vcRNA synthesis does not require host-derived primers, and the transcription termination signal is suppressed to allow for full-length RNA product synthesis. Synthesized vcRNA acts as the template for transcription of new genomes (Kolakofsky and Hacker, 1991; Schmaljohn and Hooper, 2001). LAC virus has been shown to encapsidate S mRNA, in addition to S vcRNA, suggesting that the packaging signal may be at the 5' end of the RNA (Raju and Kolakofsky, 1987).

5. Virion maturation and release. Maturation of bunyavirus virions occurs on the Golgi complex membranes where G1 and G2 glycoproteins accumulate (Matsuoka et al., 1991). Nucleocapsids assembled from newly synthesized N protein and RNA genomes interact with the glycoproteins and accumulate on the cytoplasmic side of the Golgi complex (Matsuoka et al., 1991). Mature virions form by budding into the cisternae of the Golgi complex. Vesicles containing mature virions are released from the Golgi complex and are transported to the plasma membrane. Release of the virions from cells most likely occurs by fusion of the cytoplasmic vesicles with the plasma membrane and release by exocytosis

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(Donets et al., 1977; Smith and Pifat, 1982; Rwambo et al., 1996). Previous studies on virus maturation and release have examined the mechanisms in mammalian cells. The maturation and release process may be different in mosquito cells.

G. Requirement for host cell protein synthesis during viral replication.

Because bunyaviruses use their own polymerase to transcribe and replicate their RNA genome, it should be unnecessary for host cell transcription and translation to occur. Studies of bunyavirus RNA synthesis showed that in the presence of actinomycin D (Kascak and Lyons, 1977; Obijeski and Murphy, 1977), which is an inhibitor of DNA-dependent RNA synthesis, transcription and replication could still occur, and in the presence of puromycin and cycloheximide (Eshita et al., 1985), which are inhibitors of protein synthesis, bunyavirus transcription and replication could still occur, but at reduced levels.

Studies have shown that bunyavirus replication requires ongoing protein synthesis in host cells (Abraham and Pattnaik, 1983; Pattnaik and Abraham, 1983; Patterson and Kolakofsky, 1984; Raju and Kolakofsky, 1986; Gerbaud et al., 1987). Puromycin and cycloheximide did not inhibit primary transcription when used early in infection, but when used later in the infection cycle, inhibited secondary viral transcription (Abraham and Pattnaik, 1983). Analysis of the mRNA transcripts early in infection showed that in treated cells the transcripts were truncated, suggesting that transcription terminated prematurely. The same studies in rabbit reticulocyte lysates, which permit active translation to occur, showed that viral mRNA transcription occurred without inhibition (Bellocq et al., 1987; Raju and Kolakofsky, 1987). These studies revealed that immediate translation of newly synthesized viral mRNAs was required for complete transcription to occur. When translation of the newly synthesized viral mRNAs was blocked, transcription was initiated but not

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completed, leading to premature truncation of transcripts (Bellocq and Kolakofsky, 1987). This suggests that transcription and translation of bunyavirus mRNA are coordinated (Bellocq et al., 1987; Bellocq and Kolakofsky, 1987; Vialat and Bouloy, 1992) and translation is required for mRNA and vRNA synthesis (Raju and Kolakofsky, 1986).

The translational requirement for LAC virus replication has been shown to be dependent upon cell type. In C6/36 mosquito cells, viral replication did not require coordinated translation of viral mRNA and premature transcription termination was not observed, while in vertebrate cells, ongoing translation was required, suggesting that factors present in vertebrate cells may be required for translation (Raju et al., 1989).

H. Comparison of bunyavirus replication in vertebrate and invertebrate cells.

Arboviruses are able to infect and replicate in vertebrate and invertebrate cells. Viral replication, however, in these two different systems has different effects on the host cells. In vertebrate cell culture, bunyavirus infection is highly cytopathic. In BHK-21 cells, LAC virus induces a generalized mRNA instability, host cell protein synthesis is shut down, apoptosis is induced, and the cells lyse and die (Raju and Kolakofsky, 1988, Pekosz et al., 1996). In contrast, in mosquito cells, bunyavirus infection has no cytopathic effects. Host cell protein synthesis is not shut down and viruses are able to establish noncytopathic persistent infections (Newton et al., 1981; Nicoletti and Verani, 1985; Carvalho et al., 1986; Elliott and Wilkie, 1986; Rossier et al, 1988). Persistent infection can be maintained indefinitely and can inhibit virus superinfection (Elliott and Wilkie, 1986). However, in LAC virus transovarially-infected adult mosquitoes a complete superinfection block was not exhibited. Approximately 20% of transovarially-infected female mosquitoes became orally

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superinfected after ingestion of a blood meal containing either LAC or SSH virus (Borucki et al., 1999).

It is poorly understood how bunyaviruses are able to establish noncytopathic persistent infections in invertebrate cells. In Bunyamwera virus infected mosquito cells, the virus replicated more slowly and the accumulation of viral proteins and RNAs was observed later in infection than in vertebrate cells (Elliott and Wilkie, 1986). However, in LAC infected mosquito cells, the amounts of viral RNA and proteins were greater than in vertebrate cells (Rossier et al., 1988). This suggests that cytopathic effect (CPE) was not correlated with the amounts of viral RNA and protein produced. In Bunyamwera virus persistently infected mosquito cells, defective RNAs accumulate (Scallan and Elliott, 1992). The defective RNAs may play a role in establishing and maintaining a persistent infection, although this has not been tested.

In mosquito cells, the establishment of persistent LAC virus infections was correlated with a decrease in N protein synthesis over time. It has been proposed that N protein is able to control its own synthesis by encapsidating S segment mRNAs and blocking them from translation (Hacker et al., 1989). In LAC virus infected mammalian cells, 1% to 2% of viral mRNA was encapsidated by the N protein, whereas in mosquito cells 75% of the LAC viral mRNA was encapsidated by 24 hours post-infection (Raju and Kolakofsky, 1987b). This mechanism of control of N protein synthesis was hypothesized to condition noncytopathic persistent infections (Raju and Kolakofsky, 1987b; Hacker et al., 1989). In mosquito cells, LAC virus genome replication was down regulated while mRNA transcription continued unabated. This resulted in an accumulation of N protein, which then encapsidated the viral mRNAs. Thus the N protein controls its own translation and the translation of the other viral

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proteins. This regulation led to a slow accumulation of other viral proteins (Rossier et al., 1988). Accumulation of viral RNAs and proteins was slower in mosquito cells than in mammalian cells, which could be due to less efficient expression of the polymerase in the mosquito cells (Kolakofsky and Hacker, 1991). There was a decrease in viral RNA levels in infected mosquito cells by 24 hours post-infection. The rate of LAC protein translation and genome replication were reduced even as mRNA transcription remained unchanged. This suggests a level of translational control in mosquito cells. Also, as mentioned previously, ongoing host cell translation was not required to maintain virus replication in mosquito cells but was required in mammalian cells (Raju et al., 1989). Alternatively, viral proteins may be more efficiently processed into mature virions in mosquito cells than in mammalian cells (Newton et al., 1981). Any one or any combination of these mechanisms could be the means by which LAC virus is able to establish a noncytopathic persistent infection in mosquitoes and mosquito cell culture.

Replication of bunyaviruses in their arthropod vectors mirrors that in cell culture. Most arboviruses establish noncytopathic persistent infections in their invertebrate hosts. In mosquitoes, LAC virus establishes a persistent infection for the life of the host. Virus can be detected within the mosquito continuously, while no CPE are observed in the mosquito (Beaty and Bishop, 1988). The ability of an arbovirus to infect and persist in an arthropod vector without any signs of CPE remains a mystery. LAC virus infected mosquito cell cultures do not exhibit any signs of apoptosis (DNA laddering, membrane blebbing) (Borucki et al., 2002), while LAC virus infection of mammalian cell cultures does induce apoptosis (Pekosz et al., 1996). Recently, an inhibitor of apoptosis-like gene, AtIAP1, has been

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identified in *Aedes triseriatus* (Blitvich et al., 2002), and the gene product may play a role in the differences observed in infection in the disparate systems.

The mechanisms of LAC virus transcription and replication may play a role in the different outcomes of infection in vertebrate and invertebrate cells through modulation of innate immune responses (interferon-mediated antiviral pathway, apoptosis pathway, or RNA interference). Human MxA, an interferon (IFN)-induced antiviral protein (Aebi et al., 1989), has been shown to inhibit LAC virus growth when expressed in mosquito cell culture (Miura et al., 2001), as well as in mammalian cells (Frese et al., 1996), and acts independently of other IFN-induced proteins as demonstrated in IFN α/β knockout mice (Hefti et al., 1999). Indeed, NS_S in Bunyamwera and Rift Valley fever virus infected mice inhibits the interferon-mediated antiviral pathway (Bouloy et al., 2001; Bridgen et al., 2001; Weber et al., 2001; 2002). Mosquito cells would not be affected due to their lack of interferon and thus interferon-mediated antiviral pathways. Anopheline mosquitoes do have Dicer-like RNase III enzymes (K. Keene, unpublished data; Blandin et al., 2002), which do possess the ability to recognize and degrade double-stranded RNA (dsRNA), and RNAi has been demonstrated in *Aedes aegypti*. (I. Sanchez-Vargas, unpublished data; Adelman et al., 2002; Olson et al., 2002). Although not demonstrated in *Aedes triseriatus* mosquitoes, RNAi may function in persistent infection of mosquitoes and mosquito cells. RNAi is a process by which cells recognize dsRNA and degrade cognate mRNA through a sequence specific silencing pathway (Zamore, et al., 2000; Brantl, 2002; Hannon, 2002; Martinez et al., 2002). In the proposed silencing pathway, Dicer would degrade dsRNA introduced from aberrant transcription, RNA virus infection, transfection, or designed transgene expression, and unwind the cleavage products with an ATP-dependent helicase activity. The degraded small

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interfering RNA (siRNA, 21 to 22 nucleotides in length) molecules would then be incorporated into an RNA Induced Silencing Complex (RISC), which would then cleave targeted mRNA in a sequence specific manner and establish a resistant state (Zamore, et al., 2000; Brantl, 2002; Hannon, 2002; Martinez et al., 2002). Perhaps the encapsidation of LAC viral genomic RNA and virion complementary RNA prevents the establishment of a possible RNAi pathway by masking virus-specific dsRNA allowing for virus transcription and replication to continue unabated. Recently, NS_S of tomato spotted wilt virus (TSWV, family Bunyaviridae, genus *Tospovirus*) has been demonstrated to be a suppressor of RNAi in plant cells (Takeda et al., 2002) and probably has dsRNA binding activity. Cap scavenging differences and/or preferences in vertebrate and invertebrate cells have also been hypothesized to be a cause for the different outcomes to LAC virus infection (Dobie et al., 1997).

I. Cap scavenging.

Cap scavenging has been observed in a variety of bunyaviruses including LAC (Patterson et al., 1984; Patterson and Kolakofsky, 1984; Dobie et al., 1997), SSH (Bishop et al., 1983), Germiston (Bouloy et al., 1990), Hantaan (Garcin et al., 1995), Sin Nombre (Hutchinson et al., 1996), Dugbe (Jin et al., 1993b), tomato spotted wilt (Kormelink et al., 1992; van Poelwijk et al., 1996; Duijsings et al., 1997; 2001), Bunyamwera (Jin et al., 1993a), and Uukuniemi (Simons and Pettersson, 1991) viruses. Unlike influenza viruses, bunyaviruses scavenge oligonucleotide primers from mature host mRNAs in the cytoplasm rather than from newly synthesized host mRNAs in the nucleus (Krug, 1981; Plotch et al., 1981; Leahy et al., 1997; 1998). Studies using anti-cap antibodies showed the presence of caps on scavenged primers (Hacker et al., 1990) and that LAC virus transcription can be

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stimulated with methylated cap analogs as well as naturally occurring capped mRNAs (Patterson et al., 1984). As stated previously, the capped primers are recognized and cleaved by the endonuclease activity of the L protein (Patterson et al., 1984). Expression of the L protein of Bunyamwera virus by a recombinant vaccinia virus localized the endonuclease activity to the L protein (Jin et al., 1993a).

There does appear to be a nucleotide preference at the cleavage site of the endonucleases of certain bunyaviruses. Preferences for a single nucleotide or nucleotide motifs at positions –1 to –3 of the 5' terminal extensions of viral mRNA have been observed. Germiston virus capped primers display a U or G residue at the –1 position (Bouloy et al., 1990), SSH virus an A residue (Bishop et al., 1983), and LAC virus a U residue (Dobie et al., 1997). For the phleboviruses, Rift Valley fever and Uukuniemi, a C residue was observed at the –1 position of N and NS_S mRNAs (Simons and Pettersson, 1991) as well as a C residue for the nairovirus Dugbe (Jin et al., 1993b). For the hantaviruses Hantaan and Sin Nombre, a G residue was observed at the –1 position of mRNAs (Garcin et al., 1995; Hutchinson et al., 1996). In tomato spotted wilt virus mRNAs, no specific nucleotide or nucleotide motif was observed (van Poelwijk et al., 1996), but when TSWV-infected cells were co-infected with another plant virus, alfalfa mosaic virus (AMV), a preference for capped primers with an A residue at the –1 position cleaved from the AMV mRNAs was observed (Duijsings et al., 1997; 2001). A specific full-length capped primer was observed to be preferentially cleaved in LAC infected mosquito cells and mosquito embryos (Dobie et al., 1997). In comparison of embryonating and diapausing eggs, different specific capped primers were observed (Dobie et al., 1997). The restricted heterogeneity of capped primers may have been a function of a reduced diversity in the pool of host mRNA or may have been a function of a

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preference of LAC virus for specific capped primer sequences. To date, no studies have been conducted to determine if secondary structure of host mRNAs plays a role in recognition and cleavage of capped primers from host mRNAs.

Specific primer sequences or preferred 3' terminal residues on the capped primer suggest that a specific set of host mRNAs are used as a source of capped primers. In studies that revealed the scavenged 3' terminal nonviral nucleotides were complementary to the 5' viral sequence, it was hypothesized that after hybridization of the capped primer to the template and transcription of two or three nucleotides of the viral mRNA, the viral polymerase could slip backward on the template before further elongation (Garcin et al., 1995). This would result in the observed reiteration of the 5' terminal residue(s). This mechanism, called "prime and realign" was proposed for transcription of mRNAs for Hantaan virus (Garcin et al., 1995). Single base complementarity between the capped primer and the template vRNA may be sufficient for "prime and realign" transcription (Duijsings et al., 2001). Although an interesting possibility, the prime and realign mechanism may not be a prerequisite for the initiation of transcription of all bunyaviruses.

Another possibility to account for the homogeneity of donors for cap scavenging is that late in infection, scavenging of capped primers from viral mRNAs may occur. One study showed that levels of LAC mRNA decreased as infection in mammalian cells progressed. During the early portion of the infection cycle, host cell actin mRNA amounts decreased as LAC mRNA amounts increased (Raju and Kolakofsky, 1988). As the infection continued, LAC mRNA decreased to levels similar to those of the host cell mRNAs. This result could be a function of the viral polymerase scavenging caps from its own mRNA as the pool of host mRNA is reduced or removed. This would leave the uncapped viral mRNAs

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susceptible to host cell exonuclease degradation and thus reduction in the level of viral mRNA. To prevent removal of capped primers from viral mRNAs, influenza (family Orthomyxoviridae, genus *Orthomyxovirus*) viral mRNAs are protected from cap scavenging by the complex of proteins that catalyze mRNA synthesis (Shih and Krug, 1996; Lamb and Krug, 2001). The template vRNA functions as a cofactor in cap scavenging in that binding of the polymerase proteins PB2 to the 5' end of capped cellular mRNA and PB1 to the 3' conserved end of the influenza vRNA initiates cap scavenging (Lamb and Krug, 2001). PB2 contains a tryptophan-rich domain believed to be similar to the cap-binding domain of the eIF4E translation initiation factor (Marcotrigiano et al., 1997; Matsuo et al., 1997). This binding functions to protect viral mRNAs; a second binding site for the PB2 is present just downstream from the 5' end of viral mRNA. The binding of the PB2 to this region blocks removal of caps from the viral mRNA (Shih and Krug, 1996).

In bunyaviruses, the conserved terminal sequences may act as recognition sequences for the N and L proteins, and the vRNAs may act as cofactors for cap scavenging. A similar mechanism that blocks scavenging of viral mRNA caps for influenza virus could function to protect bunyavirus mRNAs from being scavenged for capped primers in that an alternate binding site for the polymerase may be present near the 5' end of the viral mRNA. This has yet to be demonstrated as functioning in bunyavirus transcription.

J. Quantitative analysis of bunyavirus RNA species.

Recent technological advances have allowed for the direct quantitation of the three RNA species found in bunyavirus infected cells. Real-time polymerase chain reaction (PCR) using fluorescent reporter-tagged probes and primers for specific targets allows for the quantitation of RNA species (by weight, molarity, or copy number) under standard

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conditions (reviewed in Freeman et al., 1999). This technology has been used to detect and quantitate RNA of several bunyaviruses in cell culture, infected plants and mammals, and in arthropod vectors. For tomato spotted wilt virus, as few as 1,000 copies of the S RNA genomic segment were detectable in as little as 500 fg of total RNA from infected plant tissue (Roberts et al., 2000). Similar results were observed in detection of tomato spotted wilt virus S genomic RNA in laboratory- and naturally-infected thrips (Boonham et al., 2002). Following initial reverse transcription, Black Creek Canal virus S segment vcRNA could be detected in quantities as small as the equivalent of 100 $TCID_{50}$/ml (Hutchinson et al., 1998), Puumala virus genomic S RNA could be detected in quantities as small as 10 $TCID_{50}$/ml (Garin et al., 2001), and Sin Nombre virus genomic S RNA could be detected in quantities as small as 10,000 $TCID_{50}$/ml (Hutchinson et al., 1996).

A quantitative real-time analysis of RVF virus was developed to determine the effects of antiviral compounds on synthesis of viral genomic RNA (Garcia et al., 2002). Quantitative real-time assays are also being developed for diagnosis of Crimean-Congo hemorrhagic fever virus infection (M. Vincent, personal communication). To date, all of the real-time analyses developed to examine RNA levels of bunyaviruses have been focused on surveillance or diagnosis in arthropod vectors or mammalian or plant hosts, pathogenesis in vertebrate host species, or a combination of diagnosis and chemotherapy in mammalian hosts. The technology has yet to be used to study the interactions of bunyaviruses with arthropod vectors or mammalian or plant hosts at different stages of infection or disease or under different physiologic or metabolic conditions.

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K. Natural history and epidemiology of LAC virus.

1. Introduction. Arboviruses are maintained in nature in distinct cycles involving specific arthropod vectors and susceptible vertebrate hosts (Beaty and Bishop, 1988; Beaty and Calisher, 1991). In nature, arbovirus transmission cycles may overlap geographically but usually remain separate. The relationship between the arbovirus and the arthropod vector is highly evolved. The interactions of LAC virus and its natural vector *Ae. triseriatus* have been well studied both in nature and in the laboratory.

LAC virus was first isolated in 1965 from the brain of a child who died from encephalitis in La Crosse, Wisconsin, in 1960 (Thompson et al., 1965). It was identified as a California serogroup virus distinct from California encephalitis virus and named for the location of isolation. Oligonucleotide fingerprint mapping has identified three geographically distinct varieties of LAC virus: types A, B and C (Klimas et al., 1981).

2. Distribution. A number of viruses of the California serogroup have been isolated in North America. LAC virus is the etiologic agent of most of the clinical cases of California serogroup viral encephalitis (Calisher, 1994). LAC virus is distributed throughout the eastern half of the United States and states bordering the Mississippi River (Calisher, 1983; 1994). Human infections occur throughout the geographic range of LAC virus, with the majority of cases reported in the upper Midwestern states (LeDuc, 1987; Grimstad, 1988; Calisher, 1994; Rust et al., 1999; McJunkin et al., 2001). Up to 90% of LAC encephalitis cases have historically been reported in Wisconsin, Minnesota, Illinois, Indiana and Ohio (Beaty and Calisher, 1991; Griot et al., 1994), although this may be due to higher levels of surveillance and reporting rather than virus virulence or other epidemiological factors. Recently, increased infection rates have been reported in eastern Tennessee (Jones et al., 1999; Jones,

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2000; Erwin et al., 2002), North Carolina (Szumlas et al., 1996a; 1996b; 1996c), and West Virginia (Nasci et al., 2000). The natural cycle of the virus is associated with small mammals and *Ae. triseriatus* mosquitoes in deciduous hardwood forests throughout the geographic distribution. In rural, suburban, and recreational areas, humans are exposed to LAC virus when their activities place them in close proximity to LAC virus infected mosquitoes (Monath et al., 1970). Human habitation near forested areas greatly increases the risk of infection (Beaty and Calisher, 1991; Woodruff et al., 1992). Also, the presence of water holding containers, either natural or man-made, increases the risk of infection.

3. Clinical manifestations of LAC virus infection. LAC encephalitis is the most commonly reported form of pediatric arboviral encephalitis in the United States (Calisher, 1994; Rust et al., 1999). Reports of LAC virus infections range from 42 to 174 cases per year (Tsai, 1991; Griot et al., 1994), although the number of human infections is much higher than the number of reported cases. Indeed, infection with LAC virus is greatly underreported. One report estimated that the number of human infections per year exceeded 100,000 (Grimstad and Haramis, 1984). Grimstad (1988) reported that for every case reported in children less than 15 years of age, there are more than 1,000 infections. It is estimated that less than 1.5% of LAC infections are manifested clinically (Grimstad, 1988; Tsai, 1991). Epidemiological case analysis estimates that the incidence of infection in endemic regions is 20 to 30 infections per 100,000 people. This incidence far exceeds the incidence of bacterial meningitis (McJunkin et al., 2001).

Most reported cases of LAC encephalitis occur in children under the age of 12 years. The incubation period following an infectious bite of a mosquito is unknown. Most LAC virus infections are asymptomatic. A mild form of clinical disease is characterized by a

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prodromal period of 2 to 4 days with fever (38.5 to 39°C), headache, malaise, nausea and/or vomiting (Balfour et al., 1973). Fever may increase to 39.5 to 40°C by day 3 with increased lethargy and signs of central nervous system (CNS) involvement. Symptoms usually subside within 7 to 8 days, followed by full recovery without long-term neurologic sequelae.

The more severe form of the disease is marked by an abrupt onset of 39°C fever and headache followed by sudden onset of focal or generalized seizures within 12 to 24 hours. Of patients hospitalized in La Crosse, Wisconsin, with LAC encephalitis, 42% had seizures during or after the acute illness (Rust et al., 1999). If seizure activity is prolonged or unmanageable, intensive supportive care with assisted respiration may be necessary. The average hospitalization time for patients with the severe form of the disease is 12 days. Long-term neurologic sequelae have been associated with the severe form of disease. LAC infection is highly epileptogenic (Rust et al., 1999). Learning disabilities, cognitive defects, attention deficit and hyperactivity have all been associated with LAC virus infection of the CNS (Rust et al., 1999; McJunkin et al., 2001). One study showed that 36% of LAC patients had IQ scores of 79 or less (borderline intelligence to mental retardation) in follow-up tests 10 to 18 months after hospitalization (McJunkin et al., 2001). A serologic survey for LAC antibody prevalence in people institutionalized in Wisconsin for permanent mental disorders revealed that antibody prevalence was twice as high as in the general population (Gauld et al., 1979). There are also significant social and economic impacts for individuals with and families of individuals with LAC encephalitis. A recent survey of 25 cases of LAC encephalitis in western North Carolina revealed that direct and indirect cost of treatment and convalescence health care can exceed $280,000 (2001 U.S. dollars adjusted) and severe cases can exceed $3,000,000 (2001 U.S. dollars adjusted) (Utz et al., 2002).

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The case fatality rate of LAC encephalitis is low (0.3%) (Calisher, 1994). Studies from two fatal cases revealed the effects of LAC virus infection on the brain. Severe inflammation was observed in the cerebral cortex with smaller lesions in the subcortical nuclei (Thompson et al., 1965; Kalfayan, 1983). Lesions were not observed in the cerebral white matter, cerebellum, medulla oblongata, or spinal cord.

4. Natural cycle of LAC virus and *Aedes triseriatus*. The natural cycle of LAC virus involves the mosquito vector *Ae. triseriatus* (Watts et al., 1972) and the vertebrate hosts, the gray squirrel (*Sciurus carolensis*) and the eastern chipmunk (*Tamias striatus*) (Moulton and Thompson, 1971; Kziazek and Yuill, 1977; Beaty and Calisher, 1991). *Ae. triseriatus* is a woodland mosquito that breeds in water-containing vessels, especially tree holes. The mosquito is also able to use man-made water containing vessels, such as discarded tires, as breeding sites (Beaty and Calisher, 1991; Calisher, 1994).

During the active mosquito transmission season of spring and summer, LAC virus amplification and maintenance depend primarily on mammalian reservoir hosts. Humans act as tangential or incidental hosts. Virus titers in chipmunks and ground squirrels range from 1.5 to 4.6 $\log_{10}$ $SMICLD_{50}$/0.025 ml. Mammalian reservoir hosts exhibit no signs or symptoms of infection other than increased antibody titer following infection (Pantuwatana et al., 1972) and antibody prevalence rates in adults and spring-born juvenile animals can reach 100% by September in an annual transmission cycle (Gauld et al., 1974).

LAC virus can be transmitted transovarially from an infected adult female mosquito to her progeny during oogenesis (Watts et al., 1973; 1974) and can be amplified vertically even if LAC antibodies were present in the host that was fed upon. As the progeny mosquitoes emerge as adults they are infected and capable of transmitting the virus (Watts et

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al., 1974; Beaty and Thompson, 1975; 1976). Viral amplification through transovarial transmission (TOT) probably occurs during the summer mosquito season. Transovarial transmission is responsible for LAC virus maintenance during winters in temperate climates (Watts et al., 1974). LAC virus is able to overwinter in diapausing *Ae. triseriatus* eggs (Watts et al., 1974) and upon emergence in the spring, infected mosquitoes are able to initiate the virus transmission cycle (Watts et al., 1974; Beaty and Thompson, 1975; 1976). LAC virus was found in 0.6% of larvae from four field sites prior to seasonal emergence of adults and in 1.2% of adult *Ae. triseriatus* mosquitoes processed from the same area throughout the season (Beaty and Thompson, 1975). From tree holes, 0.9% (1/110) of larvae were found to be infected, while from discarded tires 5.0% (1/20) were found to be infected (Watts et al., 1974).

LAC virus can be transmitted transovarially to offspring of both sexes. Upon emergence, infected males are able to venereally transmit the virus to uninfected female mosquitoes during mating (Thompson and Beaty, 1977; Beaty and Thompson, 1978). The rates of venereal transmission are very low; only 4% of mated females become infected, but if the female has ingested a blood meal prior to mating the infection rate reaches 50% (Thompson, 1979) for unknown reasons.

Recent evidence suggests that *Aedes albopictus* is able to transovarially transmit LAC virus and may act as the primary vector of LAC virus in the southern United States (Gerhardt et al., 2001; Erwin et al., 2002). Indeed, exposure to *Ae. albopictus* mosquitoes appears to be a major risk factor for LAC virus infection in the southern United States (Jones et al., 1999; Erwin et al., 2002).

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5. Laboratory studies of LAC virus and *Aedes triseriatus* mosquitoes.

Arboviruses must be able to pass multiple infection and dissemination barriers in order to successfully infect and be transmitted by an arthropod vector (Hardy, 1988; Beaty and Bishop, 1988). Extensive laboratory studies of LAC virus and *Ae. triseriatus* interactions have provided valuable information and a model system for studying arbovirus-vector interactions (Beaty and Bishop, 1988).

a. Virogenesis following a blood meal. Early studies used immunofluorescent techniques to study the progression of LAC virus infection in *Ae. triseriatus* mosquitoes (Beaty and Thompson, 1976; 1978). The efficiency with which virus dissemination from the midgut to other tissues occurs is dependent upon both the virus and the vector (Beaty et al., 1981; 1982; Hardy, 1988; Bishop and Beaty, 1988; Paulson et al., 1989). After ingestion of a LAC virus infected blood meal, virus antigen was detected in the posterior portion of the midguts of *Ae. triseriatus* mosquitoes, with large amounts of antigen accumulation by 7 days post-infection. LAC virus was observed to spread throughout the midgut to the foregut and was believed to spread cell to cell (Beaty and Thompson, 1976; 1978). Within 10 days post-infection, LAC virus disseminated from the midgut cells to secondary tissues and organs by the hemolymph. Antigen was undetectable or only detected late in infection in hindgut and Malpighian tubule tissue (Beaty and Thompson, 1978). The virus also replicated in the heart, neural ganglia, fat body and salivary gland tissue of the mosquito (Beaty and Thompson, 1978). After infection and replication in the salivary glands of the mosquito, the virus can be transmitted to vertebrate hosts through mosquito saliva during feeding (Woodring et al., 1996). The total extrinsic incubation period, from ingestion to transmission, of LAC virus in

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Ae. triseriatus is between 7 and 16 days (Watts et al., 1972; Beaty and Thompson, 1978). Mosquitoes remain infected for life and show no adverse effects of virus infection.

Viral antigen can be detected in ovarian follicles. The ovaries become permeable following a blood meal to allow transport of nutrients into the follicle, which may also allow virus infection. Recent evidence has shown that LAC virus antigen and transcripts can be detected in ovary associated tissue as soon 2 days following an infectious blood meal (Chandler et al., 1998). It is believed that this antigen is present in the calyx or other accessory tissue and not the ovarian follicle proper. This is probably due to an artifact of feeding mosquitoes on very high-titered artificial blood meals. Virus may infect these tissues by spreading from the bursa (Chandler et al., 1998). Tracheole infection by LAC virus provides a potential route for ovary infection early after ingestion of an infectious blood meal; LAC virus could be transported directly to the ovaries by the mosquito respiratory system prior to dissemination (Chandler et al., 1998).

b. Barriers to infection. Arboviruses encounter several barriers within the mosquito that may prevent productive vector infection of tissues or cells or dissemination from one tissue to another. There is inter- and intra-specific variation in these barriers both between viruses, between vectors, and between viruses and vectors (Hardy et al., 1983; Hardy, 1988). This variation appears to be under genetic control and may vary within a mosquito population. These barriers contribute to the epidemiology of arboviral diseases. The barriers present include midgut infection (MI) and escape (ME) barriers, ovarian infection (OI) and escape (OE) barriers, and salivary gland infection (SGI) and escape (SGE) barriers (Hardy et al., 1983; Hardy, 1988; Woodring et al., 1996).

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The first barrier that must be passed for a virus is the MI barrier (Hardy et al., 1983; Hardy, 1988; Woodring et al., 1996). For LAC virus, titers in the small mammal host must exceed 3.2 log_{10} $SMICLD_{50}$/0.025 ml for the virus to efficiently infect the *Ae. triseriatus* through a blood meal, although as little as 1.8 to 2.0 log_{10} $SMICLD_{50}$/0.025 ml of virus is able to infect mosquitoes (Patrican et al., 1985). The virus must be able to infect and replicate within the midgut epithelial cells and an infection threshold must be passed in order for efficient LAC replication and dissemination into the hemocoel (Hardy et al., 1983). Many physiological and morphological changes occur in the midgut epithelial cells following ingestion of a blood meal (Houk and Hardy, 1982). The midgut and abdomen become fully distended due to the volume of blood ingested. This volume of blood ingested is controlled by physical stimuli via the ventral nerve cord (Edman and Spielman, 1988). The changes that occur allow for digestion of the blood meal and transport of the nutrients across the epithelium into the hemocoel. This nutrient transport may aid viral dissemination to organs throughout the mosquito and aid in its transmission (Hardy, 1988).

Midgut infection barriers, although frequently reported for CAL serogroup viruses, are rare for LAC virus-*Ae. triseriatus* interaction (Beaty et al., 1981; 1982; Paulson et al., 1989). However, heterologous CAL serogroup viruses are restricted in productive vector infection by midgut and salivary gland escape barriers. The genetic bases of MI and ME barriers in *Ae. triseriatus* were elucidated through the use of reassortant viruses. Reassortant viruses with LAC and SSH M RNA segments infected *Ae. triseriatus* midguts at equal rates, but the viruses with LAC M RNA segment disseminated in 98% of the infected mosquitoes, while only 26% of those containing a SSH M RNA segment disseminated (Beaty et al., 1982).

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Reassortant viruses were also used to demonstrate the presence of SGE barriers. Reassortant viruses with LAC and SSH M RNA segments infected *Ae. triseriatus* salivary glands at equal rates following intrathoracic inoculation. However, 93% of the mosquitoes infected with viruses containing a LAC M RNA segment transmitted virus while only 35% of those infected with virus containing a SSH M RNA segment transmitted virus (Beaty et al., 1981).

Viruses with the SSH M RNA segment were less likely to overcome the ME and SGE barriers following infection. These results show that the major determinants for ME and SGE in *Ae. triseriatus* mosquitoes is found on the M RNA segment.

6. Transovarial transmission of LAC virus by *Aedes triseriatus*. *Ae. triseriatus* mosquitoes overwinter in temperate regions as diapausing embryos. The ability of LAC virus to overwinter in the diapausing embryo of *Ae. triseriatus* was a major discovery in arbovirus research. Isolation of LAC virus from *Ae. triseriatus* larvae collected in tree holes in Wisconsin early in spring revealed the mechanism by which LAC virus is able to overwinter (Pantuwanta et al., 1972). Further studies in colonized *Ae. triseriatus* confirmed these results (Watts et al., 1973; 1974).

Studies were conducted to determine the anatomic basis of this phenomenon. Direct immunofluorescence was used to study virogenesis of LAC virus in transovarially-infected female and male mosquitoes (Beaty and Thompson, 1975; 1976). LAC viral antigen was detected in all infected larval tissue after metamorphosis, with the amount of viral antigen increasing with each developmental stage (Beaty and Thompson, 1976). Several larval tissues were highly permissive to LAC virus infection, including the pharynx, esophagus, pyloric ampulla and ileum (Beaty and Thompson, 1976). Newly emerged adult female

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mosquitoes contained high titers of virus in their salivary glands and were able to transmit virus immediately. Viral antigen was also detected in ovarian follicles of newly-emerged transovarially-infected adult females, demonstrating that subsequent transovarial transmission could occur directly and without an intervening infectious blood meal (Beaty and Thompson, 1976). LAC viral antigen was also detected in all organs of newly emerged adult males, including gonadal tissue and accessory glands, allowing for immediate venereal transmission (Thompson and Beaty, 1977; Beaty and Thompson, 1978).

Transovarial transmission (TOT) rates in the laboratory can far exceed those in nature. Approximately 1.2% of overwintered mosquitoes collected in tree holes in LAC endemic areas were infected (Beaty and Thompson, 1975), while in the laboratory 98% of transovarially infected females transmitted virus to their progeny (Miller et al., 1977). Although absolute direct comparison of laboratory and field studies cannot be made due to many other factors (weather, available blood source, health of mosquitoes), the results demonstrate that transovarial transmission of LAC virus by *Ae. triseriatus* female mosquitoes can be very efficient. Miller et al. (1977) showed very little variation in filial infection rates (FIR) and TOT through eight generations. The FIR remained essentially constant at a mean of 71% without an intervening infectious blood meal. There are differences in TOT efficiency in geographically distinct strains of *Ae. triseriatus*; an *Ae. triseriatus* strain from Wisconsin had a TOT rate of 50%, while strains from Connecticut and Massachusetts had lower TOT rates, 13% and 26% respectively. This suggested that the ability of *Ae. triseriatus* to transovarially transmit LAC virus is genetically controlled (Miller et al., 1982). Another field study comparing the FIR and TOT rates of *Ae. triseriatus* mosquitoes from Wisconsin and Florida showed similar results. The TOT rates for the Florida strain (78%), a

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nondiapausing strain, and the Wisconsin strain (85%), a diapausing strain, did not differ significantly. However, the FIR of the Florida strain (33%) differed significantly from the Wisconsin strain (45%) (Woodring et al., 1998). In a nondiapausing line selected from the Wisconsin strain, the TOT rates were found to be the same as wild type, but the FIR rate decreased significantly, from 45% to 34%, which was similar to the Florida strain.

Field studies of TOT and diapause (McGaw et al., 1998) demonstrated that TOT+ LAC+ embryos had a higher mortality rate (16.7%) than TOT- embryos (7.3%), although this difference was observed only after embryos emerged from diapause. Also, TOT+ embryos broke diapause earlier than TOT- embryos, suggesting that transovarially infected mosquitoes could establish transmission cycles earlier in the mosquito transmission season (McGaw et al., 1998). When two Wisconsin *Ae. triseriatus* strains were selected for high and low FIR and TOT, the TOT rates were reduced from 72% to 14% within three generations of selection, and the FIR was reduced from 18% to 3% within the same time frame (Graham et al., 1999). This study suggested that TOT is controlled by a single genetic locus within the *Ae. triseriatus* genome and that permissiveness to infection is conditioned by a dominant allele. This study also suggested that FIR is not controlled genetically but rather by factors influencing the virus (virulence, infectivity, and titer) and the mosquito (environment, nutrition, and availability of blood) (Graham et al., 1999).

L. Mosquito ovarian structure and oogenesis

1. Introduction. Ovarian structure and oogenesis have been studied in a variety of mosquitoes. Most information is from studies with *Aedes aegypti* and *Culex pipiens*, two distinct but important disease vectors. Oogenesis is a cyclical process initiated by a blood meal and terminated at oviposition of a batch of eggs (Sokolova, 1994). A newly emerged

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adult female mosquito will begin searching for a blood meal within 3 to 4 days after emergence (Edman and Spielman, 1988). The first meal ingested by an adult female is usually sugar from a nectar source, which may be necessary to replenish energy reserves (Clements, 1992).

2. Structure of the mosquito ovary. The mosquito ovary is of the polytrophic meroistic type typical of dipterans and other higher insects (King and Buning, 1985). Meroistic ovaries are characterized as having follicles consisting of an oocyte and nurse cells and the capability to produce large numbers of eggs (King and Buning, 1985).

Mosquito ovaries are present as a functional pair composed of between 50 and 500 ovarioles surrounded by an ovarian sheath. The ovarian sheath contains a membranous layer covered by a layer of muscle cells (King and Buning, 1985; Clements, 1992). Each ovariole connects to the common oviduct via epithelial tissue, or calyx, at the center of each ovary. Two or three follicles, depending on the mosquito species, and the germarium, or stem cell, comprise each ovariole (Clements, 1992). Each of the follicles in an ovariole is in a different developmental stage. The mosquito respiratory system enters the ovaries as two tracheae, which bifurcate throughout the ovaries and end as tracheoles in the ovarioles (Clements, 1992).

Each ovariole is composed of the germarium, the cell destined to become an egg, and vitellarium, which contains one or two follicles, all surrounded by the follicular epithelium (Clements, 1992). Follicles are produced by the germarium and continue to differentiate and develop as they move toward the oviduct. Nurse cells within the follicle produce nutrients that are transported into the oocyte during oogenesis. Follicular development is categorized

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as primary follicles, those that are most mature and closest to the calyx, and secondary follicles, those that are only partially developed (Clements, 1992).

3. Ovary developmental stages. Ovarian follicles are held in an arrested developmental state, or quiescence, following initial development (Clements and Boocock, 1984). Upon ingestion of a blood meal, the arrest is broken, ovaries become metabolically active and eggs begin to develop (Clements, 1992). Clements (1992) defined four distinct developmental stages of the ovaries: 1) Previtellogenic phase, 2) Initiation phase, 3) Trophic phase, and 4) Post-trophic phase. Each ovary developmental stage is separated from the next by a developmental gate, which allows for coordination of developing follicles with blood feeding and oviposition. Cycles of ovarian growth and development alternate with periods of developmental arrest (Clements and Boocock, 1984). Each arrest in development is ended with the ingestion of a blood meal, which is followed by ovarian growth and increased metabolic activity.

The previtellogenic phase is characterized by some growth followed by a resting stage. This stage has two developmental gates (germarial gate and stage I gate) that must be passed to allow for follicular separation from the germarium. The hormones 20-hydroxyecdysone and juvenile hormone provide the stimuli to pass these gates and also prepare the fat body for vitellogenesis. After the follicles have separated from the germarium, they reach a previtellogenic gate that requires a blood meal stimulus to pass (Clements, 1992).

The initiation phase immediately follows a blood meal. Follicular growth is stimulated and vitellogenesis is initiated. Ovarian ecdysteroidogenic hormone is released and stimulates the ovaries to produce and secrete ecdysone. The ecdysone is converted to 20-

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hydroxyecdysone and stimulates increased vitellogenesis. The trophic phase is the main period for vitellogenin synthesis and oocyte growth, and it ends with the formation of the endochorion. The post-trophic phase is characterized as egg maturation and formation of the exochorion. A final developmental gate is reached, and oocyte meiosis can only be completed upon fertilization by sperm passing through the micropyle at oviposition (Clements, 1992).

4. Oogenesis or egg development. Blood feeding is the major stimulus for egg development and a new group of oocytes begins to mature with each blood meal ingested by the female. Nutrition, photoperiod and environmental temperature also contribute to stimulating egg development (Clements, 1992). Shortly after blood feeding, protein synthesis is increased in fat body and ovaries, and mRNA and rRNA synthesis is increased in nurse cells (Clements, 1992). Egg yolk, to be deposited in the maturing egg, is synthesized in a process known as vitellogenesis. The egg yolk contains all of the nutrients necessary for embryogenesis to occur (from oviposition of fertilized egg to hatching). Vitellogenin (a secretory precursor to vitellin, the major protein in yolk) is synthesized by fat body, released into the hemolymph, and transported into the oocyte within the ovary. Vitellogenins bind to receptors on the surface of the ovary and are internalized into the oocyte in clathrin-coated pits via receptor-mediated endocytosis (Koller et al., 1989; Clements, 1992). Inside the oocyte, vitellogenin is crystallized into the final form of yolk protein, vitellin. Nurse cells synthesize large amounts of mRNA and rRNA that are transported into the developing oocyte. The egg yolk also contains lipids and enzymes (Clements, 1992). Follicular epithelial cells secrete the endochorion and exochorion (egg shell) in two stages and finally end egg development (Clements, 1992). The total time for egg development for mosquitoes

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varies, depending on species, but is usually completed in four to seven days. Upon depositing of eggs by the female mosquito, the follicles return to the previtellogenic phase and remain in this inactive phase until another blood meal is ingested.

Follicular development occurs synchronously within the mosquito ovary. Within one ovarian cycle, while the primary oocytes develop into mature eggs, secondary follicles develop into primary follicles, and the germarium produces new secondary follicles (Clements, 1992). Gene expression within the mosquito ovary has not been well-studied. It has been observed in *Aedes aegypti*, that during ovary quiescence after an initial blood meal, RNA levels are decreased and do not increase until another blood meal is ingested (Banks et al., 1994). Peak RNA levels in ovaries were observed between 36 and 48 hours after a bloodmeal, and peak levels of protein synthesis were observed between 48 and 72 hours (Banks et al., 1994). The molecular mechanisms that control gene expression, oogenesis, and follicular metabolism are poorly understood. Subtractive cDNA libraries from metabolically active and quiescent ovaries are needed to better understand gene regulation and expression at each developmental stage of the ovary.

The size of the egg batch of a mosquito is determined by numerous factors including mosquito species, size and quality of blood meal and the nutritional state of the female mosquito (Clements, 1992). An egg batch can range in size from 50 to 500 eggs. Upon oviposition the egg is soft, white and water permeable (Clements, 1992), but as time progresses, the egg becomes dark and hard as the chorion sclerotizes. The embryo within the egg develops into a pharate larva prior to hatching. Following completion of embryogenesis, the pharate larva remains quiescent until proper hatching stimuli are received or it enters diapause. Hatching stimuli include flooding with water, reduced oxygen content in water,

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temperature, or photoperiod (Gjullin et al., 1941; Judson, 1960). Pharate larvae hatch from the eggs, molt through a total of four larval instar stages and a pupal stage and emerge as adults (Clements, 1992). Following diapause in northern latitudes, the eggs hatch in the spring, larvae mature, and adults emerge in May or June, depending on temperature (Thompson, 1974).

M. Diapause

1. Introduction. Diapause is a state of arrested growth and development that enables a species to overwinter or estivate, or to synchronize its developmental cycle to the seasons (Mitchell, 1988; Denlinger, 2002). It is a state of reduced metabolic activity that allows survival during adverse environmental conditions, such as extremes in temperature, both heat and cold, and drought. Diapause can be distinguished from quiescence, another form of dormancy (Tauber et al., 1986). Diapause is actively induced and metabolic activity is suppressed even if the adverse environmental conditions are not present. Diapause induction involves the alteration or stoppage of certain neuroendocrine signals at specific stages of life rather than a more passive response to environmental conditions (Mitchell, 1988; Denlinger, 2002). In contrast, quiescence is imposed directly by environmental conditions and recovery from quiescence is very rapid upon removal of the adverse conditions.

The induction of diapause involves perceived environmental cues prior to and not necessarily involving adverse environmental conditions (Clements, 1995). The most common diapause signal is a change in photoperiod (the number of hours of light in a given 24-hour period) (Tauber et al., 1986). For diapause to be triggered, a critical day length must generally be experienced by the insect. In insects that undergo a diapause in winter, cold

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temperatures may also play a role in diapause induction (Mitchell, 1988; Saunders and Gilbert, 1990; Clements, 1995).

Diapause may occur at any developmental stage in insects (egg, larva, pupa, adult). Each insect species usually has one life stage during which diapause occurs, although some species are capable of entering diapause in more than one life stage (including mosquitoes) (Tauber et al., 1986). In insect species that undergo an egg stage diapause, embryos at different stages of embryonic development may enter diapause. These embryonic development stages range from preblastula stage (immediately following fertilization) to pharate (first instar) larva, although the stage at which an insect egg begins diapause is species specific (Tauber et al., 1986). Egg diapause may be triggered by stimuli received by the egg or pharate larva (as is the case with mosquitoes) or may be triggered by stimuli received by the maternal parent prior to egg laying (Tauber et al., 1986; Ikeda et al., 1993).

Mosquitoes from temperate climates undergo diapause in winter months. Mosquitoes commonly diapause in the egg, larva, or adult life stage and rarely in the pupa stage (Clements, 1995). Mosquitoes, in general, only undergo diapause in one life stage, which is species specific (Clements, 1995). However, several species of *Aedes* mosquitoes, including *Ae. triseriatus*, are able to undergo diapause in two life stages, usually in the egg and larva stages (Mitchell, 1988; Clements, 1995), although the pharate larva is the primary stage for diapause (Holzapel and Bradshaw, 1980). In mosquito species that diapause as eggs, embryogenesis must be complete and the pharate larva must be fully formed. Only the fully developed pharate first instar larva is responsive to diapause stimuli (Clements, 1995).

Installment hatching is similar to diapause in that mosquitoes may not respond to hatching stimuli. This acts as a protective mechanism that allows a portion of the eggs to

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survive long periods of drying and to be viable if successive hatching stimuli occur in between adverse environmental conditions (drought or brief winter respite). However, there is little information about installment hatching; it may be more similar to quiescence than to diapause (Clements, 1995).

2. Hormonal regulation of diapause. The hormonal regulation of diapause has not been thoroughly studied or characterized in most insect species. Environmental stimuli do induce the production and secretion of hormones from the insect neuroendocrine system. Early evidence on hormonal regulation showed that in diapausing insects there was a lack of ecdysone (Denlinger, 1985), which suggested that diapause is a deficiency state (Tauber et al., 1986). More recent evidence has shown that diapause is not due to a simple presence or lack of a specific hormone but a complex array of events leading to different metabolic, growth and developmental, behavioral and reproductive outcomes (Denlinger, 2002).

The silkmoth, *Bombyx mori*, has been used extensively to study egg diapause. Diapause is induced in this species by diapause hormone, which is produced and secreted from the subesophageal ganglion of females (Ikeda et al., 1993). Diapause hormone is produced and transported to the developing eggs during oogenesis and affects the eggs post-oviposition. The eggs from females producing diapause hormone enter diapause after the female deposits them. Xu et al. (1995a) cloned and characterized the *B. mori* diapause hormone, which is a 24-amino acid protein whose expression is temperature and stage dependent. The diapause hormone was found to be expressed only in the ovaries of females that were receiving diapause stimuli (Xu et al., 1995b). The mRNAs for diapause hormone were found to be produced in the neurosecretory cells of the subesophageal ganglion (Sato et al., 1993), with expression specific to the embryo stage in a temperature sensitive manner

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(Xu et al., 1995b). Southern blots of genomic DNA to detect genes for diapause hormones in other insects showed some cross hybridization with other species including dipterans (Xu et al., 1995b). Also, laboratory synthesized diapause hormone was able to induce egg diapause (Ikeda et al., 1993).

It is not known if diapause hormones are present in mosquitoes, nor is much known concerning the hormonal and biochemical regulation of diapause in mosquitoes.

3. Control of gene expression during diapause. Metabolic activity and protein synthesis are restricted in diapausing insects. In many insects diapause is characterized by the reduction in the total level of RNA (Tauber et al., 1986). This reduction is the result of control of gene expression in a complex fashion. The control of gene expression during diapause has been characterized in *B. mori* (Denlinger, 2002).

Total RNA level in *B. mori* diapausing eggs was lower than in non-diapausing eggs (Kurata et al., 1978). Also, the number and size of nucleoli per cell was smaller in diapausing eggs than in non-diapausing eggs (Kurata et al., 1978). When diapausing eggs were able to break diapause, the overall levels of rRNA and the size and number of nucleoli increased (Kurata et al., 1978; 1979a; 1979b).

In the tobacco hornworm, *Manduca sexta*, modified mRNA caps control translation during diapause. Non-methylated guanosine residues are present at the 5'-termini of mRNAs in diapausing individuals (Kastern and Berry, 1976). The altered 5'-guanosine residue alters the ability of the mRNA to be recognized by eukaryotic protein synthesis initiation complex and translated (Kastern and Berry, 1976). These mRNAs with altered caps are translated with much less efficiency than normal, methylated cap-containing mRNA (Kastern et al., 1982). A similar control of translation is observed in *Drosophila* mRNA in that the

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modification to the 5' cap structure prevents entry of the mRNA into the polysome and thus blocks translation (Mermod et al., 1980).

It is not clear whether a translational control occurs in diapausing *B. mori* eggs. Conflicting studies found mRNAs from diapausing *B. mori* eggs to be both not translatable (Grzelak et al., 1979) and translatable (Saito et al., 1984; 1985a; 1985b; Fujiwara et al., 1988) in *in vitro* systems.

Recent reviews have looked at gene regulation during diapause in a wide range of insect species. Several genes, mostly heat-shock proteins, have been found to be upregulated throughout diapause in the fleshfly, *Sarcophaga crassipalpis*, and include *hsp70*, *hsp23,* and *hemolin*, which encodes an antibacterial protein (Yocum et al., 1998; Rinehart et al., 2000; 2002; Denlinger, 2002). An RNAi approach is being used to determine the effects of knockout of each of these genes on the induction and maintenance of diapause. *Samui*, a gene upregulated in early diapause in *B. mori* eggs is proposed to transmit the "5 degrees C signal" for sorbitol dehydrogenase (SDH) expression in diapausing eggs, while also protecting against cold-injures in non-diapausing eggs (Moribe et al., 2001). Genes upregulated late in diapause include *sorbitol dehydrogenase* in *B. mori* (Niimi et al., 1993a; 1993b; 1996), which may aid in cold-hardiness, and *defensin* in *Drosophila spp.* (Daibo et al., 2001), which may aid in protecting diapausing larvae from bacterial or fungal infection. A variety of other genes have been shown to be upregulated at various times during diapause in a variety of species (Denlinger, 2002). Genes that have been found to be downregulated during diapause include *hsp90* in *S. crassipalpis*, as well as several genes and proteins involved in cell cycle arrest and regulation, including *proliferating cell nuclear antigen* (Tammariello and Denlinger, 1998; Rinehart and Denlinger, 2000; Denlinger, 2002).

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The control of gene expression during diapause in mosquitoes has not been investigated in detail. The molecular basis of diapause in mosquitoes could be a fruitful area of research for developing new control strategies for mosquito-borne disease.

4. Diapause in *Aedes triseriatus*. *Ae. triseriatus* diapauses in the egg stage in the northern part of the U.S. and in the larva stage in the southern part of the U.S. (Baker, 1935; Love and Welchel, 1955; Kappus and Venard, 1967). Egg diapause can occur in eggs in natural oviposition sites independent of the environmental conditions when they were deposited (Shroyer and Craig, 1980). Also, *Ae. triseriatus* eggs can be repeatedly induced into diapause, resulting in the survival of eggs for two or more winters (Beaty and Thompson, 1976). The diapause in larval stages may function as a back-up system for mosquitoes that have already hatched (Holzapel and Bradshaw, 1980). Larval diapause is characterized by a delay in the pupation of fourth instar larvae and is dependent on the slow development of the larval stages due to poor quality diet, low temperature, and shortened photoperiod (Clay and Venard, 1972).

Diapause is induced in *Ae. triseriatus* by shortened day length and decreased temperature (Kappus and Venard, 1967). Photoperiod is the major stimulus for diapause in *Ae. triseriatus* with temperature probably acting as a modifier to diapause intensity (Kappus and Venard, 1967). Unlike the maternal influence to diapause as observed in *B. mori*, the pharate larva within the egg is photosensitive (Kappus and Venard, 1967; Shroyer and Craig, 1980). The critical day length for diapause in *Ae. triseriatus* is dependent on latitude. The photoperiod was correlated to latitude between 30° and 46° N in the U.S. and found to be one hour less of critical photoperiod for every 4.2° increase in latitude (Shroyer and Craig, 1980).

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Different geographic strains of *Ae. triseriatus* differ in critical photoperiod. One strain of *Ae. triseriatus* from Florida did not diapause at all, and critical photoperiod was dependent on latitude of origin (Shroyer and Craig, 1983). In addition, *Ae. triseriatus* mosquitoes that have been colonized for long periods of time were unresponsive to short day lengths (Shroyer and Craig, 1983). *In toto*, these studies suggest that diapause is an inherited trait that is most likely regulated by a single gene. To date no specific diapause gene has been identified in mosquitoes.

Diapause termination coincides with increased photoperiod (Baker, 1935; Love and Welchel, 1955; Shroyer and Craig, 1983) and is aided by exposure to increased temperatures prior to increased photoperiod (Shroyer and Craig, 1983). This pattern of environmental stimuli is observed in nature as spring progresses.

In laboratory settings, diapause can be induced in *Ae. triseriatus* eggs by exposing them to 10-hour light, 14-hour dark cycles at 21°C for two weeks. Following diapause conditioning, eggs are unresponsive to hatching stimuli. By placing the eggs in 16-hour light, 8-hour dark cycles, diapause is terminated, and the larvae begin hatching in installments upon repeated attempts (McGaw et al., 1998).

N. Summary and research objectives.

Arboviruses continue to cause significant morbidity and mortality in humans and domestic livestock. The largest family of arboviruses is the Bunyaviridae. Arboviruses are unique in that they must be able to replicate efficiently in two distinct and very different hosts, vertebrate hosts and arthropod vectors. In vertebrate cells and hosts LAC virus can cause a cytopathic infection resulting in cell death, while in mosquito cells and vectors, LAC virus causes a persistent noncytopathic infection. In mosquitoes and mosquito cells the

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ability of the N protein of LAC virus to regulate its synthesis is one mechanism that has been proposed to allow the establishment of a persistent infection. However, the exact mechanisms for virus persistence in the mosquito vector are not understood and require further studies.

LAC virus, a bunyavirus, and its interactions with its mosquito vector, *Aedes triseriatus*, are the subject of this review and research. To understand this virus-vector interaction, studies were conducted to examine the possible role of cap scavenging by LAC virus in outcomes of infection of mammalian and mosquito cell cultures and in infected mosquito tissues. LAC virus cap scavenging has been previously studied in C6/36 cells and in *Ae. triseriatus* embryos both during embryogenesis and during diapause (Dobie et al., 1997). Previous studies did not examine cap scavenging in mammalian cells, in cultured cells from the natural vector, or in specific mosquito tissues.

The first objective of this research was to determine the molecular basis and determinants of cap scavenging *in vitro*. Experiments to characterize the cap scavenging activity of several LAC virus isolates and other California serogroup viruses in cytopathic infection of mammalian cell cultures and persistent infection of mosquito cell cultures are described in Chapter 2. The hypothesis was that scavenging specific cap sequences in mosquito cells allows the establishment of persistence, while scavenging heterogeneous cap sequences in mammalian cells results in cell death. The identification of host mRNAs targeted for cap sequences may provide insight into host genes involved in the divergent outcomes in the two different systems. The cap scavenging activity of several LAC virus isolates *in vivo* is also described in Chapter 2.

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Previous studies on LAC virus interaction with mosquito midguts and ovaries revealed that changes in LAC virus activity were correlated with changes in the mosquito. Inability to detect LAC virus mRNA and vcRNA was coincident with mosquito ovary quiescence. Following ingestion of a blood meal, the levels of LAC mRNA and vcRNA were upregulated (Chandler et al., 1996). These previous studies made use of conventional reverse-transcription polymerase chain reaction technology to examine LAC virus transcription and replication. The second objective of this research was to determine the interaction between LAC virus and persistently infected *Ae. triseriatus* mosquitoes in possible co-regulation of LAC virus activity with the host cell at different physiologic and metabolic states. The hypothesis was that quantitative analysis of viral RNAs could reveal that co-regulation of viral transcription and replication and host transcription are determinants of efficient transovarial transmission. The interaction between LAC virus and cytopathically infected mammalian cells and persistently infected mosquito cells was also examined quantitatively. New quantitative polymerase chain reaction technology was used to examine the levels of LAC virus mRNA (transcription) and vcRNA and vRNA (replication) in host cells and tissues. Experiments to investigate the possible co-regulation of LAC virus transcription and replication with host metabolic activity or physiologic state are described in Chapter 3.

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CHAPTER 2: ANALYSIS OF CAP SCAVENGING BY CALIFORNIA SEROGROUP VIRUSES IN CELL CULTURES AND MOSQUITO TISSUES

A. INTRODUCTION

Negative-sense segmented RNA viruses utilize the 5' 7-methyl guanosine cap and adjacent nucleotides from host mRNAs to prime viral mRNA transcription. In the Bunyaviridae, the L protein contains not only RNA polymerase but also methylated cap-dependent endonuclease function believed to be responsible for attaching to and cleaving the host mRNA 5' cap and adjacent nucleotides for use as a primer (Patterson et al., 1984; Patterson and Kolakofsky, 1984; Bouloy et al., 1990; Dobie et al., 1997). This "cap-scavenging" or "cap-snatching" phenomenon has been observed and studied in a variety of bunyaviruses (Bishop et al., 1983; Patterson et al., 1984; Patterson and Kolakofsky, 1984; Ihara et al., 1985; Bouloy et al., 1990; Simons and Pettersson, 1991; Kormelink et al., 1992; Jin et al., 1993; Garcin et al., 1995; van Poelwijk et al., 1996; Dobie et al., 1997; Duijsings et al., 1997; 2001), orthomyxoviruses (Krug, 1981; Plotch et al., 1981; Leahy et al., 1997; 1998), arenaviruses (Garcin et al., 1990; Raju et al., 1990; Meyer and Southern, 1994) and the tenuiviruses of plants (Huiet et al., 1993, Ramirez et al., 1995; Nguyen et al., 1997).

The influenza virus transcription initiation mechanism acts as a model of cap scavenging for other viruses. Extensive research into this mechanism has provided considerable knowledge of cap scavenging and priming of transcription (Krug, 1981;

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Shih and Krug, 1996; Lamb and Krug, 2001). The template vRNA functions as a cofactor in cap scavenging in that binding of the polymerase proteins PB2 to the 5' end of capped cellular mRNA (through a tryptophan-rich domain believed to be similar to the cap-binding domain of the eIF4E translation initiation factor) and PB1 to the 3' conserved end of the influenza vRNA initiates cap scavenging (Marcotrigiano et al., 1997; Matsuo et al., 1997; Lamb and Krug, 2001). The viral polymerase complex also protects viral mRNAs from being scavenged; PB2 also binds to a second site just downstream from the 5' end of viral mRNA, and PB2 binding to this region blocks viral mRNAs from being scavenged for caps (Shih and Krug, 1996). The influenza virus PB1 endonuclease activity preferentially cleaves host mRNA capped primer sequences at a purine residue, usually a G residue. The G residue is aligned to the terminal C residue at the 3' terminus of the vRNA and extension occurs on the 3' OH of the G residue. Some hydrogen bonding between the scavenged primer and the virion RNA template appears to be preferred.

In vertebrate cells, LAC virus transcription is initiated by capped 5' nonviral sequences from host mRNAs (Patterson et al., 1984; Hacker et al., 1990). Initiation of transcription involves the positioning of the 3' OH terminus of the capped primer sequence antiparallel to the 3' OH of the template viral genome. Hydrogen bonding between the capped primer sequence and the template viral genome has been shown to be a requirement.

Previous studies have shown that in bunyavirus infection of mammalian cells, the scavenged 5' nonviral sequences appear to be heterogeneous in nature (Bishop et al., 1983; Eshita et al, 1985; Patterson et al., 1984; Bouloy et al., 1990; Jin and Elliott, 1993), while in mosquito cells and embryos they appear to be more homogeneous, with preference for both specific 5' nonviral sequences and for a specific residue at the 3' terminus (Dobie et al.,

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1997). Preference for a single nucleotide, di-, or trinucleotide at the endonuclease cleavage sites of certain bunyaviruses has been observed. Within the *Orthobunyavirus* genus, Germiston virus 5' nonviral sequences displayed a U or G residue at the –1 position (Bouloy et al., 1990), SSH virus an A residue (Bishop et al., 1983), and LAC virus a U residue (Dobie et al., 1997). For the phleboviruses Rift Valley fever and Uukuniemi, and the nairovirus Dugbe, a C residue was preferred at the –1 position of N and NS_S mRNAs (Simons and Pettersson, 1991; Jin et al., 1993b). For the hantaviruses Hantaan and Sin Nombre, a G residue was preferred at the –1 position of mRNAs (Garcin et al., 1995; Hutchinson et al., 1996). In tomato spotted wilt virus mRNAs, no specific nucleotide or nucleotide motif was observed at the 3' end of the primer (van Poelwijk et al., 1996), but when TSWV-infected cells were co-infected with another plant virus, alfalfa mosaic virus (AMV), a preference for 5' nonviral sequences cleaved from the AMV mRNAs with an A residue at the –1 position was observed (Duijsings et al., 1997; 2001). Nucleotide or nucleotide motif preferences have not been determined for arenaviruses (Garcin et al., 1990; Raju et al., 1990; Meyer and Southern, 1994) or the tenuiviruses of plants (Huiet et al., 1993, Ramirez et al., 1995; Nguyen et al., 1997). The difference in cap scavenging observed between mammalian and mosquito cells may be indicative of the mechanism that allows for persistence in mosquito cells and death in mammalian cells (Dobie et al., 1997).

Previous studies were conducted to examine LAC virus cap scavenging activity in C6/36 cell cultures and *Ae. triseriatus* embryos both during embryogenesis and diapause (Dobie et al., 1997). In LAC infected mosquito cells and mosquito embryos, a specific 12-nucleotide 5' host sequence was preferentially cleaved (Dobie et al., 1997). Cap sequence 1, 5'-CCACTCGCCACT-3', was observed in over 76% of 5' nonviral sequences scavenged

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from C6/36 cell cultures (56% of caps scavenged through day 15 and over 91% of caps scavenged after day 15 post-infection) and in approximately 81% of 5' nonviral sequences scavenged during embryogenesis. Previous studies did not examine cap scavenging in mammalian cells, in cultured cells from the natural vector, or in adult mosquito tissues.

The studies described here were conducted to test the hypothesis that the establishment of persistent infection in mosquito cells involves the co-regulation of host and viral mRNA transcription through cap scavenging. The specificity of cap scavenging was determined in mammalian and mosquito cell cultures infected with LAC virus, in mammalian and mosquito cell cultures infected with SSH or TAH viruses, in *Aedes triseriatus* mosquitoes infected with several different LAC virus isolates, and in mammalian and mosquito cell cultures dually infected with LAC and SIN viruses. The results obtained from these studies provide insight into the potential role of cap scavenging in the co-regulation of host and virus transcription and in virus persistence. .

B. MATERIALS AND METHODS

Cells

C6/36 cells. *Aedes albopictus* cells were grown in Leibovitz L-15 medium including 10% fetal bovine serum (FBS) and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 28°C (Singh, 1967). The cells were diluted 1:10 and passed every 7 days until virus inoculation.

MAT cells. Mather *Aedes triseriatus* cells were grown in Leibovitz L-15 medium including 20% FBS and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 28°C (Rowley et al., 1984). The cells were diluted 1:6 and passed every 7 days until virus inoculation.

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BHK-21 cells. Baby hamster kidney cells were grown in Leibovitz L-15 medium including 10% FBS and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 37°C. The cells were diluted 1:5 and passed every 7 days until virus inoculation.

Vero cells. African green monkey kidney cells were grown in Leibovitz L-15 medium including 10% FBS and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 37°C. The cells were diluted 1:5 and passed every 7 days until virus inoculation.

Viruses

LAC virus wt10. LAC virus wt10 (wild type virus, passage 10) was isolated in 1965 from the brain of a patient with a fatal case of LAC encephalitis (Thompson et al., 1965). The virus had been passed three times in suckling mouse brain and six times in BHK-21 cell culture. To prepare stock virus, BHK-21 cells, which had growth medium removed and were rinsed twice with phosphate buffered saline (PBS), were infected with 1 ml of virus at a multiplicity of infection (MOI) of 0.01 for 1 hour at 37°C with gentle rocking. After virus inoculation, the inoculum was removed and replaced with growth medium. When approximately 90% of the cells exhibited cytopathic effects (CPE) and death (36 to 48 hours post-infection at 37°C), the remaining cells were scraped into the medium, fetal bovine serum was added to 50%, and the virus was stored at -70°C in 500 μl aliquots. Virus was titrated by the Karber method (1931) in Vero cells. The stock LAC virus had a titer of $10^{8.4}$ $TCID_{50}$/ml.

LAC virus TOT3 and TOT9. LAC virus TOT3 was the same virus isolated in 1965 from the brain on a patient with a fatal case of LAC encephalitis (Thompson et al., 1965). It had been passed transovarially 3 times in *Ae. triseriatus* mosquitoes. Virus was prepared and

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titrated as described above, and had a titer of $10^{8.1}$ $TCID_{50}$/ml. LAC virus TOT9 was passed 9 times transovarially in *Ae. triseriatus* mosquitoes. Virus was prepared and titrated as described above, and had a titer of $10^{8.0}$ $TCID_{50}$/ml.

LAC virus H78mp1 and H78mp2. LAC virus H78mp1 was isolated from the brain on a patient with a fatal case of LAC encephalitis in 1978 (Chandler et al., 1998) and passed once in suckling mouse brain. Virus was prepared and titrated as described above. Stock LAC virus H78mp1 had a titer of $10^{6.3}$ $TCID_{50}$/ml. LAC virus H78mp2 had undergone an additional passage in suckling mouse brain. Stock virus was prepared and titrated as described above, and had a titer of $10^{5.8}$ $TCID_{50}$/ml.

LAC virus TCF6. LAC virus TCF6 was isolated from field collected *Ae. triseriatus* mosquitoes and passed six times in BHK-21 cell culture. Stock LAC virus TCF6 had a titer of $10^{5.5}$ $TCID_{50}$/ml.

SSH virus 76-Y-315. SSH virus 76-Y-315 was originally isolated from field collected *Aedes hexodontus* mosquitoes collected at Fort Smith, Northwest Territories, Canada in 1976 (McLean, 1982), and passed six times in BHK-21 cell culture. Stock SSH virus had a titer of $10^{8.3}$ $TCID_{50}$/ml.

TAH virus. TAH virus was obtained from the Yale Arbovirus Research Unit, New Haven, CT and maintained at the Arthropod-borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO, and passed seven times in BHK-21 cell culture. Stock TAH virus had a titer of $10^{7.4}$ $TCID_{50}$/ml.

SIN virus TE3'/2J. SIN virus TE3'/2J (Raju and Huang, 1991; Hahn, et al., 1992) was obtained at the Arthropod-borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO. Virus was prepared by transfecting full-length capped TE3'/2J

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RNA into BHK-21 cells and harvesting at 48 hours post-transfection. To prepare fresh virus, growth medium was removed from C6/36 cells, cells were rinsed twice with phosphate buffered saline and infected at an MOI of 0.01 for 1 hour at 28°C with gentle rocking. The inoculum was removed and replaced with growth medium. At 7 days post-infection at 28°C, the cells were scraped into the medium and pelleted by centrifugation at 2,000 rpm at 4°C for 5 minutes, fetal bovine serum was added to 50% to the supernatant and the virus was stored at -70°C in 500 μl aliquots. Virus was titrated by the Karber method (1931). Stock SIN virus had a titer of $10^{8.0}$ $TCID_{50}$/ml.

Virus titration protocol

Virus in infectious blood meals and infected cell culture medium was determined by serial 10-fold dilution in sterile 96-well flat bottom plates with lids. Ninety μl of Leibovitz L-15 maintenance medium was aliquotted into each well except those in the first column (1). One hundred μl of sample (supernatant, infectious blood meal, etc.) was aliquotted to the wells in column 1. Serial 10-fold dilutions from column 1 to column 12 were made by transferring 10 μl from one column to the next column, and the suspension was mixed by pipetting. Pipette tips were changed between each column to prevent carry-over contamination. One hundred μl of Vero cell suspension was added to each well. The Vero cell suspension was made by rinsing Vero cells in a 100% confluent 75-cm^2 flask twice with PBS. The cells were then treated with 3 ml of trypsin (25 g/L porcine trypsin in Hank's Balanced Salt Solution) to detach them from the growth surface. Leibovitz L-15 maintenance medium was added to a final volume of 20 ml, providing sufficient cells for two full 96-well plates. After 7 days incubation, the wells of the plate were microscopically examined for CPE and cell death. Each sample was titrated on a single 96-well plate (8

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replicates). To calculate the titer, the Karber method (1931) was used. The equation was as follows:

$\log_{10}$ of $TCID_{50}$ = X-d (P-0.5)

Where X = $\log_{10}$ of the highest concentration (lowest dilution) used; d = $\log_{10}$ of dilution factor; P = $\frac{\text{sum of percent cell death at each dilution}}{100}$

Fluorescent antibody virus titration protocol

To titrate virus in dually infected cell medium, sterile 96-well flat bottom plates with lids were used. Titration plates were prepared as above. Each sample was titrated on a single 96-well plate (8 replicates). At day 7 following set up, all medium was removed from the wells. One hundred µl of cold acetone-PBS (3:1) was added to each well to fix the cells. Forty µl of 1:200 dilution of fluorescein isothiocyanate (FITC) conjugated anti-LAC mouse polyclonal antibody or 1:200 dilution of MB92 mouse anti-Sindbis polyclonal hyperimmune serum was placed in each well and incubated at 37°C for 40 minutes in a humidified chamber. Following incubation, all wells were washed twice with 100 µl of PBS. LAC plates were then briefly rinsed with distilled water and each well was overlaid with 50 µl of glycerol-PBS (3:1). Each well of plates with Sindbis infected cells was incubated with 100 µl of 1:200 dilution of biotinylated sheep anti-mouse antibody at 37°C for 40 minutes in a humidified chamber. Wells were then rinsed twice with 100 µl of PBS. Fifty µl of 1:200 dilution of fluorescein-streptavidin was added to each well and incubated 37°C for 10 minutes in a humidified chamber. Wells were then washed twice with 100 µl of PBS and overlaid with 50 µl of glycerol-PBS (3:1). Plates were observed for fluorescence with an

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Olympus BH2 epifluorescence microscope using filters for FITC. Titer was calculated as above.

Mosquitoes

Aedes triseriatus mosquitoes (AIDL strain) originated from field material collected near La Crosse, Wisconsin, in 1981 and colonized continuously at the Arthropod-borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO, at 70°F, 70% relative humidity, 16-hour light: 8-hour dark cycles (Wasieloski, 1995).

Oral infection and processing of mosquitoes

Approximately 500 adult female *Ae. triseriatus* mosquitoes (approximately 80 mosquitoes for each of the six different LAC isolates used) were provided an infectious blood meal. A 75-cm^2 flask with a confluent monolayer of BHK-21 cells was rinsed twice with PBS and infected with LAC virus wt10 at an MOI of 0.01 as described above. At 48 hours post-infection the cells were scraped into the medium. Equal parts of defribinated sheep blood and virus suspension were mixed, and provided to *Ae. triseriatus* female mosquitoes through human-scented parafilm membrane and by hanging drop technique (Wasieloski, 1995). Mosquitoes were allowed to feed for approximately 2 hours. Only females engorged to repletion were kept for future use. Engorged females were maintained in a 1 ft^3 cage at 70°F, in 70% relative humidity and 16 hours light: 8 hours dark, and allowed to mate and oviposit eggs. At 21 days post-infectious blood meal, 15 infected and 5 uninfected, blood fed female mosquitoes were harvested, and twelve infected and 2 uninfected mosquitoes were processed. Ovary and midgut tissues were dissected for RNA extraction. Dissected ovaries and midguts from individual mosquitoes were rinsed twice in sterile PBS and stored individually in 100 μl RNA lysis buffer (4M GTC solution), which

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was prepared by combining 100 g guanidinium thiocyanate, 117 ml DEPC treated water, 7 ml 0.75 M sodium citrate solution pH 7.0, 10.6 ml 10% sarcosyl, and 1.44 ml 2-mercaptoethanol.

Experimental infection of cell culture

Approximately $5x10^5$ cells in suspension were used to seed 25-cm^2 flasks. At 80-90% confluence, the growth medium was removed and cells were rinsed twice with PBS. Virus inoculum was diluted to the appropriate MOI in Leibovitz L-15 maintenance medium (1% FBS and 100 units penicillin plus 100 μg of streptomycin per ml) to a total volume of 1 ml. Virus was placed onto the cell monolayer and gently rocked for 1 hour at room temperature. Virus inoculum was removed, and cells were rinsed twice with PBS. Five ml of maintenance medium were then added to each flask. Vertebrate cells (BHK-21) were infected at an MOI of 0.01 and invertebrate cells (C6/36 and MAT) were infected at an MOI of 10. At predetermined time points following infection, the cells were harvested, scraped into the medium, aliquotted into 1.7-ml Eppendorf tubes, and centrifuged at 14,000 xG, for 5 minutes. The supernatant was removed for use in end point virus titration, and the cell pellets were stored at –70°C until ready for use. The number of cells present at the predetermined times was determined using a hemacytometer. For infected invertebrate cells, the medium was replaced with 5 ml of fresh maintenance medium every seven days until completion of the time course.

RNA extraction from cell pellets and mosquito tissues

The RNA extraction protocol used was a modified version of the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Cell pellets were pooled and dissolved in 400 μl of RNA lysis buffer. To each

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sample, 40 µl of 2M sodium acetate pH 4.5 were added, and the samples were mixed briefly by vortexing. Four hundred µl of phenol:chloroform (5:1) pH 5.0 and 160 µl of chloroform:isoamyl alcohol (25:1) were added and the samples were mixed briefly by vortexing. The samples were then incubated on ice for 10 minutes followed by centrifugation at 14,000 xG at 4°C for 10 minutes. The aqueous phase was then mixed with an equal volume of isopropanol in a clean 1.7-ml Eppendorf tube. The samples were incubated at –70°C for one hour, and then centrifuged at 14,000 xG at 4°C for 30 minutes. The supernatant was discarded, and the pellet was dissolved in 180 µl diethyl pyrocarbonate (DEPC)-treated water. To each sample, 20 µl of 3M sodium acetate pH 5.2 and 500 µl of 100% ethanol were added. The samples were incubated at –70°C for one hour, and then centrifuged at 14,000 xG at 4°C for 30 minutes. The supernatant was discarded, and the pellet was allowed to air dry. The pellet was then dissolved in 40 µl DEPC-treated water and stored at –70°C until ready for further use.

Mosquito tissues were dissolved in 100 µl of RNA lysis buffer. To each sample, 10 µl of 2M sodium acetate pH 4.5 were added and the samples were mixed briefly by vortexing. One hundred µl of phenol:chloroform (5:1) pH 5.0 and 40 µl of chloroform:isoamyl alcohol (25:1) were added, and the samples were mixed briefly by vortexing. The samples were then incubated on ice for 10 minutes followed by centrifugation at 14,000 xG at 4°C for 10 minutes. The remaining portion of the extraction protocol was the same as above with only changes in the volumes used. Instead of 180 µl diethyl pyrocarbonate (DEPC)-treated water, 20 µl of 3M sodium acetate pH 5.2 and 500 µl of 100% ethanol, the volumes added were 45 µl, 5 µl, and 125 µl, respectively. After the

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pellets were air dried, they were dissolved in 20 μl DEPC-treated water and stored at –70°C until ready for further use.

Preparation and purification of cDNA

Approximately 3 μg of total cell RNA or one-fourth of the total mosquito tissue RNA was mixed with 15 pmol of LNR reverse transcription primer (Table 2.1) and diluted to 12 μl in DEPC-treated water. The RNA samples were heated to 70°C for 10 minutes and then allowed to cool to 20°C. To each sample, 4 μl of first strand buffer (Gibco BRL), 2 μl 0.1 M DTT, and 1 μl 10 mM dNTP mix were added. Three units of reverse transcriptase, Superscript II (Gibco BRL), in DEPC-treated water, were added and the samples were incubated at 42°C for 60 minutes. The samples were then heated to 70°C for fifteen minutes to inactivate/denature the reverse transcriptase. Samples were cooled to 4°C. To each sample, 1 μl (15 units) of RNase H was added and samples were incubated at 37°C for 10 minutes. The reverse transcriptase is unable to proceed through the 5' 7-methyl guanosine cap residue of the RNA and ends cDNA synthesis at the second residue in from the 5' terminus (Tech Support, GibcoBRL, personal communication).

Table 2.1 Primers for RT and RT-PCR of LAC S RNA

Primer name	**Sequence 5'-3'**	**Bases represented**	**Use**
LNFLAC	TCAAGAGTGTGATGTCGGATTTGG	71-95 of LAC S mRNA and vcRNA	PCR
LNR	GGAAGCCTGATGCCAAATTTCTG	741-763 of LAC S genome	RT of LAC mRNA and vcRNA and PCR
LNFc3	CGCGAGAGAGCAGCCTTGGCC	231-245 of LAC S genome	PCR
LVCB	GTGTCATCCACTTGAATAC	5-23 of LAC S genome	PCR
5'	GACATCGAAAGGGGGGGGGGGG	anneals to oligo-dC tail	PCR

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cDNA was purified with the QIAquick kit from QIAGEN according to the given protocols. Five volumes of binding solution (PB) at room temperature were added to each cDNA sample and mixed thoroughly. The cDNA-binding solution mix was transferred to a QIAquick spin cartridge and centrifuged at 14,000 xG for 30 seconds, and the flow-through was discarded. Seven hundred fifty µl of wash solution (PE) was added to each cartridge and centrifuged at 14,000 xG for 30 seconds, and the flow-through was discarded. The sample cartridge was centrifuged at 14,000 xG for one additional minute to remove any excess wash solution. The sample cartridge was then transferred to a clean 1.7-ml Eppendorf tube. Thirty µl of DEPC-treated water were added to each cartridge for two minutes to allow the cDNA to elute. The samples were centrifuged at 14,000 xG for one minute for full recovery of cDNA.

Oligo-dC tailing

Oligo-dC tailing was performed according to the protocols established by Dobie, et al. (1997). Each tailing reaction consisted of 26.7 µl of DEPC-treated water, 10.0 µl of 5X tailing buffer (Gibco BRL), 1.0 µl of 1 mM dCTP, 1.3 µl BSA (1 mg/ml) and 10 µl of cDNA sample in a 0.65 ml Eppendorf tube. One µl of recombinant terminal deoxynucleotidyl transferase (rTdT) was added to each reaction, mixed by vortexing, and centrifuged briefly at 14,000 xG. The samples were incubated at 37°C for 30 minutes and then the rTdT was heat inactivated at 65°C for 10 minutes. Final composition of tailing reaction was cDNA, 1.3 µg BSA, 20 µM dCTP, 15 units rTdT and 1X tailing buffer (0.1 M potassium cacodylate pH 7.2, 10 mM $CoCl_2$, 0.2 mM DTT). Tailed cDNA samples were then purified using the QIAquick kit as described above. Tailed cDNA samples were eluted in DEPC-treated water in a volume of 20 µl.

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Initial PCR screen of tailed cDNA sample

The tailed cDNA samples were PCR amplified using three different sets of PCR primers in separate reactions. Five µl of the tailed cDNA sample was added to 45 µl of 1X PCR buffer, containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µM of each nucleotide, and 50 pmol of each primer. The PCR reaction mixture was overlaid with 50 µl of sterile mineral oil. Primer sets included LNFLAC and LNR used to ensure full-length cDNA had been reverse transcribed, LNFc3 and LVCB used as an internal LAC control, and LNFc3 and 5' used to ensure proper oligo-dC tailing had occurred (Table 2.1). The PCR program included 80°C for 30 minutes (hot start), followed by 92°C for 1 minute (denaturing), 50°C or 55°C for 30 seconds (annealing) and 72°C for 2 minutes (elongation) for 35 cycles, followed by 72°C for 10 minutes (final elongation) and then cooling to 4°C. The annealing temperature used by the LNFLAC/LNR and the LNFc3/5' primer sets was 55°C and the LNFc3/LVCB primer set was 50°C. At the beginning of the 80°C hot start, 1.5 units of *Taq* DNA polymerase was added to each tube.

Gel electrophoresis and visualization

All PCR products were analyzed by electrophoresis on 1.0% agarose gels in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Seven µl of each product was mixed with 2.0 µl of DNA loading buffer (30% v/v glycerol, 70% v/v 1X TAE, and 0.1 mg/ml bromophenol blue). Gels were electrophoresed at 125 V constant for 1.5 hours, then stained with ethidium bromide and examined under UV light. The expected size and use of each PCR product is given (Table 2.2).

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Table 2.2 Product size of initial PCR amplification			
Forward primer	**Reverse primer**	**Product size**	**Use**
LNFLAC	LNR	714 bp	Screening
LVCB	LNFc3	240 bp	Screening
5'	LNFc3	266 bp*	screening and TA cloning
M13 forward	M13 reverse	465 bp**	screening and sequencing

*Actual size 266 bp plus length of scavenged 5' nonviral sequence.
**Actual size 465 bp plus length of scavenged 5' nonviral sequence.

TA Cloning

TA cloning protocols were those supplied with the TA Cloning kit pCR2.1 from Invitrogen. Each ligation reaction contained: 4 µl of sterile water, 1 µl of 10X ligation buffer, 2 µl of pCR 2.1 vector, 2 µl of fresh PCR product (LNFc3/5'), and 1 µl of T4 DNA ligase (4.0 Weiss units) to give a total ligation volume of 10 µl. The reagents were mixed thoroughly, centrifuged briefly at 14,000 xG, and placed in a 14°C water bath overnight. After ligation was completed, *Escherichia coli* INVαF'cells were transformed with the ligation reactions. Competent cells were thawed on ice, 2.0 µl of each ligation reaction was added to a vial of competent cells, mixed by stirring, and incubated on ice for 30 minutes. The cells were then heat shocked for exactly thirty seconds in a 42°C water bath and placed on ice for 2 minutes. Two hundred fifty µl of SOC medium (see Appendix) was added to each vial and agitated in a 37°C shaker incubator for 60 minutes at 250 rpm. After incubation, 100 and 150 µl of each transformation reaction was spread onto LB agar plates containing 50 µg/ml of ampicillin and 40 µl of a 40 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution. Plates were incubated at 37°C for 18 to 24 hours. Plates were removed from the incubator and placed at 4°C for 2 to 3 hours to allow color to fully develop for blue/white screening. White colonies were selected for PCR screening.

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Selected white colonies were plated on grid plates with LB agar containing 50 µg/ml of ampicillin and grown overnight at 37°C.

Screening of colonies

Each colony was screened with one of three different primer sets (Table 2.2). The PCR program used to screen the colonies (HOTLAC) was as follows: 95°C for 5 minutes to disrupt bacterial cells, 80°C for 30 minutes for addition of *Taq* polymerase, 92°C for 1 minute for denaturing of template, 55°C or 50°C for 30 seconds for annealing of primers, and 72°C for 2 minutes for elongation for 35 cycles, a final extension at 72°C for 10 minutes, then samples were cooled to 4°C. The PCR buffer and *Taq* polymerase were the same as listed above. A small portion of each colony was placed into the PCR reaction mixture prior to overlaying with mineral oil. The PCR products were analyzed by gel electrophoresis on 1.0% agarose gels, stained with ethidium bromide, and visualized by UV light.

Selection and purification of positives for sequencing

The colonies that yielded PCR products with the following sizes were retained: LVCB and LNFc3 240 bp, 5' and LNFc3 266 bp, and M13 forward and reverse 465 bp. M13 PCR products were purified using the QIAquick PCR Purification kit from QIAGEN for sequencing. The purification was performed as above. The concentration and purity of the DNA were determined by UV light absorbance at 260nm and 280nm.

Determining sequences of 5' nonviral sequence oligonucleotides

The LNFc3-sequencing primer (Table 2.4) was used for sequencing of the PCR products. Sequencing was performed at Davis Sequencing, Davis, California, or at AIDL using an Applied Biosystems 310 genetic analyzer. For sequencing at AIDL, 3 to 10 ng of purified PCR product were mixed with 3.2 pmol of LNFc3-sequencing primer (Table 2.3), 4

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μl of Big Dye v2.0 sequencing reaction mix (Applied Biosystems) and DEPC-treated water to a final volume of 20 μl. Samples were amplified with the following thermocycler conditions: 96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes for 25 cycles followed by cooling to 4°C. For purification of sequencing reaction products, the DyeEx Spin Kit from QIAGEN was used. The columns were centrifuged at 3,000 xG for 3 minutes to pack the column and remove excess buffer. Sequencing reactions were placed onto the columns and centrifuged at 3,000 xG for 3 minutes. The flow-through was then dried in a vacuum centrifuge for 20 minutes. Dried sequencing products were resuspended in 15 μl of template suppression reagent (TSR, Applied Biosystems) heated to 95°C for 2 minutes and cooled on ice. Reactions were then stored at –20°C until ready for sequence determination. Scavenged primer sequences are reported as 5' nonviral sequence(s) throughout the remainder of this dissertation.

Table 2.3 Additional primer used for sequencing

Primer name	Sequence 5'-3'	Bases represented	Use
LNFc3-sequencing	CTTACGCGAGAGAGCAGCCTTGGCC	227-245 of the LAC S genome	sequencing

RT-PCR of capped LAC mRNA and targeted host genes

Studies were conducted to determine the proportion of certain scavenged 5' nonviral sequences by LAC virus to prime transcription of LAC S segment mRNA and the host genes contributing 5' sequences to prime transcription of LAC mRNA. Reverse transcription with was performed as above. The cDNA samples were PCR amplified using different sets of primers in separate reactions (Table 2.4). Five μl of the cDNA sample was added to 45 μl of 1X PCR buffer as described above. Primer sets and product size are provided in Table 2.4. The LNFc3 primer described previously (Table 2.1) was used as the reverse primer in each

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Table 2.4 RT-PCR of capped LAC mRNA			
Forward primer	**Sequence 5'-3'**	**Bases represented**	Product Size
CAP-A-LAC*	CAGTTACGTTAGTAGTG	cap A and nt 1-7 of LAC S segment mRNA	255 bp
CAP-B-LAC*	TTGGTCGTCATCGAGTAGTG	cap B and nt 1-7 of LAC S segment mRNA	258 bp
CAP-C-LAC*	TCACTCCCAGTAGTG	cap C and nt 1-7 of LAC S segment mRNA	253 bp
CAP-1-LAC*	CCACTCGCCACTAGTAGTG	cap 1 (Dobie et al., 1997) and nt 1-7 of LAC S segment mRNA	257 bp
CAP-2-LAC*	AGGAAAAGTAGGTAGTAGTG	cap 2 (Dobie et al., 1997) and nt 1-7 of LAC S segment mRNA	258 bp
SIN-FULL-LAC**	ATTGACGGCGTAGTAGTG	SIN TE3'/2J genomic mRNA cap and nt 1-7 of LAC S segment mRNA	256 bp
SIN-SUB-LAC**	ATAGTCAGCATAGTAGTG	SIN TE3'/2J subgenomic mRNA cap and nt 1-7 of LAC S segment mRNA	256 bp
CAP-A***	CAGTTACGTTNN	cap A with two additional degenerate bases	variable
CAP-A extension***	CGGATCGCCAGTTACGTT	cap A with an 8 base upstream extension	variable
CAP-B***	TTGGTCGTCATCGNN	cap B with two additional degenerate bases	variable
CAP-B extension***	CGGATCGCTTGGTCGTCATCG	cap B with an 8 base upstream extension	variable
CAP-C***	TCACTCCCNN	cap C with two additional degenerate bases	variable
CAP-C extension***	CGGATCGCTCACTCCC	cap C with an 8 base upstream extension	variable

*For capped LAC mRNA RT-PCR
**For SIN TE3'/2J capped LAC mRNA RT-PCR
***For targeted host gene RT-PCR

LAC specific PCR reaction and primers LNFc3 and LVCB were used as an internal LAC control (Table 2.3). Reverse transcription was performed as above with the LNR primer for

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each LAC-SIN co-infection sample and primers LNFLAC and LNR were used as an internal LAC control (Table 2.1). For targeted host genes, reverse transcription was performed as above with either Oligo-T or Oligo-T extension primers.

The PCR program and analysis of products were as described above. Modification of the primer annealing temperature to 60°C was made for LAC specific and LAC-SIN co-infection samples. For amplification of targeted host genes, the primer annealing temperature was decreased to 25 °C for the first five cycles, followed by 55 °C for the remaining 30 cycles.

PCR products from amplification of targeted host genes were cloned into pCR2.1 by TA cloning as described above. Colonies were selected and PCR screened. Plasmids from positive clones were purified by QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer's instructions and sequenced as described above, using M13 forward and reverse primers.

Cellular mRNA purification

mRNA was purified from total RNA from C6/36, MAT, and BHK-21 cell culture. Polyadenylated mRNA was derived from 200 µg of total RNA extracted from cell pellets with the single-step RNA isolation method described above, followed by the Poly (A) Pure mRNA Purification Kit from Ambion (Austin, TX) according to the manufacturer's instructions

Northern blot analysis

Approximately 3 µg of total RNA or 200 ng of purified mRNA were denatured and electrophoresed on a 1.3% agarose gel containing 0.66 M formaldehyde and MOPS buffer (0.2 M 3-N-morpholino-propanesulfonic acid, 0.05 M sodium acetate, 1 mM EDTA pH 7.0).

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RNA was then transferred to a nylon membrane (BrightStar-Plus, Ambion) and cross-linked by UV irradiation.

Blots were prehybridized in 7% sodium dodecyl sulfate-250 mM disodium phosphate (pH 7.0) for 30 minutes at 35°C. DNA probes complementary to cap A, cap B, cap C, and *Ae. triseriatus* actin mRNA (Table 2.5) were synthesized (LifeTechnologies, Invitrogen) and end labeled with [γ-^{32}P] ATP using T4 DNA polynucleotide kinase (KinaseMax, Ambion). A 100 ng portion of the probe was added to the hybridization buffer and the blots were allowed to hybridize overnight at 35°C. The blots were washed twice with 6X SSC (0.90 M NaCl plus 0.090 M sodium citrate) at 35°C and exposed to X-ray film for 16 and 96 hours (with intensifying screens) at -70°C or to phosphorimager screens for 4, 24, and 96 hours (with intensifying screens).

Table 2.5 Northern blot analysis probes

Probe	Sequence 5'-3'	Bases represented	Tm
Cap A	AACGTAACTG	complement to nt 1-10 of Cap sequence A	-28°C
Cap B	CGATGACGACCAA	complement to nt 1-13 of Cap sequence B	-14°C
Cap C	GGGAGTGA	complement to nt 1-8 of Cap sequence C	-33°C
Actin mRNA	TCAGGTAGTCGGTCAGAT	nt 384-401 of *Ae. triseriatus* actin mRNA (B. Kempf, unpublished)	10°C

Tm were calculated by using thermodynamic basis sets for nearest neighbor interactions (Breslauer et al., 1986; Freier et al., 1986).

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C. RESULTS

1. Priming of LAC virus wt10 transcription in C6/36 (*Aedes albopictus*) cell culture. The 5' nonviral sequences scavenged for transcription by LAC virus wt10 throughout a 42 day time course post-infection in C6/36 mosquito cell cultures were cloned and sequenced. A total of 176 5' nonviral sequences were determined, 73.3% from day 18 post-infection or later. Of these, 49 (27.8%) were observed only a single time and were designated as unique. The longest 5' nonviral sequence was 24 nucleotides in length, while the shortest was 3 nucleotides in length. The average length of the 176 5' nonviral sequences was approximately 11.0 nucleotides, and G or C residues comprised 42.3% of all nucleotides (Table 2.6).

A predominant 5' nonviral sequence was observed throughout the time course of infection in the C6/36 cell culture. This was designated cap A (5'-CAGTTACGTT-3'), and was obtained 93 times (52.8%) throughout the time course. After LAC virus had established a persistent infection by day 18, cap A was obtained 74 times (57.4%). In total, 79.6% of all cap A sequences were obtained after day 18 of persistent infection (Table 2.6).

A second predominant 5' nonviral sequence was obtained, but with much less frequency than cap A. This 5' nonviral sequence, designated cap B (5'-TTGGTCGTCATCG-3'), was obtained 17 times (4.0%) throughout the time course. After LAC virus had established a persistent infection by day 18, cap B was obtained 16 times (4.9%). In total, 94.1% of all cap B sequences were obtained after day 18 of persistent infection (Table 2.6).

Similar to previous studies (Dobie et al., 1997), a large proportion of 5' nonviral sequences contained a T residue at the 3'-terminus and associated viral sequences contained

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Table 2.6 5′ nonviral sequences scavenged by LAC virus wt10 from C6/36 cell culture. Sequences observed more than once designated in parentheses.

Day p.i.	Cap sequence	Viral sequence
Published viral sequence		agtagtgtactccacttgaatactttg
1	CAATCCTCGA	agtagtgtaccccacttgaatactttg
3	CAGTTACGTT	agtagtgcccccgcccaaaatactttg
3	CAGTTACGTT(x6)	agtagtgtaccccacttgaatactttg
3	CAGTTCCGTT	agtagtgtaccccacttgaatactctg
3	CAGTTACGTT	aatagtgtacccccccttgaatactttg
3	CTCATTCGCGAT	agtagtgtactccacttgaatactttg
3	TTGGTCGTCATCG	agtagtgtaccccacttgaatactttg
5	CAGTTACGTT	agtagtgtaccccacttgaatactttg
5	ATCTTGTTGTG	agtagtgtaccccacttgaatactttg
7	CAGTTACGTT(x2)	agtggtgtaccccacttgaatactttg
9	AGGGGGT	agtagtgtactccacttgaatactttg
9	CAGTTACGTT(x2)	agtagtgtaccccacttgaatactttg
9	CAGTATTCACT	gtagtgtactccccttgaatactttg
9	ATTCTCGCGAC	gtagtgtaccccacttgaatactttg
9	TTTCTACGAGT	gtagtgtaccccacttgaatactttg
9	ATCAGTACAGCT	agtagtgtactccacttgaatactttg
9	TATCTTTCACCC	gtagtgtaccccacttgaatactttg
9	CTCTTTCCGGCTT	agtagtgtactccacttgaatactttg
9	TCATTCTTCGTAGT	agtagtgtactccacttgaatactttg
9	AGCTGCAGTAAACC	agtagtgtaccccacttgaatactttg
9	AGAACCGAGCGTAGT	agtagtgtactccacttgaatactttg
9	GTTTCTGTTTCCTTTTG	gtagtgtaccccacttgaatactttg
9	TGGGGAGTTTTTCACTGGAGTAGT	gtagtgtaccccacttgaatactttg
11	TTTCTGAAGT	agtagtgtaccccacttgaatactttg
11	TTCCAGCGAGT	agtagtgtactccacttgaatactttg
11	AGTCTCAGTTCA	agtagtgtactccacttgaatactttg
11	TTTCTGGTTGGTGT	agtagtgtactccacttgaatactttg
11	TATGTGAAGTTCGTCGTAGT	agtagtgtactccacttgaatactttg
13	TTT	agtgtactctacttgaatactttg
13	TGG	gtagtgtaccccacttgaatactttg
13	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
13	AGTCGATTCTGT	agtagtgtactccacttgaatactttg

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Table 2.6 (Continued)

Day p.i.	Cap sequence	Viral sequence
15	CAGTTACGTT(x2)	agtagtgtaccccacttgaatactttg
15	ACATGTTATTT	agtagtgtactccacttgaatactttg
15	TCTTTCGCTTC	gtagtgtaccccacttgaatactttg
15	AAGAATGCCTCCTCG	agtagtgtaccccacttgaatactttg
18	CTGGAGG	gtactccacttgaatactttg
18	TCAGTCCTC	agtagtgtactccacttgaatactttg
18	CAGTTACGTT	agtagtgtaccccacttgaatactttg
18	TTTTTATTCA	agtagtgtaccccacttgaatactttg
18	CTCAAGTGTC	agtagtgtaccccacttgaatactttg
18	CGAACTTCTTT	agtagtgtaccccacttgaatactttg
18	TGCAGTTACGTT	agtagtgtaccccacttgaatactttg
18	TGCTGTGAACAT	agtagtgtattccacttgaatactttg
18	ATCAGTACAGCTT	agtagtgtactccacttgaatactttg
18	TTGGTCGTCATCG	agtagtgtaccccacttgaataccttg
18	TTAGAAAGCATTTT	gtagtgtaccccacttgaatactttg
21	CAGTTGCGTT	agtagtgtaccccacttgaatactttg
21	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
21	TGTCAGACTAG	agtagtgtaccccacttgaatactttg
21	CGTCGCGTTGT	agtagtgtactccacttgaatactttg
21	ACAGTCCTACTCGT	gtagtgtaccccacttgaatactttg
24	CAGTTACTTT	agtagtgtaccccacttgaatactttg
24	CAGTTACGTT(x3)	agtagtgtaccccacttgagtactttg
24	AGCAGACAGAGT	agtagtgtaccccacttgaatactttg
24	TGCTGTGAACAT	agtagtgtactccacttgaatactttg
24	TTGGTCGTCATCG(x2)	agtagtgtaccccacttgaatactttg
24	CTTTCCGAGTACT	agtagtgtactccacttgaatactttg
24	ATTACCTCGCTAACCTACA	agtagtgtactccacttgaatactttg
27	CAGTTACGTT	agtagtgtaccccacttggatactttg
27	CAGTTACGTT	agtagtgtaccccacttgagtactttg
27	CAGTTACGTT	agtagtgtaccccgcttgagtactttg
27	CAGTTACGTT(x2)	agtagtgtaccccacttgaatactttg
27	TCTTACATAT	agtagtgtaccccacttgaatactttg
27	AGAGTGGAGC	agtagtgtacaccacttgaatacattg
27	AGACGAGCCAAA	agtagtgtaccccacttgaatactttg
27	TTGGTCGTCATCG(x3)	agtagtgtaccccacttgaatactttg
27	ACTCCATCCAAAATAGT	agtagtgtacttcacttgaatactttg
30	CAGTTACGTT(x38)	agtagtgtaccccacttgaatactttg
30	CAGTTACGCT	agtagtgtaccccacttgaatactttg

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Table 2.6 (Continued)

Day p.i.	Cap sequence	Viral sequence
33	CGGTT	agtagtgtactccacttgaatactttg
33	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
33	TTCAGTTTCGT	agtagtgtaccccacttgaatactttg
33	AGAGCAGCCAAT	agtagtgtactccacttgaatactttg
33	TTGGTCGTCATCG(x7)	agtagtgtaccccacttgaatactttg
33	ATTACCTCGCTAACCTACA	agtagtgtactccacttgaatactttg
36	AGTTCCGTT	agtagtgtaccccacttgaatactttg
36	CAGTTACGTT(x2)	agtagtgtaccccacttgaatactttg
36	ACAAAGTAAA	agtagtgtaccccacttgaatactttg
36	TTGTTTAGTCCA	agtagtgtactccacttgaatactttg
36	CTGGTCGTCATCG	agtagtgtaccccacttgaatactttg
39	TCGG	actccacttgaatactttg
39	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
39	AGCACAATTTAT	gtagtgtaccccacttgaatactttg
39	ATCAGTACAGCTT(x3)	agtagtgtactccacttgaatactttg
39	TTGGTCGTCATCG	agtagtgtaccccacttgaatactttg
39	TGAGACGGCCCACCAG	gtagtgtactccacttgaatactttg
42	CAGTTACGTT(x15)	agtaatgtaccccacttgaatactttg
42	TGCGGTCGCCGG	gtagtgtactccacttgaatactttg
42	TTGGTCGTCATCG(x3)	agtagtgtaccccacttgaatactttg

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a T to C transition from the published sequence at position 11 of the viral mRNA. Of the 5' nonviral sequences, 133 (75.6%) ended in a 3'-terminal T residue. Within the associated viral mRNA, 142 (80.7%) sequences contained the T to C transition at position 11 (Table 2.6).

LAC virus replication in C6/36 cells was characterized by end point titration assay. LAC virus titer in the cell culture medium increased to $10^{7.69}$ $TCID_{50}$/ml by day 9, decreased to $10^{3.44}$ $TCID_{50}$/ml by day 27, and then plateaued between $10^{5.50}$ $TCID_{50}$/ml and $10^{6.00}$ $TCID_{50}$/ml by day 33 (Figure 2.1).

2. Priming of LAC virus wt10 transcription in MAT (*Aedes triseriatus*) cell culture. The 5' nonviral sequences scavenged for transcription by LAC virus wt10 throughout a 42 day time course post-infection in MAT mosquito cell culture were cloned and sequenced. A total of 89 5' nonviral sequences were identified, 55.1 % from day 18 post-infection or later. Of these, 4 (4.5%) were observed only a single time and designated as unique. The longest 5' nonviral sequence was 19 nucleotides in length, while the shortest was 10 nucleotides in length. The 89 5' nonviral sequences had an average length of approximately 10.6 nucleotides, and G or C residues comprised 41.3% of all nucleotides (Table 2.7).

Cap A (5'-CAGTTACGTT-3') predominated in infection of MAT cell culture. It was obtained 70 times (78.7%) during the 42-day infection, and 35 times (71.4%) after day 18 post-infection. In total, 50.0% of all cap A sequences were obtained after the establishment of persistent infection (Table 2.7). Cap B (5'-TTGGTCGTCATCG-3') was also obtained, but with much less frequency than cap A. This 5' nonviral sequence was observed 3 times (4.5%) throughout the 42-day infection. After establishment of persistence

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LAC titers in C6/36 cell culture

Virus in supernatant (log TCID $_{50}$/ml/day)

9.00 8.00 7.00 6.00 5.00 4.00 3.00 2.00 1.00

0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45

Days post infection

LAC wt10

Figure 2.1 Titration of LAC virus in the cell culture medium of infected C6/36 cells. Each data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

Table 2.7 5′ nonviral sequences scavenged by LAC virus wt10 from MAT cell culture. Sequences observed more than once designated in parentheses.

Day p.i.	Cap sequence	Viral sequence
Published viral sequence		agtagtgtactccacttgaatactttg
1	CAGTTACGTT	agtagtgtaccccacttgaatactttg
1	CAGTTACGTT	agtggtgtaccccacttgaatactttg
1	CAGTTACGTT	agcagtgtaccccacttgagtactttg
3	CAGTTACGTT(x12)	agtagtgtaccccacttgaatactttg
3	CAGTTACGTT(x6)	agtggtgtaccccacttgaatactttg
3	CAGTTACGTT	ggtggtgtaccccacttgaatactttg
5	TGCTGTGAACAT	agtagtgtattccacttgaatactttg
7	CGAACTTCTTT	agtagtgtaccccacttgaatactttg
7	ATTACCTCGCTAACCTACA	agtagtgtactccacttgaatgctttg
9	CAGTTACGTT(x9)	agtggtgtaccccacttgaatactttg
9	TTGGTCGTCATCG	agtagtgtaccccacttgaatactttg
9	ATTACCTCGCTAACCTACA	agtagtgtactccacttgaatgctttg
11	CAGTTACGTT	agtagtgtaccccacttgaatactttg
11	CAGTTACGTT	agtagtgtaccccacttgaatactctg
15	CAGTTACGTT	agtggtgtaccccacttgaatactttg
15	CAGTTACGTT	agtagtgtaccccacttgaatactttg
18	CAGTTACGTT(x8)	agtagtgtaccccacttgaatactttg
18	CAGTTACGTT(x3)	agtggtgtaccccacttgaatactttg
18	CAGTTGCGTT	agtagtgtaccccacttgaatactttg
18	CAGTTACGTT	agaagtgtaccccacttgaatactttg
18	ATTACCTCGCTAACCTACA	agtagtgtactccacttgaatgctttg
24	CAGTTACGTT(x2)	agtggtgtaccccacttgaatactttg
24	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
24	CAGTTACGTT	agtagcgtaccccacttgaatactttg
24	CAAGTTGCGCT	agtagtgtaccccacttgaatactttg
24	CGGAACTTCTTT	agtagtgtaccccacttgaatactttg
24	TTGGTCGTCATCGx2)	agtagtgtaccccacttgaatactttg
27	CAGTTACGTT	agtagtgtaccccacttgaatactttg
30	CGAACTTCTTT	agtagtgtaccccacttgaatactttg

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Table 2.7 (Continued)

Day p.i.	Cap sequence	Viral sequence
33	CAGTTACGTT(x6)	agtagtgtaccccacttgaatactttg
33	CAGTTACGTT	agtagtgtaccccacttgaatactctg
33	CAGTTACGTT	agtggtgtaccccacttgaatactttg
33	CGTCGCGTTGT	gtagtgtactccacttgaatactttg
33	CGAACTTCTTT	agtagtgtaccccacttgaataccttg
36	TTGGTCGTCATCG	agtagtgtaccccacttgaatactttg
42	CAGTTACGTT(x6)	agtagtgtaccccacttgaatactttg
42	CAGTTACGTT	agtagtgtatcccacttgaatactttg
42	CAGTTACGTT	agtggtgtaccccacttgaatactttg
42	CGGTTACGTT	agtagtgtaccccacttgaatactttg
42	TTCAGTTTCGT(x3)	agtagtgtaccccacttgaatactttg

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by LAC virus by day 18, cap B was observed 3 times (6.1%). In total, 75.0% of all cap B sequences were obtained after the establishment of persistent infection (Table 2.7).

Similar to the results obtained from C6/36 cell culture, a large proportion of 5' nonviral sequences contained a T residue at the 3'-terminus and associated viral sequences contained a T to C transition at position 11 of the mRNA. Of the 5' nonviral sequences, 83 (93.3%) ended in a 3'-terminal T residue. Within the associated viral mRNA, 84 (94.4%) sequences contained the T to C transition at position 11 (Table 2.7).

Following infection of MAT cells, LAC virus titer in the cell culture medium increased to $10^{7.44}$ $TCID_{50}$/ml by day 3 and then decreased to $10^{3.81}$ $TCID_{50}$/ml by day 15. Two plateaus in virus titer were observed. The first occurred between day 18 and day 27 post-infection, when titers ranged between $10^{6.94}$ $TCID_{50}$/ml and $10^{7.19}$ $TCID_{50}$/ml. The second occurred between day 30 and day 39 post-infection, when titers ranged between $10^{3.25}$ $TCID_{50}$/ml and $10^{3.94}$ $TCID_{50}$/ml (Figure 2.2).

3. Priming of LAC virus wt10 transcription in BHK-21 mammalian cell culture. The 5' nonviral sequences scavenged for transcription by LAC virus wt10 throughout a 48 hour infection of BHK-21 mammalian cell culture were cloned and sequenced. A total of 155 5' nonviral sequences were identified. Approximately 17.4% (27) of the 5' nonviral sequences were observed only a single time and designated as unique. The 5' nonviral sequences ranged in length from 18 nucleotides to 3 nucleotides. The average length of the 155 capped primers was 10.6 nucleotides, 43.3% being either G or C residues (Table 2.8).

Cap A (5'-CAGTTACGTT-3') predominated during BHK-21 cell infection. It was observed 114 times (73.5%) during the 48 hours of virus growth (Table 2.8). Cap B (5'-TTGGTCGTCATCG-3') was obtained twice (1.3%) throughout the time course (Table 2.8).

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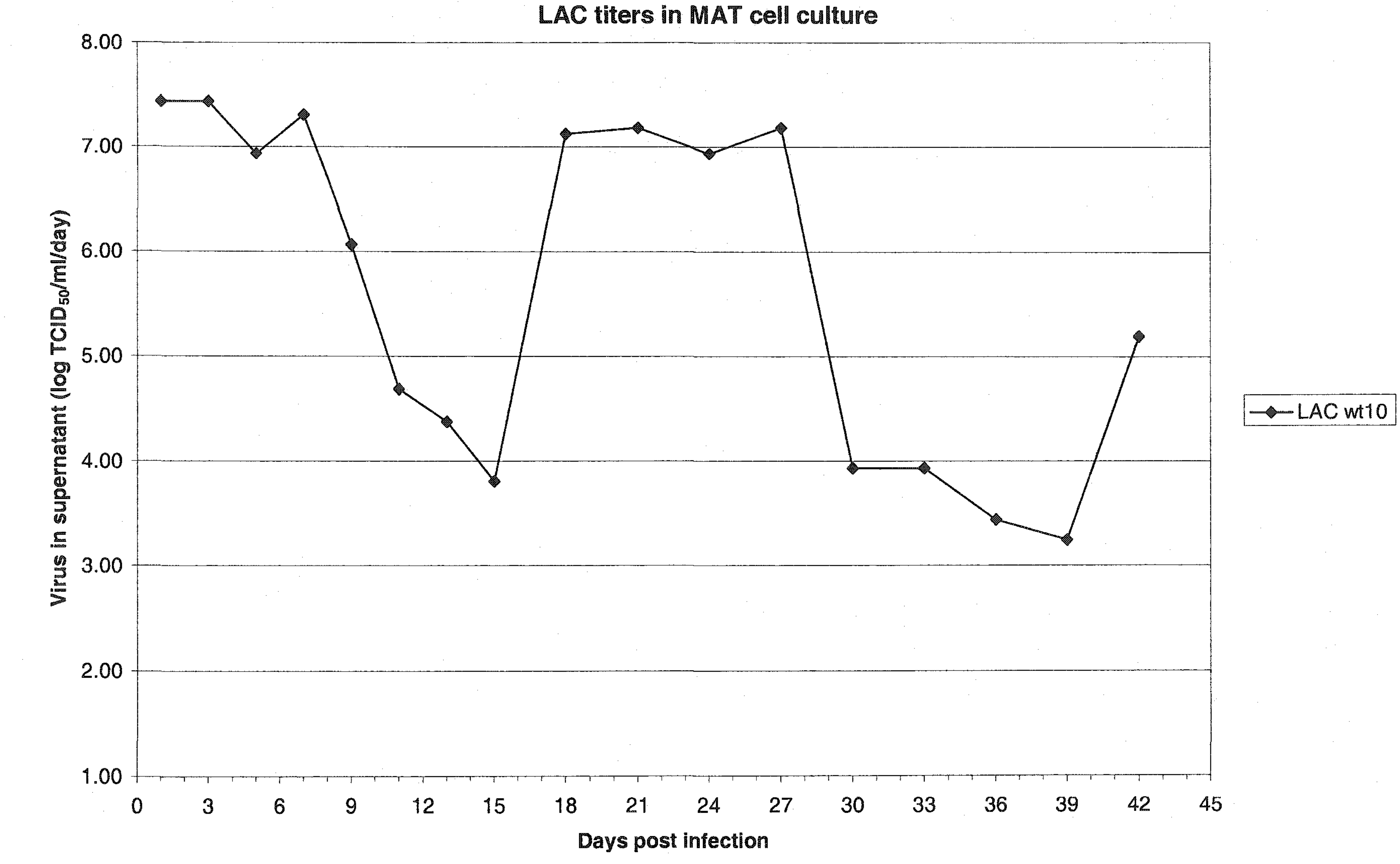

Figure 2.2 Titration of LAC virus in the cell culture medium of infected MAT cells. Each data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

Table 2.8 5′ nonviral sequences scavenged by LAC virus wt10 from BHK-21 cell culture. Sequences observed more than once designated in parentheses.

Time p.i.	Cap sequence	Viral sequence
Published viral sequence		agtagtgtactccacttgaatactttg
4h	CTTTCCGCC	agtagtgtaccccacttgaatactttg
4h	CAGTTACGTT(x11)	agtagtgtaccccacttgaatactttg
4h	CAGTTACGTT	agtactgtaccccacttgaatactttg
4h	CAGTTACGCT	agtagtgtaccccacttgaatactttg
4h	CAGTTACCTT	agtagtgtaccccacttgaatactttg
4h	CAGTTACGTT	agtagtgtaccccacttgaatacattg
4h	TTCCAGCGAGT	agtagtgtactccacttgaatactttg
4h	AGAACCGAGCGTAGT	agtagtgtactccacttgaatactttg
8h	CAGTTACGTT	agtagtgtagcccacttgaatactttg
8h	CAGTTACGTT(x9)	agtagtgtaccccacttgaatactttg
8h	ACCGAGCGTAGT(x2)	agtagtgtactccacttgaatactttg
12h	TTT	agtgtactctacttgaatactttg
12h	TCACTCCC	agtagtgtaccccacttgaatactttg
12h	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
12h	CAGTTACGTT	agtagtgcaccccacttgaatactttg
12h	CGTCTGCGCGT	agtagtgtaccccacttgaatactttg
12h	CTGAGGTGTAGT	agtagtgtaccccacttgaatactttg
12h	TTGGTCGTCATCG	agtagtgtaccccacttgaatactttg
12h	AGAACCGAGCGTAGT	agtagtgtactccacttgaatactttg
12h	AGTGCTCGGTTTCTG	agtagtgtactccacttgaatactttg
12h	ATTCCGGGCCTCCCCT	agtgtaccccacttgaatactttg
20h	AAAATGAAA	agtggtgtaccccacttgaatactttg
20h	CAGTTACGTT	agtggtgtaccccacttgaatactttt
20h	CAGTTACGTT	agtggtgtaccccacttgaatactttg
20h	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg
20h	ACCGGAGCGTAGT	agtagtgtaccccacttgaatactttg
20h	AGTCTCAGTTCAACCT	agtgtactccacttgaatactttg
24h	ATCCGGCGG	agtagtgtaccccacttgaatactttg
24h	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
24h	CAGTTACGTT	ggtagtgtaccccacttgaatactttg
24h	CAGTTACGTT	agtggtgtaccccacttgaatactttg
24h	TTTCCTGTTCT	gtagtgtaccccacctgaatactttg
24h	CTCAGCTCCATT	agtagtgtaccccacttgaatacattg
24h	ACTCTCAGTTCA	agtagtgtactccacttgaatactttg
24h	CGTTTCCGCTTCCG	agtagtgtaccccacttgaatactttt
24h	ATCGGAGCTCCGGTAGT	gtagtgtaccccacttgaatactttg

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Table 2.8 (Continued)

Time p.i.	Cap sequence	Viral sequence
28h	CAGTTACGTT(x9)	agtagtgtaccccacttgaatactttg
28h	CAGTTACGTT	agtagtgtactccacttgaatactttg
28h	CGGTTACGTT	agtagtgtaccccacttgaatactttg
28h	TGATTCCGTCCCGT	gtagtgtaccccacttgaatactatg
32h	CAGTTACGTT(x9)	agtagtgtaccccacttgaatactttg
32h	AGAACCGAGCGTAGT	agtagtgtactccacttgaatattttg
36h	AATAAGTGT	agtagtgtaccccacttgaatactttg
36h	CAGTTTCGTT	agtagtgtaccccacttgaatactttg
36h	CAGTTACGTT(x7)	agtagtgtaccccacttgaatactttg
36h	TTGGTCGTCATCG	agtagtgtaccccacttgaatactttg
36h	CGGATCAGTACAGCT	agtagtgtactccacttgaatactttg
36h	CGAGAGCCGCAGTGTAGT	agtagtgtactccacttgaatactttg
40h	CAGTTACGTT(x7)	agtagtgtaccccacttgaatactttg
40h	CAGTTACGTT	agcagtgtaccccacttgaatactttg
40h	ACATGTTATTT	agtagtgtactccacttgaatactttg
40h	CAGTTCTTATTT	agtgcaccccacctgaatactttg
40h	AGAACCGAGCGTAGT	agtagtgtactccacttgaatactttg
44h	CGGTTACGTT	agtagtgtaccccacttgaatactttg
44h	CAGTTACGTT(x17)	agtagtgtaccccacttgaatactttg
44h	CAGTTACGTT(x2)	agtagcgtaccccacttgaatactttg
44h	CAGTTACGTT	agtaatgtaccccacttgaatactttg
44h	CAGTTACGTT	agtagtgtaccccacttgaatacttag
48h	CAGTTACGTT(x11)	agtagtgtaccccacttgaatactttg
48h	CAGTTACGTT	ggtagtgtgccccacttgaatactttg
48h	CAGTTACGCT	agtagtgtaccccacttgaatactttg
48h	AGAACCGAGCGTAGT	agtagtgtactccacttgaatactttg

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Similar to the results obtained in C6/36 and MAT cell cultures, a large proportion of the 5' nonviral sequences contained a T residue at the 3'-terminus (144 5' nonviral sequences, 92.9%) and associated viral sequences contained a T to C transition at position 11 of the mRNA (138 associated viral sequences, 89.0%) (Table 2.8).

Following LAC virus infection of BHK-21 cells, LAC virus titer in the cell culture medium increased to $10^{4.25}$ $TCID_{50}$/ml by 4 hours post-infection and then plateaued between $10^{5.81}$ $TCID_{50}$/ml and $10^{6.75}$ $TCID_{50}$/ml between 8 and 40 hours post-infection. The virus titer increased to $10^{7.88}$ $TCID_{50}$/ml by 48 hours post-infection (Figure 2.3).

4. Effect of cell type on host mRNA targeted to prime virus transcription and on viral mRNA. The 5' nonviral sequences scavenged by LAC virus wt10 in each cell type were compared to each other. The results are presented in Table 2.9. Certain of the 5' nonviral sequences observed during LAC virus infection of mosquito and mammalian cells were scavenged in more than one cell type. The two predominant 5' nonviral sequences (Cap A and B) were observed in each of the three cell types. Five 5' nonviral sequences were observed in both C6/36 cells and MAT cells and four 5' nonviral sequences were observed in both C6/36 cells and BHK-21 cells (Table 2.10). Several of the associated viral mRNA sequences from C6/36 cells (13/176, 7.4%) and BHK-21 cells (2/155, 1.3%) had single base truncations at the 5' terminus. Additionally, four (2.6%) of associated viral mRNA sequences from BHK-21 cells contained three-base 5' truncations. In contrast, only a single (1.1%) associated viral mRNA sequence from MAT cells had a single base 5' truncation.

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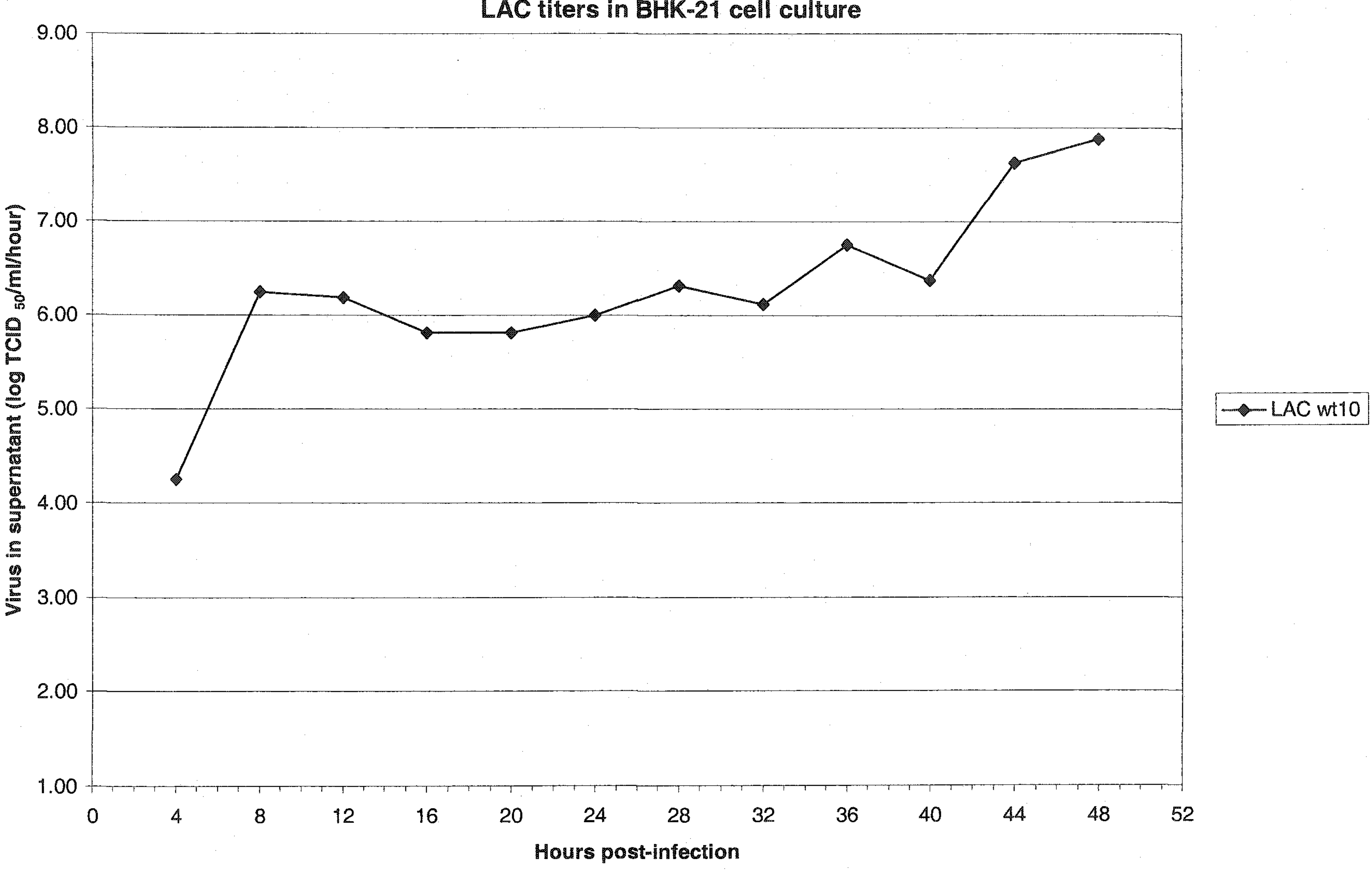

Figure 2.3 Titration of LAC virus in the cell culture medium of infected BHK-21 cells. Each data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

Table 2.9. Comparison of 5' nonviral sequences scavenged by LAC virus wt10 in each cell type. Characteristics of the 5' nonviral sequences in each cell type are given, along with the overall characteristics after combining the results.

	C6/36	MAT	BHK-21	Overall
Number of Caps	176	89	155	420
Unique caps	49 (11.7%)	4 (1.0%)	27 (6.4%)	80/420 (19.0%)
Longest Cap	24 nt	19 nt	18 nt	24 nt
Shortest Cap	3 nt	10 nt	3 nt	3 nt
Average length	11.0 nt	10.6 nt	10.6 nt	10.7 nt
Cap GC%	42.3%	41.3%	43.3%	42.5%
	(818/1934 nt)	(389/941 nt)	(709/1638 nt)	(1916/4513 nt)
5'CAGTTACGTT3'				
Cap A	93/176	70/89	114/155	277/420
	(52.8%)	(78.7%)	(73.5%)	(66.0%)
After day 18	74/129	35/49	N/A	109/178
	(57.4%)	(71.4%)	N/A	(61.2%)
5'TTGGTCGTCATCG3'				
Cap B	17/176	4/89	2/155	23/420
	(4.0%)	(4.5%)	(1.3%)	(5.5%)
After day 18	16/129	3/49	N/A	19/178
	(4.7%)	(6.1%)	N/A	(10.7%)
T to C transition at viral position 11	142/176 (80.7%)	84/89 (94.4%)	138/155 (89.0%)	364/420 (86.7%)
Caps ending in Terminal 3' T	133/176 (75.6%)	83/89 (93.3%)	144/155 (92.9%)	360/420 (85.7%)

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Table 2.10 5′ nonviral sequences from LAC virus mRNA observed in multiple cell types. Percentage of sequences per cell type are given in parentheses. Sequences observed in only one cell type are not listed.

Cap Sequence	C6/36 cells	MAT Cells	BHK-21 Cells
CAGTTACGTT	yes(52.8%)	yes(78.7%)	yes(73.5%)
TTGGTCGTCATCG	yes(4.0%)	yes(4.5%)	yes(1.3%)
TTT	yes(0.6%)	no(0.0%)	yes(0.6%)
ACATGTTATTT	yes(0.6%)	no(0.0%)	yes(0.6%)
CGAACTTCTTT	yes(0.6%)	yes(3.4%)	no(0.0%)
CGTCGCGTTGT	yes(0.6%)	yes(1.1%)	no(0.0%)
TTCAGTTTCGT	yes(0.6%)	yes(1.1%)	no(0.0%)
TTCCAGCGAGT	yes(0.6%)	no(0.0%)	yes(0.6%)
TGCTGTGAACAT	yes(1.1%)	yes(1.1%)	no(0.0%)
AGAACCGAGCGTAGT	yes(0.6%)	no(0.0%)	yes(5.3%)
ATTACCTCGCTAACCTACA	yes(1.1%)	yes(2.2%)	no(0.0%)

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5. Priming of SSH and TAH virus transcription in mosquito and mammalian cell cultures. To determine if SSH and TAH virus-infected cell cultures scavenged the same caps as LAC virus wt10, 5' nonviral sequences scavenged by SSH and TAH viruses were cloned and sequenced from C6/36 and MAT cell cultures at day 33 post-infection, and BHK-21 cell culture at 24 hours post-infection.

From SSH infected cell cultures, a total of 45 5' nonviral sequences were determined, 10 from C6/36 cells, 10 from MAT cells, and 25 from BHK-21 cells. All of the 5' nonviral sequences obtained from SSH virus-infected cell cultures were observed more than once. The longest 5' nonviral sequence was 10 nucleotides in length, while the shortest was 8 nucleotides in length in each of the cell types. In C6/36 cell cultures, the 10 5' nonviral sequences averaged 9.4 nucleotides; 45.7% of nucleotides were either G or C residues. In MAT cell cultures, the 10 5' nonviral sequences averaged 9.6 nucleotides; 43.8% of nucleotides were either G or C residues. In BHK-21 cell cultures, the 25 5' nonviral sequences averaged 9.9 nucleotides; 40.7% of nucleotides were either G or C residues (Tables 2.11 and 2.12).

Similar to the results of LAC virus wt10 infected cells shown previously, the same predominant 5' nonviral sequence was observed during infection in each cell type. Cap A (5'-CAGTTACGTT-3') was observed 7 times (70.0%) in C6/36 cell cultures, 8 times (80.0%) in MAT cell cultures, and 24 times (96.0%) in BHK-21 cell cultures (Table 2.10). Cap B (5'-TTGGTCGTCATCG-3') was not observed in any of the SSH virus infected cell cultures (Table 2.10). However, a new predominant 5' nonviral sequence was obtained. This new 5' nonviral sequence, designated cap C (5'-TCACTCCC-3') was observed 3 times (30.0%) in

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Table 2.11 5′ nonviral sequences scavenged by SSH and TAH viruses from C6/36, MAT, and BHK-21 cell cultures. Sequences observed more than once designated in parentheses.

Virus	Cell	Cap Sequence	Viral sequence
Published viral sequence*			agtagtgtactccacttgaatactttg
SSH	C6/36	TCACTCCC(x3)	gtagtgtaccccacttgaatactttg
SSH	C6/36	CAGTTACGTT(x6)	agtagtgtaccccacttgaatactttg
SSH	C6/36	CAGTTACGTT	agtagcgtaccccacttgaatactttg
SSH	MAT	TCACTCCC(x2)	gtagtgtaccccacttgaatactttg
SSH	MAT	CAGTTACGTT(x8)	agtagtgtaccccacttgaatactttg
SSH	BHK-21	TCACTCCC	gtagtgtaccccacttgaatactttg
SSH	BHK-21	CAGTTACGTT(x22)	agtagtgtaccccacttgaatactttg
SSH	BHK-21	CAGTTACGTT	agtggtgtaccccacttgaatactttg
SSH	BHK-21	CAGTTACGTT	agtagtgaaccccacttgaatactttg
TAH	C6/36	TCACTCCC(x6)	gtagtgtaccccacttgaatactttg
TAH	C6/36	CAGTTACGTT(x17)	agtagtgtaccccacttgaatactttg
TAH	C6/36	TCAGTTACGTT(x2)	agtagtgtaccccacttgaatactttg
TAH	C6/36	CGTTTCCGCTTCCGT	agtagtgtaccccacttgaatactttg
TAH	MAT	TCACTCCC(X4)	gtagtgtaccccacttgaatactttg
TAH	MAT	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
TAH	MAT	CGGTTACGTT	agtagtgtaccccacttgaatactttg
TAH	MAT	TAGTTACGTT	agtagtgtaccccacttgaatactttg
TAH	MAT	ACAGTTACGTT	agtagtgtaccccacttgaatactttg
TAH	BHK-21	TCACTCCC(x4)	gtagtgtaccccacttgaatactttg
TAH	BHK-21	CAGTTACGTT(x8)	agtagtgtaccccacttgaatactttg

*The published viral sequence of nucleotides 1-27 of the S segment mRNA is identical for LAC, SSH, and TAH viruses.

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Table 2.12 Comparison of 5' nonviral sequences scavenged by SSH and TAH viruses from C6/36, MAT, and BHK-21 cell cultures. Characteristics of the 5' nonviral sequences in each cell type are given, along with the overall characteristics after combining the results.

SSH

	C6/36	MAT	BHK-21	Overall
Caps	10	10	25	45
3'T	7/10 (70.0%)	8/10 (80.0%)	24/25 (96.0%)	39/45 (86.7%)
T11C	10/10 (100.0%)	10/10 (100.0%)	25/25 (100.0%)	45/45 (100.0%)
CAP A	7/10 (70.0%)	8/10 (80.0%)	24/25 (96.0%)	39/45 (86.7%)
CAP B	0/10 (0.0%)	0/10 (0.0%)	0/25 (0.0%)	0/45 (0.0%)
CAP C	3/10 (30.0%)	2/10 (20.0%)	1/25 (4.0%)	6/45 (13.3%)
GC%	43/94nt (45.7%)	42/96nt (43.8%)	101/248nt (40.7%)	186/438nt (42.5%)
Ave. length	9.4 nt	9.6 nt	9.9 nt	9.7 nt
Unique	0/10 (0.0%)	0/10 (0.0%)	0/25 (0.0%)	0/45 (0.0%)

TAH

	C6/36	MAT	BHK-21	Overall
Caps	26	10	12	48
3'T	20/26 (76.9%)	6/10 (60.0%)	8/12 (66.7%)	34/48 (70.8%)
T11C	26/26 (100.0%)	10/10 (100.0%)	12/12 (100.0%)	48/48 (100.0%)
CAP A	17/26 (65.4%)	3/10 (30.0%)	8/12 (66.7%)	28/48 (58.3%)
CAP B	0/26 (0.0%)	0/10 (0.0%)	0/12 (0.0%)	0/48 (0.0%)
CAP C	6/26 (23.1%)	4/10 (40.0%)	4/12 (33.3%)	14/48 (29.2%)
GC%	115/255nt (45.1%)	44/93nt (47.3%)	52/112nt (46.4%)	211/460nt (45.9%)
Ave. length	9.8 nt	9.3 nt	9.3 nt	9.6 nt
Unique	1/26 (3.8%)	3/10 (30.0%)	0/12 (0.0%)	4/48 (8.3%)

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C6/36 cell cultures, twice (20.0%) in MAT cell cultures, and once (4.0%) in BHK-21 cell cultures (Tables 2.11 and 2.12).

Similar to the previous results with LAC virus wt10, a large proportion of 5' nonviral sequences contained a T residue at the 3'-terminus and associated viral sequences contained a T to C transition at position 11 of the viral mRNA. Of the 5' nonviral sequences, 7 (70.0%) in C6/36 cell cultures, 8 (80.0%) in MAT cell cultures, and 24 (96.0%) in BHK-21 cell cultures ended in a 3'-terminal T residue. Within the viral mRNA, 10 sequences (100.0%) in C6/36 cell cultures, 10 sequences (100.0%) in MAT cell cultures, and 25 sequences (100.0%) in BHK-21 cell cultures contained the T to C transition at position 11 (Tables 2.11 and 2.12).

From TAH infected cell culturess, a total of 48 5' nonviral sequences were identified, 26 from C6/36 cell cultures, 10 from MAT cell cultures, and 12 from BHK-21 cell cultures. One 5' nonviral sequence (3.8%) from C6/36 cell cultures, 3 (30.0%) from MAT cell cultures, and none from BHK-21 cell cultures were observed only once and designated unique. The longest 5' nonviral sequence found in TAH virus infected MAT cell cultures was 15 nucleotides in length, while the shortest was 8 nucleotides in length. In C6/36 cell cultures, the 26 5' nonviral sequences averaged 9.8 nucleotides; 45.1% of nucleotides were either G or C residues. In MAT cell cultures, the 10 5' nonviral sequences averaged 9.3 nucleotides; 47.3% of nucleotides were either G or C residues. In BHK-21 cell cultures, the 12 5' nonviral sequences averaged 9.3 nucleotides; 46.4% of nucleotides were either G or C residues (Tables 2.11 and 2.12).

Similar to the results of LAC virus wt10 and SSH virus, cap A (5'-CAGTTACGTT-3') was the predominant 5' nonviral sequence, observed 17 times (75.4%) in C6/36 cell cultures, 3 times (30.0%) in MAT cell cultures, and 8 times (66.7%) in BHK-21 cell cultures

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(Table 2.10). Cap B (5'-TTGGTCGTCATCG-3') was not observed in any of the TAH virus-infected cell culturess (Table 2.10). Interestingly, cap C (5'-TCACTCCC-3') was observed 6 times (23.1%) in C6/36 cell cultures, 4 times (40.0%) in MAT cell cultures, and 4 times (33.3%) in BHK-21 cell cultures (Tables 2.11 and 2.12).

Similar to the previous results with LAC virus wt10 and SSH virus, a large portion of TAH 5' nonviral sequences contained a T residue at the 3'-terminus and associated viral sequences contained a T to C transition at position 11 of the viral mRNA. Of the 5' nonviral sequences, 20 (76.9%) in C6/36 cell cultures, 6 (60.0%) in MAT cell cultures, and 8 (66.7%) in BHK-21 cell cultures ended in a 3'-terminal T residue. Within the associated viral mRNA, 26 sequences (100.0%) in C6/36 cell cultures, 10 sequences (100.0%) in MAT cell cultures, and 12 sequences (100.0%) in BHK-21 cell cultures contained the T to C transition at position 11 (Tables 2.11 and 2.12).

All SSH and TAH viral mRNA sequences associated with cap C (5'TCACTCCC-3') contained 1 base (20/20, 100.0%) truncation at the 5' terminus. The single base truncation at the 5' terminus of viral mRNA associated with cap sequences ending in a C residue observed in SSH and TAH infected cell cultures was not observed in LAC infected cell cultures.

6. Priming of LAC virus isolate transcription in mosquito tissues. To determine if LAC virus wt10 had the same cap scavenging specificity as other LAC virus isolates, ovaries and midguts from persistently infected *Ae. triseriatus* mosquitoes were dissected and analyzed for 5' nonviral sequences. LAC virus wt 10 cap scavenging specificity was compared to five different LAC virus isolates including: LAC H78mp1, LAC H78mp2, LAC TOT3, LAC TOT9, and LAC TCF 6.

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A total of 148 5' nonviral sequences were obtained from tissues infected with the six different LAC virus isolates. There were 28 5' nonviral sequences (13 ovary, 15 midgut) from LAC virus wt10 infected female *Ae. triseriatus* mosquitoes, 26 5' nonviral sequences (11 ovary, 15 midgut) from LAC H78mp1 infected female *Ae. triseriatus* mosquitoes, and 20 5' nonviral sequences (10 ovary, 10 midgut) from LAC H78mp2 infected female *Ae. triseriatus* mosquitoes. From female *Ae. triseriatus* mosquitoes infected with either LAC TOT3 or LAC TOT9, 25 (15 ovary, 10 midgut) and 29 (11 ovary, 18 midgut) 5' nonviral sequences were obtained, respectively. From LAC TCF6 infected female *Ae. triseriatus* mosquitoes, 20 (10 ovary, 10 midgut) 5' nonviral sequences were obtained. In total 70 5' nonviral sequences were obtained from ovary tissue and 78 from midgut tissue (Tables 2.13 and 2.14). The 5' nonviral sequence length ranged from 8 to 15 nucleotides. All ovary 5' nonviral sequences were 8 to 10 nucleotides in length, and all midgut 5' nonviral sequences ranged in length from 10 to 15 nucleotides (Tables 2.13 and 2.14). Similar to experiments in LAC virus infected cell culture, the GC% for the 5' nonviral sequences scavenged in ovaries ranged from 40.0% to 45.9% and from midguts ranged from 39.6% to 43.4%. The average length of 5' nonviral sequences scavenged in ovaries was 9.9 nucleotides, and in midguts, 10.2 nucleotides.

As in the previously described results, cap A (5'-CAGTTACGTT-3') was the predominant 5' nonviral sequence in all tissues with all LAC virus isolates. Cap A was observed in 9 ovary 5' nonviral sequences and 15 midgut 5' nonviral sequences from LAC virus wt10 infected mosquitoes. Cap A was observed in 11 ovary 5' nonviral sequences and 10 midgut 5' nonviral sequences from LAC H78mp1 infected mosquitoes. Cap A was observed in 10 ovary 5' nonviral sequences and 9 midgut 5' nonviral sequences from LAC

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Table 2.13 5′ nonviral sequences scavenged by LAC virus isolates from ovary and midgut tissues from infected *Ae. triseriatus* mosquitoes. Sequences observed more than once designated in parentheses. Percentage of total sequences per virus and tissue type is given.

Virus Tissue	Cap Sequence	%	Viral sequence
Published viral sequence			agtagtgtactccacttgaatactttg
H78mp1			
Ovary	CAGTTACGTT(x11)	100	agtagtgtaccccacttgaatactttg
Midgut	CAGTTACGTT(x10)	76.9	agtagtgtaccccacttgaatactttg
Midgut	CAGCTACGTT	7.7	agtagtgtaccccacttgaatactttg
Midgut	AGCAGTTACGTT(x2)	13.3	agtagtgtaccccacttgaatactttg
Midgut	TTGGTCGTCATCG(x2)	13.3	agtagggtaccccacttgaatactttg
H78mp2			
Ovary	CAGTTACGTT(x8)	80.0	agtagtgtaccccacttgaatactttg
Ovary	CAGTTACGTT	10.0	agtagtgtaccacacttgaatactttg
Ovary	CAGTTACGTT	10.0	agtagtgtaccccacttgaatacttag
Midgut	CAGTTACGTT(x9)	90.0	agtagtgtaccccacttgaatactttg
Midgut	CAGTTACCTT	10.0	agtagtgtaccccacttgaatactttg
TOT 9			
Ovary	CAGTTACGTT(x9)	81.8	agtagtgtaccccacttgaatactttg
Ovary	CAGTTACGCT	9.1	agtagtgtaccccacttgaatactttg
Ovary	CGTTTCCGCTTCCGT	9.1	agtagtgtaccccacttgaatactttg
Midgut	CAGTTACGTT(x15)	83.3	agtagtgtaccccacttgaatactttg
Midgut	CAGTCACGTT	5.6	agtagcgtaccccacttgaatactttg
Midgut	CAGTTACGTT	5.6	agtagtgtaccccgcttgaatactttg
Midgut	CAGTTACGTT	5.6	agtagtgtaccccacttgaatactctg
TOT 3			
Ovary	CAGTTACGTT(x14)	93.3	agtagtgtaccccacttgaatactttg
Ovary	CAGTTACGTT	6.7	agtagtgtgccccacttgaatactttg
Midgut	CAGTTACGTT(x9)	90.0	agtagtgtaccccacttgaatactttg
Midgut	ACAGTTACGTT	10.0	agtagtgtaccccacttgaatgctttg
TCF 6			
Ovary	CAGTTACGTT(x8)	80.0	agtagtgtaccccacttgaatactttg
Ovary	CAGTTACGTT	10.0	agtagtgtaccccacttgaatacattg
Ovary	CAGTTACGTT	10.0	agtagtgtaccccacttgaatacttca
Midgut	CAGTTACGTT(x8)	80.0	agtagtgtaccccacttgaatactttg
Midgut	TTGGTCGTCATCG(x2)	20.0	agtaggataccccacttgaatactttg
WT 10			
Ovary	TCACTCCC(x4)	30.8	gtagtgtaccccacttgaatactttg
Ovary	CAGTTACGTT(x9)	69.2	agtagtgtaccccacttgaatactttg
Midgut	CAGTTACGTT(x15)	100	agtagtgtaccccacttgaatactttg

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Table 2.14 Comparison of 5' nonviral sequences scavenged by LAC virus isolates in ovary and midgut tissues from infected *Ae. triseriatus* mosquitoes. Characteristics of 5' nonviral sequences in each tissue are given, along with overall characteristics after combining results.

	WT 10	H78mp1	H78mp2	TOT 3	TOT 9	TCF 6	Overall
Cap sequences							
Total	28	26	20	25	29	20	148
Ovary	13	11	10	15	11	10	70
Midgut	15	15	10	10	18	10	78
Longest Cap (in nucleotides)							
Ovary	10	10	10	10	10	10	10
Midgut	10	13	10	11	15	13	15
Shortest Cap (in nucleotides)							
Ovary	8	10	10	10	10	10	8
Midgut	10	10	10	10	10	10	10
GC%							
Total	44.3%	41.9%	40.0%	39.8%	41.7%	41.7%	41.6%
Ovary	45.9%	40.0%	40.0%	40.0%	43.5%	40.0%	41.6%
Midgut	40.0%	43.1%	40.0%	39.6%	40.6%	43.4%	41.7%
Caps ending in terminal 3' T							
Total	24/28	24/26	20/20	25/25	29/29	18/20	144/148
Ovary	13/13	11/11	10/10	15/15	11/11	10/10	70/70
Midgut	15/15	13/15	10/10	10/10	18/18	8/10	74/78
T to C transition at viral position 11							
Total	28/28	26/26	20/20	25/25	29/29	20/20	148/148
Ovary	13/13	11/11	10/10	15/15	11/11	10/10	70/70
Midgut	15/15	15/15	10/10	10/10	18/18	10/10	78/78
Cap A 5'CAGTTACGTT3'							
Total	24	20	19	24	26	18	132
Ovary	9	11	10	15	9	10	64
Midgut	15	10	9	9	17	8	68
Cap B 5'TTGGTCGTCATCG3'							
Total	0	2	0	0	0	2	4
Ovary	0	0	0	0	0	0	0
Midgut	0	2	0	0	0	2	4
Cap C 5'TCACTCCC3'							
Total	4	0	0	0	0	0	4
Ovary	4	0	0	0	0	0	4
Midgut	0	0	0	0	0	0	0
Unique caps							
Total	0	1	1	1	3	0	6
ovary	0	0	0	0	2	0	2
midgut	0	1	1	1	1	0	4

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H78mp2 infected mosquitoes. Cap A was observed in 15 ovary 5' nonviral sequences and 9 midgut 5' nonviral sequences from LAC TOT3 infected mosquitoes. Cap A was observed in 9 ovary 5' nonviral sequences and 17 midgut 5' nonviral sequences from LAC TOT9 infected mosquitoes. Cap A was observed in 10 ovary 5' nonviral sequences and 8 midgut 5' nonviral sequences from LAC TCF6 infected mosquitoes. Overall, cap A comprised 91.4% of all 5' nonviral sequences from ovaries (64 out of 70), 87.2% from midguts (68 out of 78), and 89.2% overall (132 out of 148) (Tables 2.13 and 2.14).

Cap B (5'-TTGGTCGTCATCG-3') was observed in midgut tissue following infection with two of the LAC virus isolates. Two of the 5' nonviral sequences from LAC H78mp1 and two of the LAC TCF6 5' nonviral sequences were cap B. Overall, cap B comprised 5.1% of all 5' nonviral sequences scavenged from midguts (4 out of 78), and 2.7% of both tissues (4 out of 148) (Tables 2.13 and 2.14).

Cap C (5'-TCACTCCC-3') was observed in ovary tissue following infection with LAC virus wt10. Four (26.7%) of the LAC virus wt10 5' nonviral sequences were cap C. Overall, cap C comprised 5.7% of all 5' nonviral sequences scavenged from ovaries (4 out of 70), and 2.7% from both tissues (4 out of 148) ((Tables 2.13 and 2.14).

Single or unique 5' nonviral sequences were observed in midguts infected with four of the LAC virus isolates and in ovaries infected with one of the LAC virus isolates. A unique 5' nonviral sequence was observed in 1 midgut infected with LAC H78mp1, H78mp2, TOT3, and TOT9 infected mosquitoes, and in 2 ovary 5' nonviral sequences from LAC TOT9 infected mosquitoes. Overall, unique 5' nonviral sequences comprised 2.9% (2/70) of all 5' nonviral sequences scavenged from ovaries, 5.1% (4/78) from midguts, and 4.1% (6/78) overall (Tables 2.13 and 2.14).

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Similar to the results of cap scavenging analyses from cell culture, a large proportion of the 5' nonviral sequences ended in a T residue at the 3' terminus and exhibited the T to C transition in the viral mRNA sequence at position 11. All of the associated viral mRNA sequences from 5' nonviral sequences scavenged in the ovaries and the midguts by all LAC virus isolates exhibited the T to C transition in the viral mRNA sequence at position 11. All of the 5' nonviral sequences scavenged by all of the LAC virus isolates in the ovaries ended with a T residue at the 3' terminus. Of the 5' nonviral sequences scavenged by the LAC virus isolates in the midguts, only 2 of 13 (15.4%) LAC H78mp1 and 2 of 10 (10.0%) LAC TCF6 5' nonviral sequences did not end with the 3' terminal T residue. In the midguts, 94.9% (74 out of 78) ended with the 3' terminal T residue, and 97.3% (144 out of 148) ended with the 3' terminal T residue (Tables 2.13 and 2.14).

Only four (2.7%) of the associated viral mRNA sequences from 5' nonviral sequences from infected mosquito tissues contained single base truncations at the 5' terminus. All of those sequences were associated with cap C (5'-TCACTCCC-3') and the viral mRNA contained a 5' terminal G residue. This cap C and viral mRNA 5' G residue were observed in LAC virus wt10 infected ovaries.

7. Confirmation of presence of 5' nonviral sequences on viral mRNA species. RT-PCR was performed using observed 5' nonviral sequences paired with LNR as primers to amplify sequences from total RNA isolated from LAC infected MAT cell cultures. Initial reverse transcription was performed using LNR primer specific for the 3' terminus of the LAC virus S segment mRNA (Table2.1). PCR amplification was performed using an internal LAC primer, LVCB, and one of five different primers that combined observed 5' nonviral sequence and the first 7 nucleotides from the 5' terminus of LAC virus S segment

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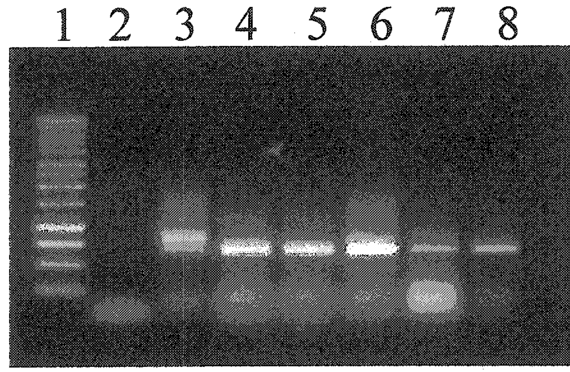

1 = standard
2 = PCR Blank
3 = + control (1/4 vol.)
4 = CAP-A 5'-CAGTTACGTT-3'
5 = CAP-B 5'-TTGGTCGTCATCG-3'
6 = CAP-C 5'-TCACTCCC-3'
7 = CAP-1 5'-CCACTCGCCACT-3'
8 = CAP-2 5'-AGGAAAAGTAGGT-3'

Figure 2.4 RT-PCR amplification of LAC virus S segment mRNA from MAT cell cultures using sequences derived from scavenged 5' nonviral sequences as forward primers. The RT-PCR amplification products were electrophoresed on 1.0% agarose 1X TAE gels and the contents of each lane are given above. Positive control was cDNA made from LAC S segment mRNA and amplified with LNFc3 and LVCB primers (Table 2.1)

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mRNA. Amplification products of the expected sizes (Table 2.4) were obtained from each of the reactions. The 5' nonviral sequences from this study, caps A, B, and C, gave products that stained with greater intensity than the 5' nonviral sequences from the previous study, caps 1 and 2 (Dobie et al., 1997) (Figure 2.4). It appears that cap C primer gives the most intensely staining product but these results may not reflect abundance.

8. LAC virus and SIN virus co-infection in C6/36 and MAT cell cultures. Studies were conducted to determine if LAC virus would scavenge 5' nonviral sequences from SIN virus mRNA in dually infected C6/36 and MAT cell cultures. Viruses were used to infect cells under two different infection regimens. In regimen A, the cell cultures were co-infected simultaneously with LAC and SIN viruses, and in regimen B, LAC virus was allowed to establish a persistent infection for 18 days prior to infection with SIN virus.

Overall, in C6/36 cell cultures co-infected with LAC and SIN viruses, 71 5' nonviral sequences were obtained. A single SIN-derived 5' sequence (5'-ATAGTCAGCAT-3') was observed at day 3 in cells co-infected using regimen A (1.4%). This 5' sequence is exactly the 5' distal eleven nucleotides from mRNAs transcribed from the first and second subgenomic promoters of SIN TE3'/2J virus (Table 2.15). The longest 5' nonviral sequence was 20 nucleotides in length and the shortest was 2 nucleotides in length. The GC% was 42.1% and the average length of the 5' sequences was 10.9 nucleotides. Of the 5' nonviral sequences, 42 (59.2%) were observed only a single time and designated as unique (Table 2.14). Cap A (5'-CAGTTACGTT-3') was the predominant 5' nonviral sequence observed throughout infection of C6/36 cell cultures (27 of 71, 38.0%) (Table 2.15). Cap B (5'-TTGGTCGTCATCG-3') and cap C (5'-TCACTCCC-3') were not observed in the co-infected C6/36 cell cultures. A large proportion of 5' nonviral sequences contained a T

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Table 2.15 5′ nonviral sequences scavenged by LAC virus in C6/36 cell cultures co-infected with SIN virus. Sequences observed more than once designated in parentheses.

Time point	Cap sequence	Viral sequence
Regimen A		
Published LAC viral sequence		agtagtgtactccacttgaatactttg
SIN genomic 5′ sequence		attgacggcgtagtacacactattgaa
SIN subgenomic 5′ sequence		atagtcagcatagtacatttcatctga
Day 3	CAT	agtagtgtactccacttgaatactttg
Day 3	**ATAGTCAGCA**	agtagtgtaccccacttgaatactttg
Day 3	AACAGCATCTGT	agtagtgtactccacttgaatactttg
Day 3	ACGCACCAGTAG	gtagtgtaccccacttgaatactttg
Day 3	ATTCGCCAACTGC	gtagtgtaccccacttgaatactttg
Day 3	ACCTGTTGCACGT	agtagtgtactccacttgaatactttg
Day 3	AATCCGTGTGCGT	gtagtgtactccacttgaatactttg
Day 3	ATTCATCTCACTGT	agtagtgtactccacttgaatactttg
Day 9	CTTTCATTAC	agtagtgtaccccacttgaatactttg
Day 9	AGTCGTCAGTC	agtagtgtaccccacttgaatactttg
Day 9	ACTCAGCTCTTG	gtagtgtaccccacttgaatactttg
Day 9	ACACTTCGTTCTT	gtagtgtaccccacttgaatactttg
Day 9	ATGTTGATTCTTGT	agtagtgtaccccacttgaatactttg
Day 9	ATTTAGTGTGCGTATT	agtagtgtactccacttgaatactttg
Day 18	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
Day 18	ACATTCGCTGT	agtagtgtaccccacttgaatactttg
Day 18	ATTAAGTAAAGT	gtagtgtaccccacttgaatactttg
Day 18	ATTCTTTCGCTTC	gtagtgtaccccacttgaatactttg
Day 18	ACTTTCAGGCGCCG	agtagtgtaccccacttgaatactttg
Day 24	CGGGCG	agtagtgtactccacttgaatactttg
Day 24	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
Day 33	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
Day 33	AAAGTGGAAA	agtagtgtactccacttgaatactttg
Day 33	AGTTGTGCTGC	agtagtgtactccacttgaatactttg
Day 42	CG	gtagtgtaccccacttgaatactttg
Day 42	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg

ATAGTCAGCA from SIN 5′ subgenomic sequence

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Table 2.15 **(Continued)**

Regimen B		
Day 18+8h	CATTTCGTTGA	agtagtgtactccacttgaatactttg
Day 18+8h	ACAATCACAATT	tagtgtaccccacttgaatactttg
Day 18+8h	TGAAAAACGGCCTG	gtagtgtactccacttgaatactttg
Day 18+8h	CGGACAGTTCCCGGT	agtagtgtaccccacttgaatactttg
Day 18+8h	CTTTTTTTTTCTCTC	gtagtgtaccccacttgaatactttg
Day 18+8h	TCTGTCCCACGTTTCGG	gtagtgtaccccacttgaatactttg
Day 18+16h	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
Day 18+16h	ATTCCAAATCGT	agtagtgtaccccacttgaatactttg
Day 18+16h	ATTTTTTTCGTATTCGTAGT	gtagtgtaccccacttgaatactttg
Day 18+24h	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg
Day 18+32h	TGA	tagtgtactccacttgaatactttg
Day 18+32h	CAGTTACGTT(x2)	agtagtgtaccccacttgaatactttg
Day 18+32h	CAACAGTCTTAT	agtagtgtaccccacttgaatactttg
Day 18+32h	ACCCCAGTCAGT	agtagtgtactccacttgaatactttg
Day 18+32h	CAGTTCAATTTCCA	agtagtgtaccccacttgaatactttg
Day 18+40h	CGG	agtagtgtaccccacttgaatactttg
Day 18+40h	CGGGT	agtagtgtactccacttgaatactttg
Day 18+40h	CAGTTACGTT	agtagtgtaccccacttgaatactttg
Day 18+40h	ACTCAGTCGATTG	agtagtgtaccccacttgaatactttg
Day 18+40h	CTTTTTGATTGTTTT	gtagtgtaccccacttgaatactttg
Day 18+48h	CGGG	gtagtgtaccccacttgaatactttg
Day 18+48h	CAGTTACGT	agtagtgtaccccacttgaatactttg
Day 18+48h	ATTAGTCAACT	agtagtgtaccccacttgaatactttg
Day 18+48h	AATATCAGTCCG	gtagtgtaccccacttgaatactttg
Day 18+48h	AAGAAAGGTTGTT	agtagtgtactccacttgaatactttg
Day 18+48h	AAAAAAGGTTCCT	agtagtgtactccacttgaatactttg
Day 18+48h	TGAGAGCAGCTCTCC	gtagtgtaccccacttgaatactttg

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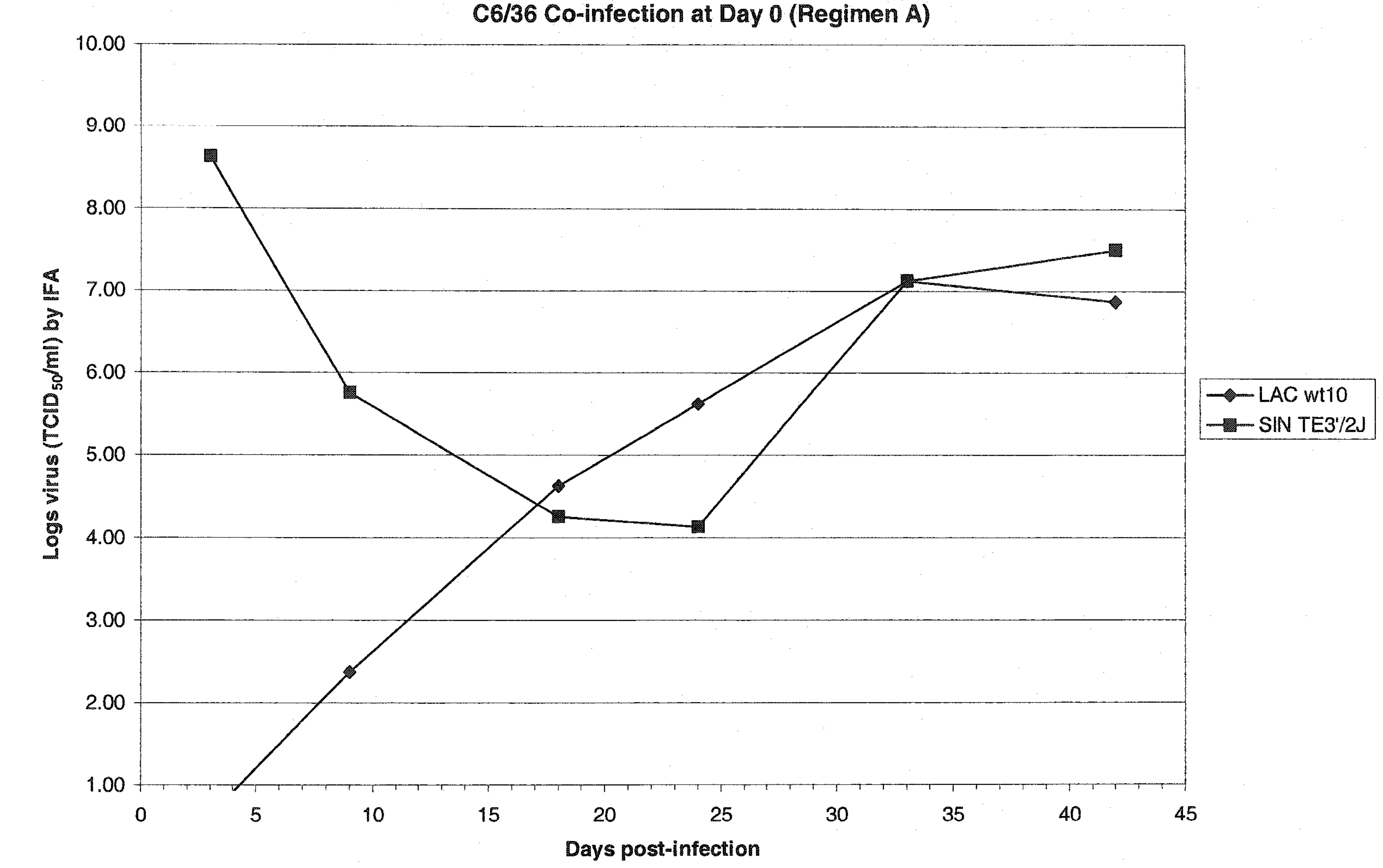

Figure 2.5 Titers of LAC and SIN viruses in C6/36 cell cultures co-infected at day 0 (Regimen A). Each data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

residue at the 3'-terminus and associated viral sequences contained a T to C transition at position 11 of the viral mRNA. Of the 5' nonviral sequences, 49 (69.0%) ended in a 3'-terminal T residue, and of the associated viral mRNA, 55 (77.5%) sequences contained the T to C transition at position 11 (Table 2.15).

Following dual infection of C6/36 cells using regimen A, LAC virus titer was low at day 3 post-infection (below $10^{1.00}$ $TCID_{50}$/ml) and steadily increased to a plateau of approximately $10^{7.00}$ $TCID_{50}$/ml by day 33 post-infection. The SIN virus titer was approximately $10^{8.50}$ $TCID_{50}$/ml within 3 days post-infection and decreased to a stable titer of approximately $10^{4.00}$ $TCID_{50}$/ml by day 18 post-infection. Late in the infection, SIN virus titers began to rise and reached $10^{7.00}$ $TCID_{50}$/ml by day 33 and $10^{7.50}$ $TCID_{50}$/ml by day 42 post-infection (Figure 2.5).

Following dual infection of C6/36 cells using regimen B, LAC virus titer reached $10^{7.00}$ $TCID_{50}$/ml by day 5 post-infection. As LAC virus established a persistent infection in the cells, the titer decreased and stabilized at $10^{6.00}$ $TCID_{50}$/ml by day 18. Following infection with SIN virus at day 18, the titer of LAC virus decreased to $10^{3.00}$ $TCID_{50}$/ml within 8 hours. By 16 hours after co-infection, the titer of LAC virus began to increase and reached approximately $10^{8.50}$ $TCID_{50}$/ml by 32 hours after co-infection. The titers of LAC virus stabilized to $10^{7.50}$ $TCID_{50}$/ml by 40 hours after co-infection. SIN virus titers reached approximately $10^{6.00}$ $TCID_{50}$/ml by 16 hours after co-infection and then gradually decreased to $10^{4.25}$ $TCID_{50}$/ml by 48 hours after co-infection (Figure 2.6).

Overall, in MAT cell cultures co-infected with LAC and SIN viruses, 68 5' nonviral sequences were determined. No SIN-derived 5' sequences were observed (Table 2.16). The longest 5' nonviral sequence obtained was 15 nucleotides in length and the shortest was 2

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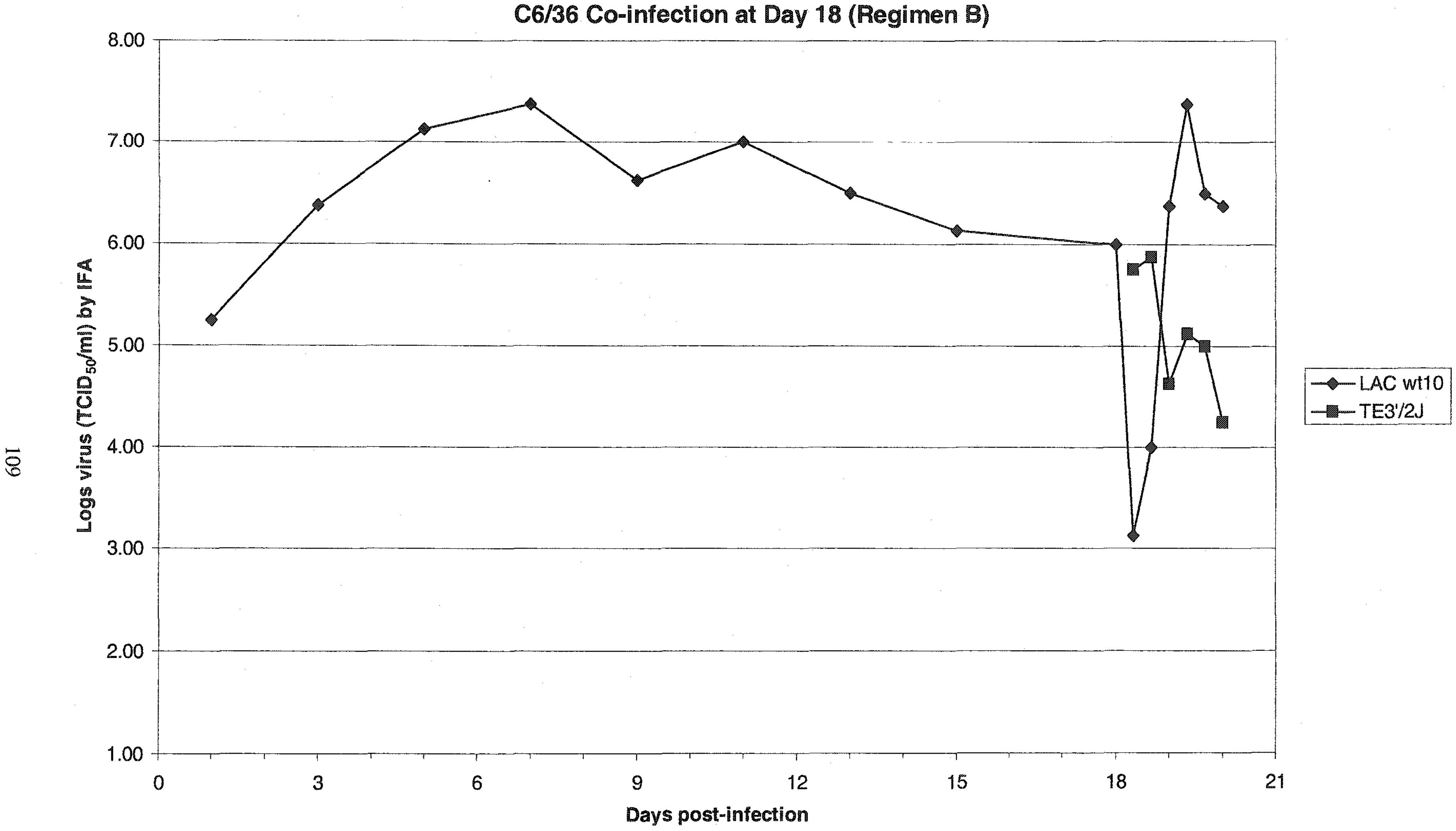

Figure 2.6 Titers of LAC and SIN viruses in C6/36 cell cultures after establishment of LAC virus persistence at 18 days (Regimen B). Each data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

Table 2.16 5′ nonviral sequences scavenged for LAC virus transcription in MAT cell cultures co-infected with SIN virus. Sequences observed more than once designated in parentheses.

Time point	Cap sequence	Viral sequence
Regimen A		
Published LAC viral sequence		agtagtgtactccacttgaatactttg
SIN genomic 5′ sequence		attgacggcgtagtacacactattgaa
SIN subgenomic 5′ sequence		atagtcagcatagtacatttcatctga
Day 3	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
Day 3	ATTTTAAGACTTGTA	agtagtgtactccacttgaatactttg
Day 9	CAGTTACGTT(x6)	agtagtgtaccccacttgaatactttg
Day 9	CGGATTAGTACTCCT	gtagtgtaccccacttgaatactttg
Day 18	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg
Day 18	TCAGTTACGTT	agtagtgtaccccacttgaatactttg
Day 24	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
Day 24	ATTTTCAGAC	tagtgtactccacttgaatactttg
Day 33	AG	agtaatgtaccccacttgaatactttg
Day 33	CAGTTACGTT(x3)	agtagtgtaccccacttgaatactttg
Day 33	AAAAATCAAATTC(x2)	agtagtgtaccccacttgaatactttg
Day 42	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg
Regimen B		
Day 18+8h	TG	agtagtgtaccccacttgaatactttg
Day 18+8h	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
Day 18+16h	CGG	agtagtgtaccccacttgaatactttg
Day 18+16h	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg
Day 18+24h	TG	agtagtgtaccccacttgaatactttg
Day 18+24h	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg
Day 18+24h	CAGTTACGTT	agcagtgtaccccacttgaatactttg
Day 18+32h	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg
Day 18+40h	CGG	agtagtgtaccccacttgaatactttg
Day 18+40h	CAGTTACGTT(x4)	agtagtgtaccccacttgaatactttg
Day 18+48h	CGGG	gtagtgtaccccacttgaatactttg
Day 18+48h	CAGTTACGTT(x5)	agtagtgtaccccacttgaatactttg

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nucleotides in length. The GC% was 39.5% and the average length of the 5' sequences was 9.6 nucleotides. Of the 5' nonviral sequences, 5 (7.4%) were only observed a single time and designated as unique (Table 2.16). Cap A predominated throughout infection, and was observed 56 times (82.4%). Cap B and cap C were not observed in the co-infected MAT cell cultures. A large proportion (58/68, 85.3%) of 5' nonviral sequences contained a T residue at the 3'-terminus and associated viral sequences (66/68, 97.1%) contained a T to C transition at position 11 of the viral mRNA (Table 2.16). Similar results were obtained after separation of the 5' nonviral sequences in MAT cell cultures into regimen A and B.

Following dual infection of MAT cells using regimen A, LAC virus titer was low at day 3 post-infection (below $10^{2.00}$ $TCID_{50}$/ml) and steadily increased to a plateau of approximately $10^{7.00}$ $TCID_{50}$/ml by day 33 post-infection. The SIN virus titer was approximately $10^{7.00}$ $TCID_{50}$/ml within 3 days post-infection and decreased to approximately $10^{4.00}$ $TCID_{50}$/ml by day 18 post-infection. Late in the infection, SIN virus titers began to rise and reached approximately $10^{6.00}$ $TCID_{50}$/ml by day 24 and remained stable through day 42 post-infection (Figure 2.7).

Following dual infection of MAT cells using regimen B, LAC virus titer reached $10^{8.00}$ $TCID_{50}$/ml by day 7 post-infection. As LAC virus established a persistent infection in the cell culture, the titer decreased and stabilized between $10^{6.00}$ $TCID_{50}$/ml and $10^{7.00}$ $TCID_{50}$/ml by day 18. Following infection with SIN virus at day 18, the titer of LAC virus decreased to $10^{3.00}$ $TCID_{50}$/ml within 8 hours of co-infection. By 16 hours after co-infection, the titer of LAC virus began to increase and reached approximately $10^{8.50}$ $TCID_{50}$/ml by 32 hours after co-infection. The titers of LAC virus began to decrease by 40 hours after co-infection and reached approximately $10^{5.50}$ $TCID_{50}$/ml. SIN virus titers reached

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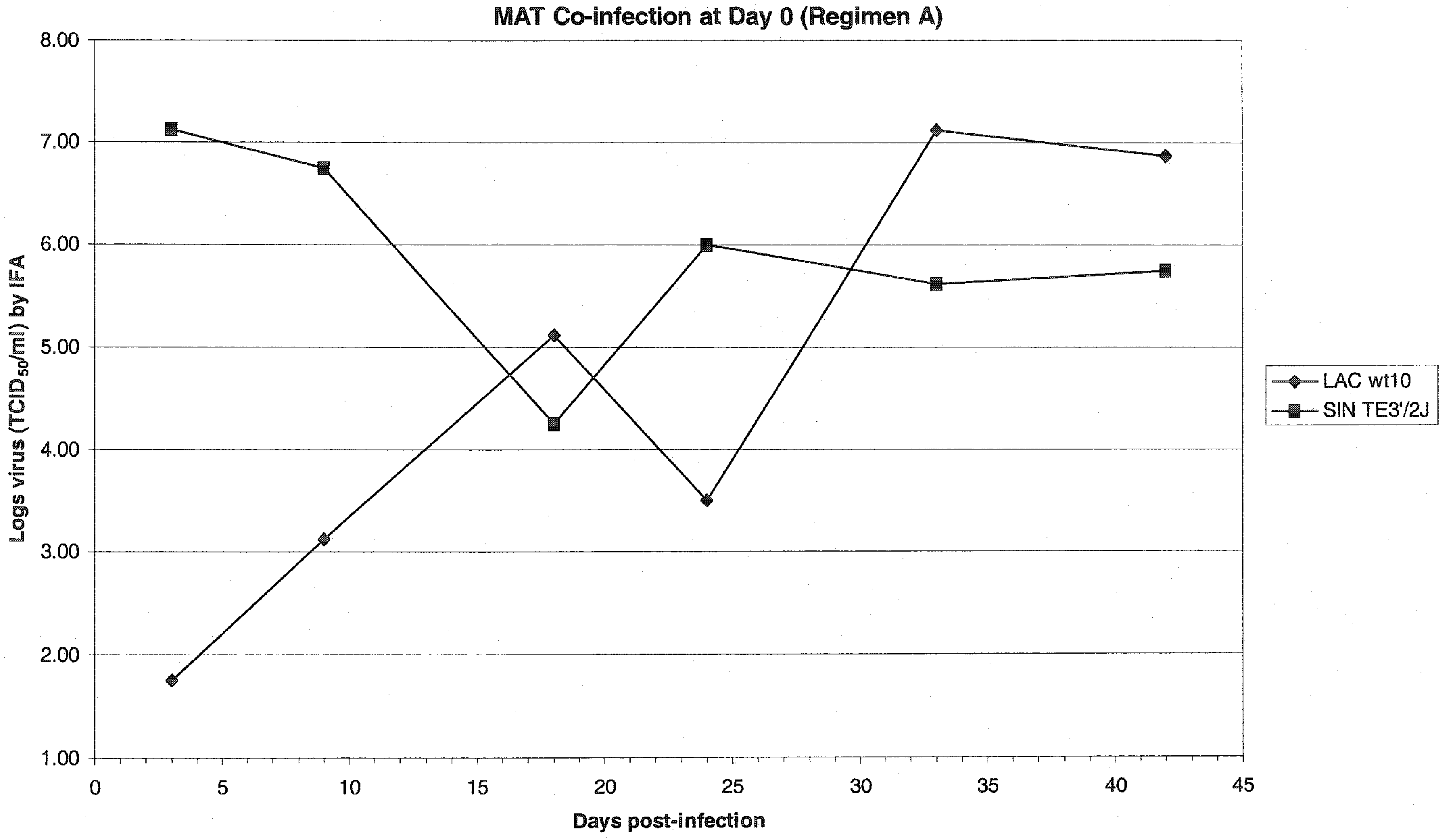

Figure 2.7 Titers of LAC and SIN viruses in MAT cell cultures co-infected at day 0 (Regimen A). Each data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

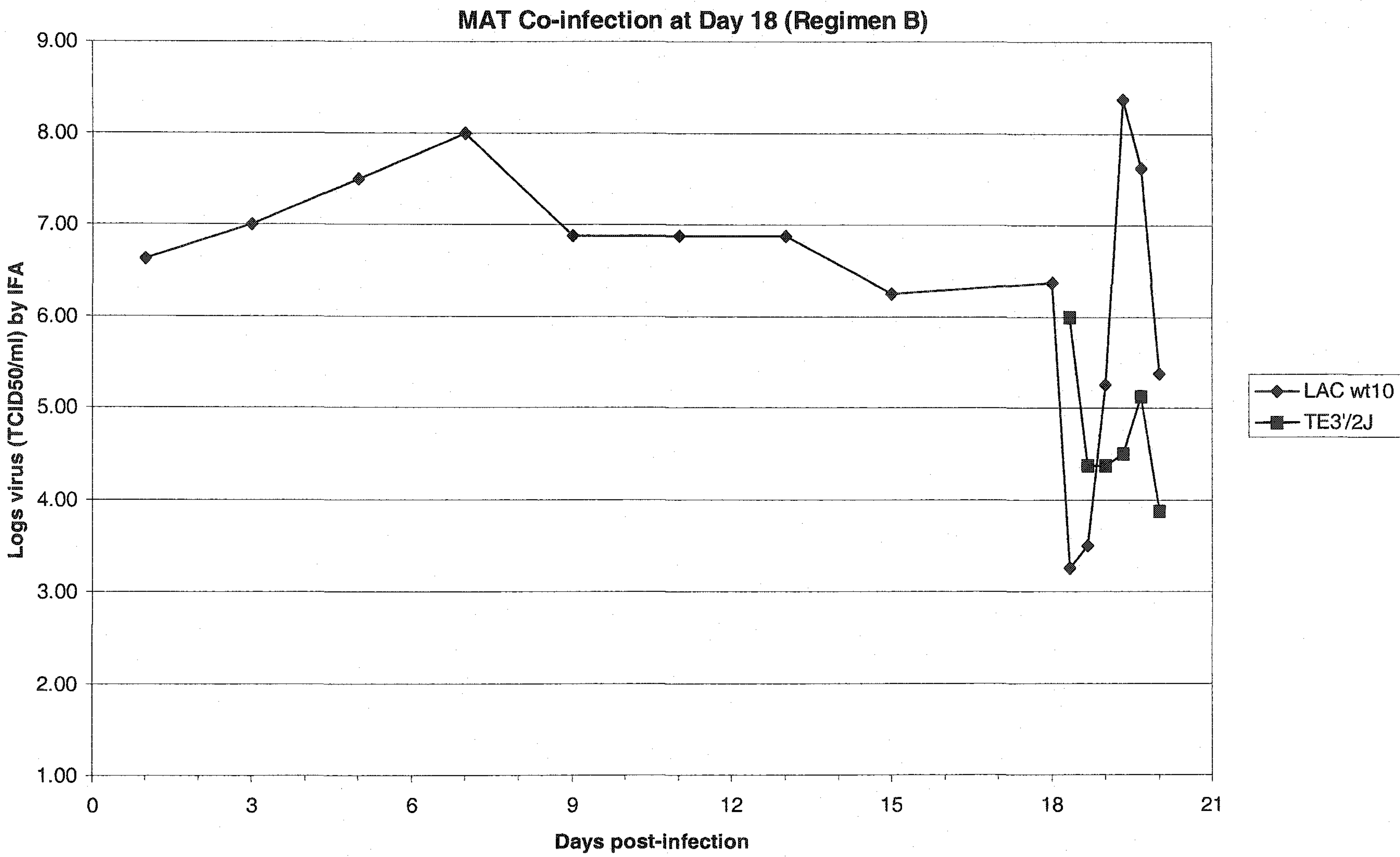

Figure 2.8 Titers of LAC and SIN viruses in MAT cell cultures after establishment of LAC virus persistence for 18 days (Regimen B). Each data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

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approximately $10^{6.00}$ $TCID_{50}$/ml by 8 hours after co-infection and then gradually decreased to approximately $10^{4.00}$ $TCID_{50}$/ml by 48 hours after co-infection (Figure 2.8).

Sixteen (22.5%) of the associated viral mRNA sequences from dually infected C6/36 cells contained single base truncations at the 5' terminus (8 each from regimen A and B), while only 2 (2.9%) from MAT cells contained single base truncations (1 each from regimen A and B).

9. Scavenged SIN caps used to prime transcription of LAC virus mRNA. Since only one of 139 cloned LAC mRNAs from mosquito cell cultures co-infected with SIN virus had a SIN-derived 5' sequence, a different approach was used to determine the potential scavenging of SIN mRNA 5' sequences for LAC transcription. RT was performed on total RNA isolated from the dually infected cells using LNR primer specific for the 3' terminus of the LAC virus S segment mRNA (Table 2.1) and PCR amplification was performed with the LNFc3 reverse primer and one of two different forward primers that combined the 5' sequences of either the SIN genomic RNA or the first and second subgenomic mRNAs with the 5' distal 7 nucleotides from the LAC virus S segment mRNA. Products of the expected size (256 bp) were obtained from each primer set (Table 2.4). For the SIN TE3'/2J genomic RNA primer, a PCR product was observed on all but day 33 and day 42 of infection regimen A, while for the SIN TE3'/2J subgenomic RNA primer, products were observed at day 33 and day 42 of infection regimen A and were less intensely-stained (Figure 2.9). PCR products were obtained for each time point for each SIN TE3'/2J capped primer of infection regimen B. Small amounts of PCR products were observed in MAT cell cultures for SIN TE3'/2J genomic RNA primers at day 18 of infection regimen A, and for SIN TE3'/2J subgenomic mRNA primers at days 18, 33, and 42 of infection regimen A. PCR products

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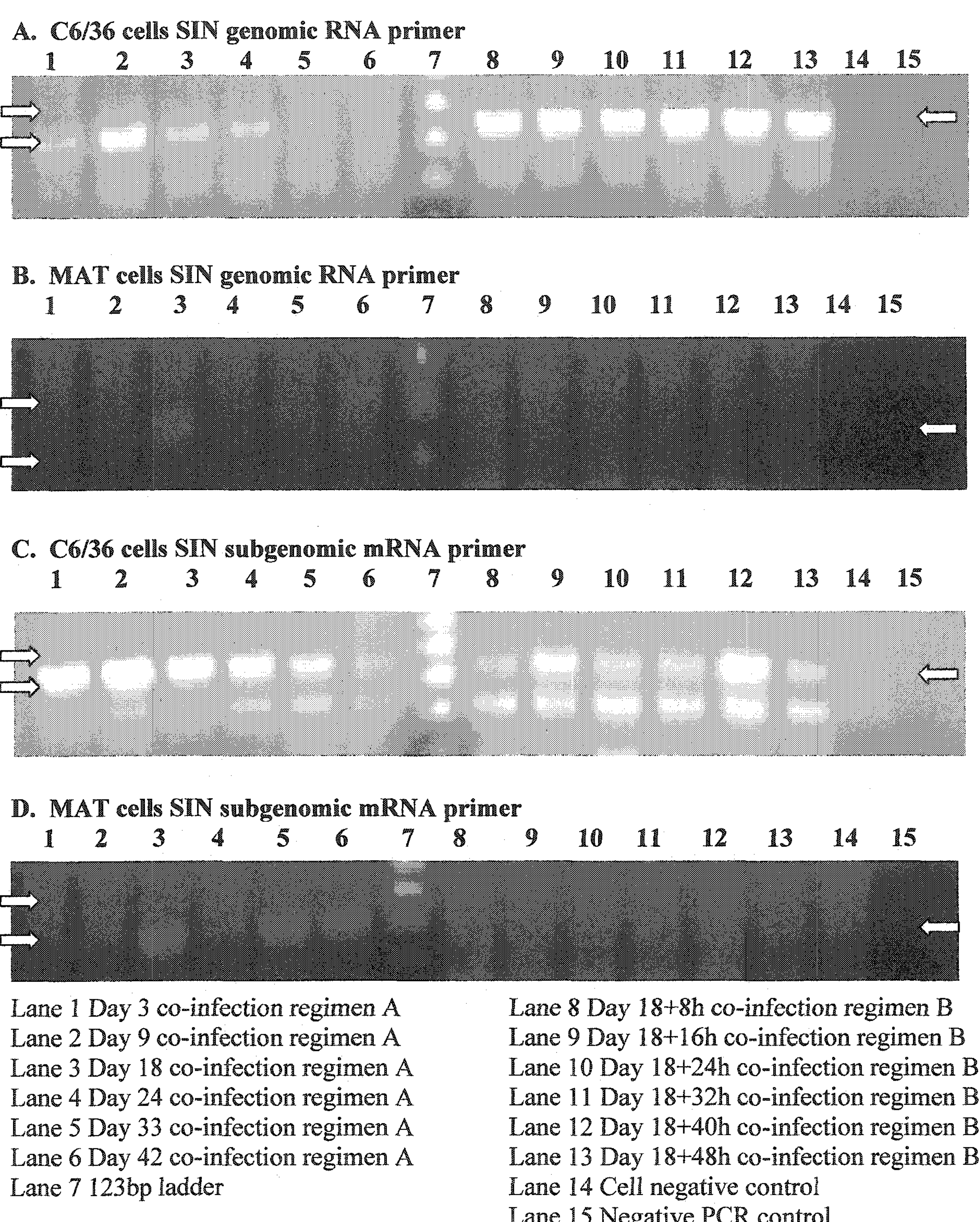

Lane 1 Day 3 co-infection regimen A
Lane 2 Day 9 co-infection regimen A
Lane 3 Day 18 co-infection regimen A
Lane 4 Day 24 co-infection regimen A
Lane 5 Day 33 co-infection regimen A
Lane 6 Day 42 co-infection regimen A
Lane 7 123bp ladder
Lane 8 Day 18+8h co-infection regimen B
Lane 9 Day 18+16h co-infection regimen B
Lane 10 Day 18+24h co-infection regimen B
Lane 11 Day 18+32h co-infection regimen B
Lane 12 Day 18+40h co-infection regimen B
Lane 13 Day 18+48h co-infection regimen B
Lane 14 Cell negative control
Lane 15 Negative PCR control

Figure 2.9 RT-PCR amplification of LAC virus S segment mRNA using forward primers derived from SIN TE3'/2J primers and LNR reverse primer. RT-PCR amplification products were electrophoresed on 1.0% agarose 1X TAE gels and contents of each lane are given above. Right arrows designate the 256bp expected product, left arrows designate the 246 and 369 bp bands of the 123 bp ladder. The additional band is a shadow.

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were not observed in MAT cell cultures in any of the samples from infection regimen B for either genomic or subgenomic SIN TE3'/2J primers (Figure 2.9).

10. Analysis of host messages scavenged for 5' nonviral sequences. Northern blot analysis was performed on total RNA and mRNA from uninfected C6/36, MAT, and BHK-21 cell cultures to identify potential host genes targeted by cap scavenging. Hybridization probes were designed as exact complements to caps A, B, and C and radiolabeled with [γ-^{32}P] ATP. The radiolabeled probes were hybridized to the RNAs cross-linked to nylon membranes. Northern blot analysis revealed a detectable band in BHK-21 cell culture total RNA, but not in mRNA, when probed for message containing cap B. A band was detectable after 24 and 96 hour exposure to phosphoimager screens and had a size of approximately 1.0 Kb (data not shown). No bands were detected in either C6/36 or MAT total RNA or mRNA with cap B probe, or in any RNA from any cell type with either cap A or cap C probes (data not shown).

Sixty different clones containing inserts of varying sizes were isolated following RT-PCR and cloning of RNA from C6/36, MAT, and BHK-21 cell cultures. RT-PCR was performed with primers specific for caps A (5'-CAGTTACGTT-3'), B (5'-TTGGTCGTCATCG-3'), and C (5'- TCACTCCC-3'). The inserts ranged in size from approximately 0.35 Kb to 2.00 Kb. Approximately 30 clones with inserts of approximately 0.80 Kb or larger were sequenced.

11. Database search for host messages targeted for 5' nonviral sequences. Nucleotide sequence identity to data entered in GenBank was determined for all sequenced inserts by conducting BLAST searches (Altschul et al., 1997) at the National Center for Biotechnology Information website through the National Library of Medicine and National

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Institutes of Health (http://www.ncbi.nlm.nih.gov). Between 600 and 800 nucleotides of sequence from each insert was used for each nucleotide sequence identity search. Following nucleotide sequence identity searches, each insert sequence was searched, using the same software, for protein domain homology to known protein sequences.

Targeted host genes for cap A and B sequences from C6/36 and MAT cell cultures demonstrated high nucleotide sequence identity with either *Aedes albopictus, Aedes aegypti*, and *Anopheles gambiae* cytochrome oxidase mitochondrial genes (E scores ranging from perfect match to $1e^{-100}$ and $1e^{-190}$) or had no significant homology or identity to any known sequence. Protein domain searches revealed that the sequences with nucleotide sequence identity to cytochrome oxidase mitochondrial genes had protein domain homology to *Aedes aegypti* cytochrome c oxidase subunit I (E scores of e^{-111} and e^{-120}) or to *Anopheles quadrimaculatis* ATP synthase subunit 6 (E scores of $6e^{-84}$ and $9e^{-82}$). Cap C sequences from C6/36 and MAT cell cultures demonstrated high nucleotide sequence identity with *Aedes albopictus* 16S ribosomal RNA gene (E scores of $4e^{-10}$, $4e^{-19}$, and $2e^{-20}$) or had nucleotide sequence identity to a tyrosine phosphatase (E score of $3e^{-12}$). Protein domain searches revealed that the sequences with identity to *Aedes albopictus* 16S ribosomal RNA gene had homology to an *Anopheles gambiae* PEST strain 16S ribosomal RNA gene (E scores of $5e^{-8}$ and $9e^{-12}$). An E score is the number of matches one would "expect" to see when searching within a database of a given size. An E score of 1 or approaching 1 assigned to a match is interpreted as meaning that in a database of the current size one might expect to observe 1 match with a similar score simply by chance alone. An E score that is much less than 1 can be interpreted as a more "significant" match or an exact match (Appendix).

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Targeted host genes from cap A and C sequences from BHK-21 cell culture demonstrated high sequence identity to *Mus musculus* ubiquitin-conjugating enzyme (E score of $3e^{-90}$), *Bos taurus* LN1 mRNA (E score of $5e^{-6}$), or 16s ribosomal RNA gene (E scores of $8e^{-94}$, $4e^{-22}$, $1e^{-18}$, and $5e^{-6}$). The sequence with identity to the *Bos taurus* LN1 mRNA had protein domain homology to the *Mus musculus* hypothetical protein XP_164548, however, the remaining sequences did not demonstrate protein domain homologies to any known protein sequences. Cap B sequences from BHK-21 cell culture had no sequence identity to any known sequence, and when analyzed for protein domain homology none was detected.

D. DISCUSSION

The remarkable ability of LAC virus to infect and persist in mosquitoes and mosquito cell cultures, but to cause CPE and cell death in mammalian cell cultures, suggests co-regulation of viral transcription and replication with host transcription in mosquito cells. The studies described in this research were conducted to investigate the possible co-regulation of viral activity with host transcription. A persistent infection is noncytopathic for the life of the mosquito or mosquito cells and can occur immediately following infection. It has been demonstrated that within 24 hours post-infection of mosquito cells, the rate of genome and N protein synthesis were severely reduced while mRNA levels remained high (Rossier et al., 1988).

Specific attention was given to four main questions: what are the determinants and molecular basis of cap scavenging *in vitro* and *in vivo*; does cap scavenging in mammalian cell culture condition cell death; what are the host messages scavenged for 5' nonviral sequences; will LAC virus preferentially scavenge 5' nonviral sequences from mRNA of another, co-infecting, unrelated virus? To elucidate the answers to these questions, cap

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scavenging activity was characterized for several LAC virus isolates and other California serogroup viruses in cytopathic infection of mammalian cell cultures and persistent infection of mosquitoes and mosquito cell cultures. Attempts were also made to identify host mRNAs targeted for removal of 5' nonviral sequences. The hypothesis of abundant messages versus specific messages being targeted by cap scavenging was also explored by northern blot analysis and co-infection experiments.

Several conclusions can be drawn from this study. Cap scavenging is not the major determinant between the differing outcomes observed in persistent infection of mosquitoes and mosquito cells and in cytolytic infection of mammalian cells, although it may contribute to the differences observed. Cap scavenging was examined in mosquito and mammalian cell cultures and in mosquito tissues. Similar cap scavenging preferences were observed in mosquito and mammalian cells, although it appeared to be more restricted in cells from *Ae. triseriatus* mosquitoes. Similar cap scavenging preferences were observed in *Ae. triseriatus* midgut and ovary tissues. A preference for a specific 5' nonviral sequence was observed, and predominant 5' nonviral sequences were demonstrated to be present at the 5' terminus of LAC mRNAs. Specific 3' nucleotide(s) were observed to be preferred in the scavenged 5' nonviral sequence.

In this study, a large number of LAC virus S segment mRNA 5' terminal sequences were determined in mosquito and mammalian cell cultures. Of the 420 5' nonviral sequences from LAC virus wt10 infected C6/36, MAT, and BHK-21 cell cultures, 19% were unique sequences. The major 5' nonviral sequence, cap A (5'-CAGTTACGTT-3'), was observed in 66% of the S segment mRNA from the three cell types. A second 5' nonviral sequence, cap B (5'-TTGGTCGTCATCG-3'), was observed in 5.5% of the mRNA from the three cell

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types (Section C.1-3). Similar to the previous study, a predominant 5' nonviral sequence was observed and specificity of cap scavenging was observed throughout infection. The majority (85.7%) of 5' nonviral sequences had a 3' terminal T residue, and the associated viral mRNA sequence contained a T to C transition at position 11 (86.7%). The GC content of scavenged 5' nonviral sequences in this study (42.5%) was considerably lower than in the previous study (Dobie et al., 1997) (64%) (Section C.1-3). However, the GC content of several animal genomes has been determined, including 35% in *Anopheles gambiae*, 41% in *Drosophila melanogaster*, 38% in *Homo sapiens*, and 42% in *Mus musculus* (Holt et al., 2002). The GC content of the 5' nonviral sequences in this study does coincide, although GC content to mRNA would be more relevant. Slight differences in RNA extraction (RNAMATRIX versus isopropanol and ethanol precipitations) or cDNA purification (GlassMax versus QIAquick) protocols may have contributed to the differences observed, although this seems unlikely. Differences in C6/36 cell cultures (passage history, long-term storage, and overall health) may also have contributed to the observed differences. The observation of a T to C transition at position 11of viral mRNA may not be of significance. It may be the result of a stable mutation within the genome of the LAC virus isolate used compared to a published sequence or the mutation may be in the sequence of one of the published LAC isolates. Recent sequence analysis of two LAC isolates used in this study (GenBank accession numbers NC 004110 and AF528167) revealed a C residue at position 11 of the viral mRNA.

The 5' nonviral sequence heterogeneity was observed more frequently in C6/36 *Ae. albopictus* cell culture (71 different 5' nonviral sequences out of 176, 40.3%) and BHK-21 cell culture (41 different 5' nonviral sequences out of 155, 26.5%) than in MAT *Ae. triseriatus* cell culture (17 different 5' nonviral sequences out of 89, 19.1%) (Section C.1-3).

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This result suggests that in cells from the natural vector, cap scavenging is more restrictive in host messages targeted for 5' nonviral sequences, while in cells from non-vector mosquitoes and mammals, cap scavenging is more broadly active. Additionally, more single base truncations were seen in C6/36 and BHK-21 cells compared to MAT cells. LAC viral mRNA, and potentially genomic template, could be more stable in cells from the natural vector.

The specificity for similar 5' nonviral sequences in mammalian and mosquito cell cultures (Table 2.9) suggests that differences in cap scavenging specificity might not be responsible for differences in infection outcomes between mosquitoes and mosquito cell cultures and mammalian hosts and cell cultures. Because similar 5' nonviral sequences were observed in two divergent cell types, mosquito and mammal, a message or group of messages conserved within the animal kingdom could be targeted by the virus for 5' nonviral sequences. Portions of the 5' end of eucaryotic mRNAs are conserved for binding to the 18S rRNA ribosomal binding site (Lewin, 1980). The relative abundance and functions of the targeted host mRNAs were not determined in this study. Additionally, AGT, GT, T, and CCC residue motifs were observed in many of the scavenged sequences. These residues match residues and motifs within the first six to eleven bases of the LAC viral mRNA sequence, or complement the first six to eleven bases of the 3' end of LAC viral genome sequence. The complementary nucleotide motifs could be utilized in a prime and realign mechanism for the initiation of transcription as described previously. The LAC virus L protein (RNA-dependent RNA polymerase) may specifically target host messages containing these motifs within the immediate sequence of the 5' end of mRNA. In vitro analysis of cap scavenging with purified L protein and synthetic mRNAs with or without these motifs would

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allow for examination of specific motifs and location within the 5' termini of mRNA and specificity of cap scavenging.

Similar results were obtained from SSH and TAH virus infected C6/36, MAT, and BHK-21 cells. It appears that within the CAL serogroup, more closely related (LAC and SSH) and more distantly related (LAC and TAH) viruses have similar cap scavenging specificity. Additionally, a new 5' nonviral sequence, cap C (5'-TCACTCCC-3'), was observed in all cell types infected with either SSH or TAH virus (Table 2.10). This new motif was observed in 21.5% of all 5' nonviral sequences in SSH and TAH infected cell cultures (Section C.5). There does not appear to be a difference in 5' nonviral sequence specificity between LAC virus and other viruses of the CAL serogroup for cap A. Differences were observed for the specificity of caps B and C. The presence of cap C (5'-TCACTCCC-3') at the 5' terminus of viral mRNA correlated with single base truncations in both SSH and TAH infected mosquito and mammalian cells (Table 2.10). These truncations may be the result of altered prime and realign transcription initiation due to the string of C residues at the 3' terminus of the 5' nonviral sequence. The first nucleotides of the genomic template able to be used in this hypothesized mechanism are bases 10 to 13 from the 3' terminus and could lead to the observed mutation in viral mRNA. Interestingly, viral mRNA sequence primed with either cap A and B was never associated with nucleotide truncations at the 5' terminus.

A large number of capped primer sequences were obtained from *Ae. triseriatus* ovaries and midguts infected with one of six different LAC virus isolates. Similar to cap scavenging studies in cell culture, cap A was the predominant 5' nonviral sequence observed in ovaries (91.4%) and midguts (87.2%) (Section C.6). Cap B was observed only in midgut

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tissue (5.1%) and cap C was observed in only ovaries (5.7%). This may be the result of differential gene expression in the different tissues. A high degree of homogeneity of 5' nonviral sequences was observed; only 2.9% of ovary and 5.1% of midgut 5' nonviral sequences were unique and different from caps A, B, or C (Table 2.11). Similar to the results from cap scavenging in cell culture, GC content of 5' nonviral sequences was low (41.6% ovaries, 41.7% midguts). The majority of 5' nonviral sequences ended in a 3' terminal T residue (100% ovaries, 94.9% midguts) and all of the associated viral mRNA sequences contained a T to C transition at position 11 (Section C.6). These results demonstrated that cap scavenging in mosquito tissues has the same specificity as in cell culture. Also, virus passage, either through transovarial transmission in mosquitoes (TOT3 and TOT9), or through cell culture or mouse brain (TCF6, H78mp1, and H78mp2) does not affect cap scavenging specificity. Potential mutations in the L gene acquired over time with passage may affect cap scavenging specificity. The sequences of the L genes of the different LAC isolates used in this study were not determined. Additionally, few viral mRNA mutations were observed in mosquito tissues compared to cell culture. Single base truncations were only observed in LAC wt10 infected ovaries (Table 2.11). These results support the hypothesis that LAC viral mRNA, and potentially its genome, is more stable in tissues and cells from the natural vector.

RT-PCR analysis of LAC S mRNA revealed that 5' nonviral sequences observed in viral mRNA from cell cultures and mosquito tissues were present at the 5' ends of LAC mRNA. The design of the primers included individual 5' nonviral sequences along with minimal LAC mRNA 5' sequence. Interestingly, amplification products of the three predominant 5' nonviral sequences from this study, caps A, B, and C, were of much brighter

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intensity (Figure 2.4) compared to the two predominant 5' nonviral sequences from the previous study, caps 1 and 2 observed during embryogenesis and deep diapause (Dobie et al., 1997). Of the three 5' nonviral sequences from this study used in RT-PCR analysis, cap C had the most intensely staining amplification product followed by cap A. This may not be significant because of the different GC content and length of the primers upstream of the LAC sequence and this assay was not designed to be quantitative. All of the predominant 5' nonviral sequences from this and the previous study were demonstrated to be present at the 5' ends of LAC mRNA.

Attempts to identify host mRNAs targeted by the LAC virus RNA-dependent RNA polymerase were problematic due to the low GC% in the 5' nonviral sequences. Northern blot analysis using radiolabeled hybridization probes specific for caps A, B, and C, and *Ae. triseriatus* actin mRNA revealed only weak signals at 24 and 96 hour exposure to phosphorimager screens. Cap B probe identified a potential message of approximately 1.0 kb in length from BHK-21 total RNA but not mRNA. Caps A and C, as well as *Ae. triseriatus* actin mRNA probes did not identify any specific targets in either BHK-21 total RNA or mRNA. Cap A, B, and C probes and *Ae. triseriatus* actin mRNA probe did not identify any specific targets in total RNA or mRNA from C6/36 or MAT cells.

Attempts at RT-PCR amplification of host mRNAs targeted for cap scavenging revealed several potential host messages. Potential messages amplified from uninfected C6/36 and MAT cells with caps A and B as primers had sequence identity to mitochondrial genes, specifically, cytochrome c oxidase (Section C.11). Previous attempts to identify host messages targeted by cap scavenging also revealed mitochondrial cytochrome c oxidase genes (Blitvich et al., 2001). Amplification of mitochondrial gene transcripts using

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scavenged sequences as primers may not be significant. The mitochondrial genes observed are normally transcribed and translated completely within the mitochondria. It is important to note that release of cytochrome c from the mitochondria is involved in triggering apoptosis (Saikumar et al., 1999; Reed, 2000). The cellular location and abundance of mitochondrial transcripts could change during infection. If during infection mitochondria breakdown, mitochondrial mRNAs may be present in the cytoplasm for scavenging. Additionally, mitochondrial mRNAs are uncapped. Products amplified from C6/36 and MAT cells with cap C primers were identified as transcripts of 16S ribosomal RNA genes. Additionally, a tyrosine phosphatase mRNA was identified with cap C primer in MAT cells. Products from BHK-21 cells with cap A primers were also identified as transcripts from a 16S ribosomal RNA gene, and as a ubiquitin-conjugating enzyme gene from *Mus musculus*; no potential messages were identified with cap B primers; and products with cap C primers were identified as 16S ribosomal RNA gene transcripts and LN1 mRNA from *Bos taurus* (Section C.11). The identification of cytochrome c oxidase transcripts was most likely the result of low stringency annealing temperatures for the initial 5 cycles during amplification. Those products identified as 16S ribosomal RNA are most likely not of significance, first because they would not provide caps and are of mitochondrial origin and second because only 40 to 50 bases of the sequence aligned to those of the ribosomal genes. The remaining 600 to 800 bases of sequence from each of those inserts had no significant identity or homology to any known sequence. They may contain the messages of interest, but due to limited available information, they could not be identified. Different protocols and reaction conditions may aid in the identification of host messages targeted by cap scavenging.

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Studies of co-infection by LAC virus wt10 and SIN TE3'/2J of cultured mosquito cells demonstrated that LAC virus rarely scavenged abundant SIN virus 5' mRNA sequences to prime transcription of LAC viral mRNAs, and revealed an interesting dynamic between the two distinct viruses. In C6/36 cell cultures, both during co-infection at day 0 and SIN superinfection at day 18 after LAC infection, heterogeneity of LAC 5' nonviral sequences was more pronounced (59.2%) (Table 2.12) than in MAT cells (7.4%) (Table 2.13). Cap A was the predominant 5' nonviral sequence in both cell types (38% in C6/36 and 82.4% in MAT cell culture). The majority of 5' nonviral sequences possessed 3' terminal T residues (67.6% in C6/36 and 70.6% in MAT cell culture), and the associated viral mRNAs contained a T to C transition at position 11 (77.5% in C6/36 and 97.1% in MAT cell culture) (Section C.8). A SIN TE3'/2J 5' nonviral sequence was observed on a single LAC mRNA. This 5' nonviral sequence was scavenged in C6/36 cell culture at day 3 post-infection and was scavenged from either the first or second subgenomic SIN mRNA (Table 2.12). The lack of heterogeneity in MAT 5' nonviral sequences and continued targeting of specific host mRNAs also suggests that in cells from the natural vector, cap scavenging is more restrictive to certain host messages, while in cells from non-vector mosquitoes, SIN co-infection only made cap scavenging differences more pronounced. These results were unexpected. A preference for SIN 5' mRNA sequences priming transcription of LAC mRNAs was expected due to the large amounts of SIN mRNA present within 48 hours after infection (Adelman, 2000).

The identification of a single SIN 5' mRNA sequence used for priming of transcription of LAC mRNA prompted determination whether LAC mRNA transcription utilized SIN 5' sequences. RT-PCR analysis of LAC S mRNA revealed that both SIN virus

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genomic and subgenomic mRNA 5' sequences were present at the 5' ends of LAC mRNA in C6/36 cells at nearly every time point post-infection following both co-infection procedures (Figure 2.9). Genomic 5' sequence was not utilized late in co-infection (days 33 and 42), and subgenomic 5' sequences were only weakly utilized at day 42 after co-infection. In MAT cell cultures, a PCR product was observed for SIN genomic 5' sequence at day 18 after co-infection, and subgenomic 5' sequence at days 18, 33, and 42 after co-infection (Figure 2.9). SIN genomic and subgenomic 5' sequences were not observed in any other samples. These results suggest that in MAT cell cultures, although SIN reached titers comparable to those in C6/36 cell cultures (maximum titers of $10^{8.63}$ $TCID_{50}$/ml and $10^{5.88}$ $TCID_{50}$/ml in C6/36 cells versus $10^{7.13}$ $TCID_{50}$/ml and $10^{6.00}$ $TCID_{50}$/ml in MAT cells in regimen A and B, respectively), priming of LAC virus mRNA transcription remained specific for the cellular 5' nonviral sequences used during single infection (Figures 2.5-2.8). As observed in LAC virus wt10 infected C6/36 and MAT cells, a higher proportion of viral mRNA sequences in dually infected C6/36 cells contained single base truncations at the 5' terminus of the LAC-encoded sequence than in dually infected MAT cells. These results also suggest that LAC virus cap scavenging for transcription in cells and tissues from the natural vector has a higher fidelity with respect to the viral RNA than in other cell lines or mosquitoes.

The titration of both LAC and SIN viruses in the cell culture medium revealed the interaction of the two viruses within infected cells. LAC virus replication was transiently inhibited when dually infected with SIN virus. After titers of SIN virus decreased, LAC replication returned to previous levels (Figure 2.5 and 2.7). If LAC virus had established a persistent infection prior to superinfection of SIN virus, LAC virus titers rapidly (within 8 hours) decreased after infection by SIN virus. LAC virus titers rapidly recovered following

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superinfection (Figure 2.6 and 2.8). The most likely explanation for SIN virus inhibiting the initial replication or rapidly depressing established replication of LAC virus is that SIN virus transcription and replication occur at a much faster rate than LAC virus. This high level of SIN transcription and replication would reduce substrates for macromolecular synthesis (amino acids and ribonucleotides) for LAC virus biosynthesis. Additionally, SIN virus titers in C6/36 and MAT cell culture media appeared to have a biphasic or possibly cyclic pattern following co-infection with regimen A. If SIN infection were allowed to proceed longer, a cyclical mode of viral replication may have been observed.

Northern blot analysis of potential host messages targeted by cap scavenging and the co-infection of LAC and SIN in mosquito cells also examined the abundance versus specificity of cap scavenging. The inability to detect or only weakly detect host messages by northern blot analysis of total RNA and mRNA from host cells suggests that the host messages are not targeted by cap scavenging due to abundance; rather, cap scavenging is very specific. Additionally, infection with SIN virus results in large quantities of SIN virus mRNAs. A single SIN 5' sequence was observed. This supports the hypothesis that cap scavenging targets specific capped host mRNAs rather than abundant mRNAs.

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CHAPTER 3: QUANTITATIVE ANALYSIS OF LAC VIRUS TRANSCRIPTION AND REPLICATION *IN VITRO* AND *IN VIVO*

A. INTRODUCTION

Reverse transcription-polymerase chain reaction (RT-PCR) was previously used in our laboratory to investigate the potential for co-regulation of LAC virus transcription and replication in tissues of infected *Aedes triseriatus* female mosquitoes (Chandler et al., 1996). In infected mosquitoes, when the ovaries became quiescent after a blood meal and oviposition, LAC mRNA and vcRNA declined to undetectable amounts, but vRNA remained detectable, by RT-PCR. Following a second blood meal and activation of another gonadotrophic cycle, synthesis of LAC mRNA and vcRNA was induced and RNA achieved amounts similar to those observed prior to quiescence. These results suggested that the synthesis of the three LAC virus RNA species was co-regulated with the metabolic activity of the mosquito ovary. However, quantitative analysis would have been more revealing of the co-regulation of virus transcription and replication with host metabolic activity.

Real-time quantitative-polymerase chain reaction (Q-PCR) has allowed researchers to directly calculate the quantity of a specific nucleic acid by weight, concentration, and/or copy number in a sample. Fluorescent reporter-tagged probes allow for multiplex reactions and generation of standard curves for quantitating several targets in one reaction (Freeman et al, 1999). Real-time quantitative assay techniques have been used for diagnostics in field and laboratory settings, drug efficacy studies, and molecular analysis and pathogenesis of virus

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interactions with host cells. Q-PCR assays are now widely used for rapid diagnosis using West Nile virus-infected human clinical specimens, avian samples, and field-collected mosquitoes (Lanciotti et al., 2000). Real-time quantitative assays have been developed and used for *in vitro* and *in vivo* studies of several members of the Bunyaviridae including tomato spotted wilt virus (Roberts et al., 2000; Boonham et al., 2002), Black Creek Canal (Hutchinson et al., 1998), Puumala (Garin et al., 2001), and Sin Nombre (Hutchinson et al., 1996) hantaviruses, and Rift Valley fever virus (Garcia et al., 2001). The sensitivity of these assays is remarkable, detecting an equivalent of 10-100 $TCID_{50}$/ml of the respective virus genomes and as little as 1,000 copies of individual RNA species both in cell cultures, vertebrate tissues, and insect tissues. These studies have applied Q-PCR in diagnostic and pathogenesis examinations of bunyavirus-infected animal and plant samples and to determine effectiveness of antiviral treatment in bunyavirus infections. Q-PCR technology has yet to be used to study the molecular interactions between bunyaviruses and arthropod, mammalian, or plant host cells. Changes in host physiologic and metabolic activity may affect the co-regulation of bunyavirus replication and transcription in host cells.

In this dissertation research the potential co-regulation of LAC virus transcription and replication with metabolic activity of *Ae. triseriatus* ovaries and midguts was examined by Q-PCR. The hypothesis was that quantitative analysis of viral RNAs would reveal that co-regulation of viral transcription and replication and host transcription are determinants of efficient transovarial transmission. The titers of infectious virus in medium of infected mosquito and mammalian cell cultures were compared with intracellular amounts of all three LAC virus RNA species. Traditional RT-PCR sensitivity was compared to the sensitivity of Q-PCR. These studies were conducted to provide insight into the co-regulation of LAC virus

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transcription and replication with host cell activity in persistently infected *Ae. triseriatus* mosquitoes at different metabolic or physiologic states.

B. MATERIALS AND METHODS

Cells

C6/36 cells. *Aedes albopictus* cells were grown in Leibovitz L-15 medium including 10% fetal bovine serum (FBS) and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 28°C (Singh, 1967). The cells were diluted 1:10 and passed every 7 days until virus inoculation.

MAT cells. Mather *Aedes triseriatus* cells were grown in Leibovitz L-15 medium including 20% FBS and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 28°C (Rowley et al., 1984). The cells were diluted 1:6 and passed every 7 days until virus inoculation.

BHK-21 cells. Baby hamster kidney cells were grown in Leibovitz L-15 medium including 10% FBS and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 37°C. The cells were diluted 1:5 and passed every 7 days until virus inoculation.

Vero cells. African green monkey kidney cells were grown in Leibovitz L-15 medium including 10% FBS and 100 units penicillin per ml plus 100 μg of streptomycin per ml in 25-cm^2 flasks at 37°C. The cells were diluted 1:5 and passed every 7 days until virus inoculation.

Virus

LAC virus wt10. LAC virus wt10 (wild type virus, passage 10) was isolated in 1965 from the brain of a patient with a fatal case of LAC encephalitis (Thompson et al., 1965). The virus had been passed three times in suckling mouse brain and six times in BHK-21 cell

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culture. To prepare stock virus, BHK-21 cells, which had growth medium removed, and were rinsed twice with phosphate buffered saline (PBS), were inoculated with 1 ml of virus at a multiplicity of infection (MOI) of 0.01 for 1 hour at 37°C with gentle rocking. The inoculum was then removed and replaced with growth medium. When approximately 90% of the cells exhibited cytopathic effects (CPE) and death (36 to 48 hours post-infection at 37°C), the remaining cells were scraped into the medium, fetal bovine serum was added to 50%, and the virus was stored at -70°C in 500 μl aliquots. Virus was titrated by the Karber method (1931). The stock LAC virus had a titer of $10^{8.4}$ $TCID_{50}$/ml.

Virus titration protocol

Virus in infectious blood meals and infected cell culture medium was determined by serial 10-fold dilution $TCID_{50}$ as described in Chapter 2, Section B, pp. 62-63.

Mosquitoes

Aedes triseriatus mosquitoes (AIDL strain) originated from field material collected near La Crosse, Wisconsin, in 1981 and colonized continuously at the Arthropod-borne and Infectious Diseases Laboratory, Colorado State University, Fort Collins, CO, at 70°F, 70% relative humidity, 16-hour light: 8-hour dark cycles (Wasieloski, 1995).

Oral infection and preparation of tissues from mosquitoes

Approximately 500 adult female *Aedes triseriatus* mosquitoes were provided an infectious blood meal as previously described (Chapter 2, section B, pp. 64-65). An additional 200 female mosquitoes were provided a noninfectious blood meal simultaneously. At predetermined times post-infectious blood meal, 15 infected mosquitoes and 5 uninfected females were harvested. Twelve infected mosquitoes and 2 uninfected mosquitoes were processed. Ovary and midgut tissues were dissected for RNA extraction, and head tissues

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were processed for fluorescent antibody analysis for viral antigen (See below). Dissected ovaries and midguts from individual mosquitoes were rinsed twice in sterile PBS and stored individually in 100 μl RNA lysis buffer. At day 30 post-infection, the remaining infected and uninfected mosquitoes were divided into two groups each. One group of infected and uninfected mosquitoes was provided a second meal of noninfectious blood through a human-scented parafilm membrane. The other group of infected and uninfected mosquitoes was provided 10% sucrose (w/v) with 0.1% green food color (v/v) in sterile distilled water through a human-scented parafilm membrane.

At predetermined times following the second meal, 15 infected mosquitoes and 5 uninfected females were harvested. Twelve infected mosquitoes and 2 uninfected mosquitoes were processed. Ovary and midgut tissues were dissected for RNA extraction, and head tissues were processed for fluorescent antibody analysis for viral antigen (See below). Dissected ovaries and midguts from individual mosquitoes were rinsed twice in sterile PBS and stored individually in 100 μl RNA lysis buffer. RNA lysis buffer (4M GTC solution) was prepared by combining 100 g guanidinium thiocyanate, 117 ml DEPC treated water, 7 ml 0.75 M sodium citrate solution pH 7.0, 10.6 ml 10% sarcosyl, and 1.44 ml 2-mercaptoethanol.

Direct detection of viral antigen by immunofluorescent analysis

Mosquito heads were severed from the bodies and placed on acid washed slides. Each head was squashed on a slide, and fixed in cold acetone for 10 minutes. Approximately 100 μl of 1:200 dilution of fluorescein isothiocyanate (FITC) conjugated anti-LAC mouse polyclonal antibody F92 (Chandler, 1995) were placed on each tissue and incubated at 37°C for 40 minutes in a humidified chamber. Following antibody binding, the slides were rinsed

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twice with PBS for 10 minutes, then rinsed briefly in distilled water. Coverslips were mounted in PBS-glycerol (1:3 v/v) and samples were observed for fluorescence with an Olympus BH2 epifluorescence microscope using filters for FITC.

Experimental infection of the respective cell cultures

Approximately 5.0×10^5 cells in suspension were used to seed 25-cm^2 flasks. At 80-90% confluence, the growth medium was removed and cells were rinsed twice with PBS. Virus inoculum was diluted to the appropriate MOI in Leibovitz L-15 maintenance medium (1% FBS and 100 units penicillin plus 100 μg of streptomycin per ml) to a total volume of 1 ml. Vertebrate cells (BHK-21) were infected at an MOI of 0.01 and invertebrate cells (C6/36 and MAT) were infected at an MOI of 10. Virus inoculum was placed onto the cell monolayer and gently rocked for 1 hour at room temperature. Virus inoculum was removed, and cells were rinsed twice with PBS. Five ml of maintenance medium was then added to each flask. At predetermined time points following infection, the cells were harvested, scraped into the medium, aliquotted into 1.7-ml Eppendorf tubes, and centrifuged at 14,000 xG for 5 minutes. The supernatant was removed for use in end point virus titration, and the cell pellets were stored at –70°C until ready for use. The number of cells present at each time point was enumerated by hemacytometer counts. For infected invertebrate cells, the medium was replaced with 5 ml of fresh maintenance medium every seven days until completion of the time course.

RNA extraction from cell pellets and mosquito tissues

RNA extraction from mosquito tissue and cell pellets was by a modified version of the single-step method by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) previously described (Chapter 2, Section B, pp. 65-67).

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Reverse transcription

Approximately 3 μg of total cell RNA or one-fourth of the total mosquito tissue RNA were mixed with 15 pmol of two reverse transcription primers and diluted to 12 μl in DEPC-treated water. The RNA samples were heated to 70°C for 10 minutes and then allowed to cool to 20°C. To each sample, 4 μl of first strand buffer (Gibco BRL), 2 μl 0.1 M DTT, and 1 μl 10 mM dNTP mix were added. Three units of reverse transcriptase, Superscript II (Gibco BRL), in DEPC-treated water were added, and samples were incubated at 42°C for 60 minutes. Samples were then heated to 70°C for fifteen minutes to inactivate the reverse transcriptase. Samples were cooled to 4°C. To each sample, 1 μl (15 units) of RNase H was added, and samples were incubated at 37°C for 10 minutes. Three primers were used, each in combination with *Ae. triseriatus* actin reverse primer, for the initial reverse transcription. LAC cDNAs were made simultaneously with actin cDNA in sets of two to control for any error introduced in reverse transcriptase efficiency, pipetting error, or variation in reaction conditions. Each cDNA pair included a reverse transcription primer for *Ae. triseriatus* actin mRNA with one of the three primers for the LAC RNA species (LNR for mRNA, LNFLAC for vRNA, or LVCQ for vcRNA) (Table 3.1 and Figure 3.1).

Table 3.1 Primers for RT and RT-PCR of LAC S RNA

Primer name	**Sequence 5'-3'**	**Bases represented**	**Use**
LNFLAC	TCAAGAGTGTGATGTCGGATTTGG	71-95 of LAC S mRNA and vcRNA	RT of LAC vRNA and PCR
LNR	GGAAGCCTGATGCCAAATTTCTG	741-763 of LAC S vRNA	RT of LAC mRNA and vcRNA and PCR
LVCQ	GTGTGCTCCACTGAATACA	962-980 of LAC S vRNA	RT of LAC vcRNA
LVC	TTTTGCTGTCCCCTACCACC	892-911 of LAC S vRNA	PCR
Actin Reverse	TCAGGTAGTCGGTCAGAT	384-401 of *Ae. triseriatus* actin mRNA (B. Kempf, unpublished)	RT of *Ae. triseriatus* actin mRNA and PCR
Actin Forward	TCCAGAGCAAGAGAGGTA	1-19 of *Ae. triseriatus* actin mRNA (B. Kempf, unpublished)	PCR

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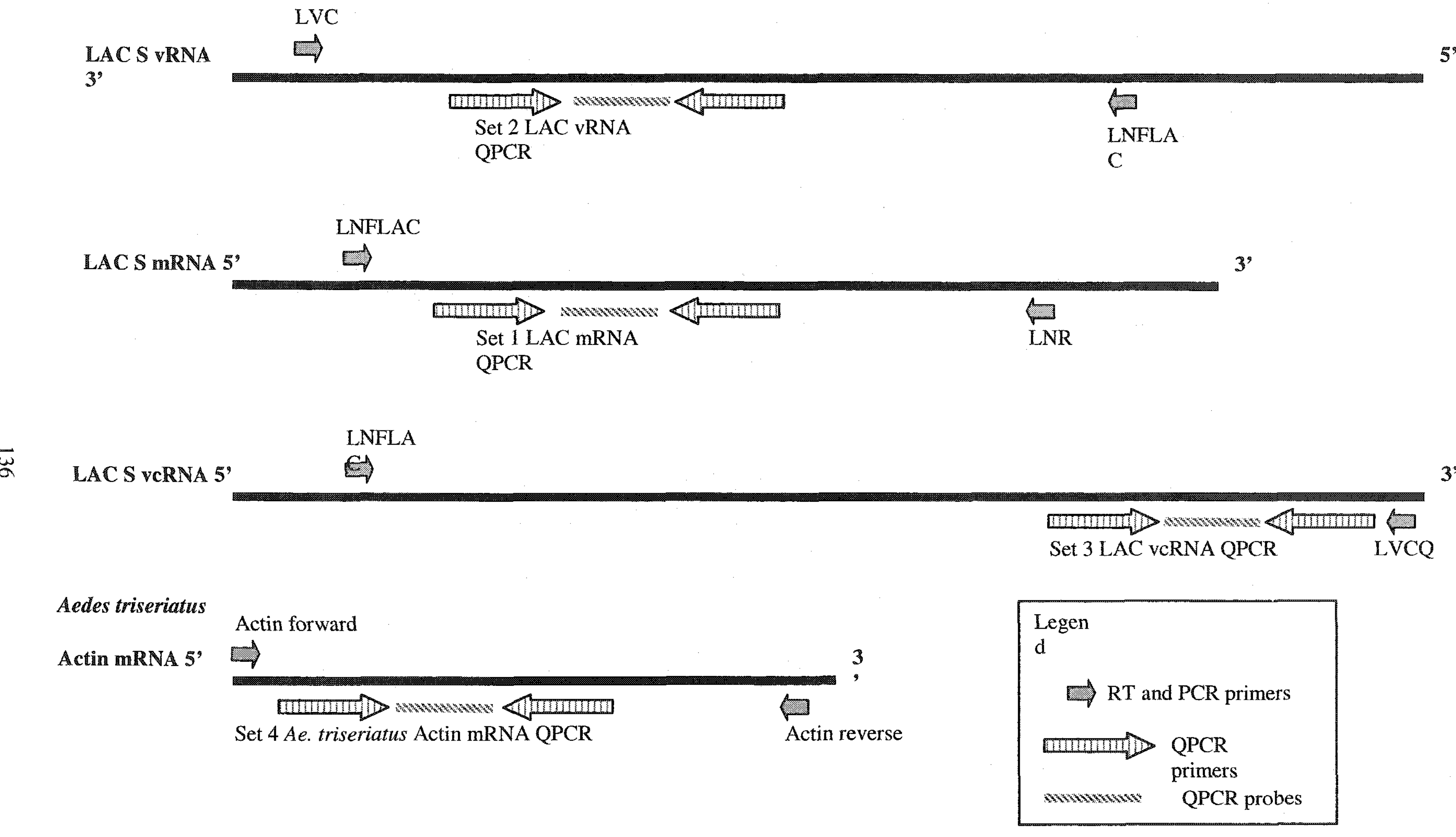

Figure 3.1 RT-PCR primers and Q-PCR primers and probes locations on the target RNA species. Strategy used to amplify and quantitate each LAC S segment RNA species and *Ae. triseriatus* actin mRNA by Q-PCR. The sequence and positions of each primer and probe are given in Tables 3.1 and 3.3.

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RT-PCR protocol for comparison to quantitative analysis

The cDNA samples from the reverse transcription were PCR amplified in separate reactions using four different sets of primers. One-fourth (5.0 µl) of the cDNA sample was added to 45 µl of 1X PCR buffer containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 200 µM of each nucleotide, and 50 pmol of each primer. The PCR reaction mixture was overlaid with 50 µl of sterile mineral oil. The amplification target and products are listed in Table 3.2. The PCR program was as follows: 80°C for 30 minutes (hot start), followed by 92°C for 1 minute (denaturing), 55°C for 30 seconds (annealing) and 72°C for 2 minutes (elongation) for 25 cycles, followed by 72°C for 10 minutes (final elongation) and then cooled to 4°C. At the 80°C hot start temperature, 1.5 units of *Taq* DNA polymerase were added to each tube. Serial dilutions of known copy number plasmids were PCR amplified under the same reaction conditions to determine lower detection limits.

Table 3.2 Primer pairs for LAC virus RT-PCR

RT primer	PCR primers	RNA detected	Product size
LNFLAC	LVC	LAC S vRNA	860 bp
LNR	LNFLAC	LAC S mRNA and vcRNA	715 bp
LVCQ	LNFLAC	LAC S vcRNA	910 bp
Actin Reverse	Actin forward	*Ae. triseriatus* actin mRNA	401 bp

Gel electrophoresis and visualization

All PCR products were analyzed by electrophoresis on 1.0% agarose gels in 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Seven µl of each product was mixed with 2.0 µl of DNA loading buffer (30% v/v glycerol, 70% v/v 1X TAE, and 0.1 mg/ml bromophenol blue). Gels were electrophoresed at 125 V constant for 1.5 hours. Gels were then stained with ethidium bromide and examined under UV light.

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Q-PCR primer and probe design

Four TAQMAN primer and probe sets were designed using Applied Biosystems Primer Express software, version 1.0: one for each of the RNA species being analyzed. Primer and probe placement are based on the sequence of LAC S genome segment or on the sequence of *Ae. triseriatus* actin mRNA (B. Kempf, unpublished data) (Table 3.3). All of the primers and probes were designed to prevent overlapping during hybridization with the target molecule while maintaining close proximity within the sequence. The vcRNA specific primers and probes were designed to include the proposed end site of the LAC S segment mRNA with the probe spanning the site and primers upstream and downstream of the site (Figure 3.1).

Q-PCR primer and probe optimization

The concentrations and reaction conditions for the TAQMAN primers and probes were optimized. The forward and reverse primers (50, 300, and 900 nM concentrations) were used to amplify $1x10^9$ copies of the appropriate control plasmid in a 50 µl reaction containing 1X core PCR buffer, 5mM magnesium chloride, 800 µM dNTP mix, 250 nM TAQMAN probe, 2.5 units SureStart *Taq* DNA polymerase, and 50, 300, or 900 nM of forward and reverse primers. The target molecules were amplified using an Applied Biosystems 7700 sequence detection system thermocycler with the following reaction conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 95°C for 15 seconds and 60°C for 1 minute (40 cycles). The optimum concentrations were chosen by determining the lowest cycle threshold (C_T) at which amplification was detected compared to the overall strength of the reaction (ΔR_n) (Table 3.4).

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Table 3.3 Primers and probes for real-time quantitative PCR				
Set	**Forward 5'-3'**	**Bases represented**	**Tm in °C**	**RNA detected**
1	CCTTGCTGCAGTTAGGATCTTCTT	nt 186-209 of LAC S vRNA	59	LAC S mRNA and vcRNA
2	GGTTAGCCTTCCTCTCTGGCTTA	nt 268-246 of LAC S mRNA and vcRNA	59	LAC S vRNA
3	GGTTATCCAAAAGGGTTTTCTTAAGG	nt 845-870 of LAC S vRNA	60	LAC S vcRNA
4	AGCACGGTATCATCACCAACTG	nt 52-73 of *Ae. triseriatus* actin mRNA (B. Kempf, unpublished)	59	*Ae. triseriatus* actin mRNA
Set	**Reverse 5'-3'**	**Bases represented**	**Tm in °C**	**RNA detected**
1	CCACTCTCCAAATTTAGGGTTAGC	nt 285-262 of LAC S vRNA	58	LAC S mRNA and vcRNA
2	CCTTGCTGCAGTTAGGATCTTCTT	nt 186-209 of LAC S vRNA	59	LAC S vRNA
3	TAGTTTTTTGCTGTCCCTACCA	nt 935-913 of LAC S vRNA	59	LAC S vcRNA
4	CAACGCGCAGCTCATTGT	nt 126-109 of *Ae. triseriatus* actin mRNA (B. Kempf, unpublished)	58	*Ae. triseriatus* actin mRNA
Set	**Probe 5'-3'**	**Bases represented**	**Tm in °C**	**RNA detected**
1	FAM-AGGCCAAGGCTGCTCTCTCGCGTA-TAMRA	nt 224-247 of LAC S vRNA	70	LAC S mRNA and vcRNA
2	VIC-CAGCCTTGGCCTTTGCGGCATT-TAMRA	nt 235-214 of LAC S mRNA and vcRNA	70	LAC S vRNA
3	FAM-CCCACAAAAATAGCAGCTAAATGGGTG-TAMRA	nt 874-910 of LAC S vRNA	65	LAC S vcRNA
4	TET-TGATATGGAGAAGATCTGGCACCACACCT-TAMRA	nt 77-105 of *Ae. triseriatus* actin mRNA (B. Kempf, unpublished)	68	*Ae. triseriatus* actin mRNA

To determine the optimum concentration of the TAQMAN probe, 50, 100, 150, 200, and 250nM concentrations were used to detect $1x10^9$ copies of the appropriate control plasmid in 50 μl of the same reaction mixture with optimized forward and reverse primer concentrations. The reactions were amplified using an Applied Biosystems 7700 sequence

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detection system thermocycler. The optimum concentrations were chosen by determining the lowest cycle threshold (C_T) at which amplification was detected compared to the overall strength of the reaction (ΔRn) (Table 3.4).

Table 3.4 Optimum primer and probe concentration for real-time quantitative PCR		
Primer	**Singleplex concentration**	**Multiplex concentration**
LAC mRNA forward	50 nM	50 nM
LAC mRNA reverse	300 nM	300 nM
LAC vRNA forward	50 nM	50 nM
LAC vRNA reverse	300 nM	300 nM
LAC vcRNA forward	300 nM	300 nM
LAC vcRNA reverse	300 nM	300 nM
Actin forward	50 nM	50 nM
Actin reverse	300 nM	200 nM
Probe	**Singleplex concentration**	**Multiplex concentration**
LAC mRNA	200 nM	200 nM
LAC vRNA	200 nM	200 nM
LAC vcRNA	200 nM	200 nM
Actin	200 nM	200 nM

Optimization of multiplex Q-PCR

After optimization of each primer and probe set for Q-PCR, optimization for multiplex was performed. Since amplification using the primer and probe sets for the three LAC RNA species was more important than that of the actin mRNA internal control, the concentration of the primers for the actin portion of the assay was limited to avoid competition. A primer limitation titration was performed by testing a range of concentrations below the optimum (300, 250, 200, 150, 100, 50 and 25 nM). The optimum limited primer concentration resulted in the lowest ΔR_n with a constant C_T. The primer concentration was further optimized by comparing multiplex and singleplex amplification of serial dilutions of the *Ae. triseriatus* actin control plasmid. For multiplex amplification the concentration of actin primers remained at 50 nM for the forward primer and was decreased to 200 nM for the reverse primer (Table 3.4).

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Q-PCR analysis

One-fourth of each cDNA reaction was used for Q-PCR analysis. Samples were amplified in 50 μl with the same reaction mixture containing optimized forward and reverse primer and probe concentrations. The cDNAs were amplified using an Applied Biosystems 7700 sequence detection system thermocycler with the same reaction conditions. Serial 10-fold dilutions of known copy number control plasmids ($1x10^{12}$ to $1x10^{4}$ copies) were amplified simultaneously with the unknown samples to generate standard curves.

Graphical analysis of log_{10} of copy number of the known plasmids versus cycle threshold (C_T) was used to generate a linear regression equation for each RNA species. Log_{10} values for unknown copy number were then generated based on the linear regression equation. Actual copy number was then generated by calculating 10 to the power of each log value. To normalize the values, the actin mRNA data were set as equal within each sample. To obtain accurate LAC mRNA copy numbers, the copy number generated for each vcRNA was subtracted from that of each mRNA copy number. Copy number per cell from infected cell culture was determined by dividing the copy number obtained from Q-PCR by the number of cells present per flask.

Statistical analysis

Comparisons of the copy number of each LAC RNA species in each tissue at each time point were performed using SAS statistical analysis software. To determine the significance of differences between time points as well as between blood-fed and sugar-fed mosquitoes, the specific SAS code was used (Appendix). The "USER INPUT" portion of the SAS code signifies the positions within the program that names and data values were

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entered prior to submission and processing of the program. Scheffe's t-test (1953) and Tukey's t-test (1994) were performed based on their controlled conservative comparisons.

C. RESULTS

1. Q-PCR analysis of LAC RNA species in infected C6/36 cells. The copy numbers of LAC virus S segment vRNA, mRNA, and vcRNA in infected C6/36 cells were determined at predetermined time points. Each RNA species could be detected as early as day 1 post-infection and was detectable throughout the time course of infection. Virus titration by end point assay revealed that following infection, LAC virus titer in the cell culture medium increased to a peak of $10^{7.25}$ $TCID_{50}$/ml by day 14 and then decreased to a plateau of $10^{5.00}$ $TCID_{50}$/ml by day 28. The copy number of LAC S segment mRNA increased to a maximum of 2.7×10^4 copies per cell on day 9 post-infection and then stabilized between 1.0 and 1.5×10^4 copies per cell by day 14 post-infection. The copy number of LAC S segment vRNA increased to a maximum of 5.1×10^4 copies per cell on day 6 post-infection and then stabilized between 1.6 and 1.8×10^4 copies per cell by day 9 post-infection. The copy number of the LAC S segment vcRNA increased to a maximum of 6.6×10^3 copies per cell on day 6 post-infection and then stabilized between 1.2 and 1.5×10^3 copies per cell by day 9 post-infection. At each time point throughout the course of infection, the quantity of LAC vRNA exceeded that of LAC mRNA, which exceeded the quantity of vcRNA. As the copy number of each RNA species stabilized, the titer of LAC virus in the cell culture supernatant also stabilized (Figure 3.2 and Table 3.5 A).

2. Q-PCR analysis of LAC RNA species in infected MAT cells. The copy numbers of LAC virus S segment vRNA, mRNA, and vcRNA in infected MAT cells were determined at predetermined time points. Each RNA species could to be detected as early as

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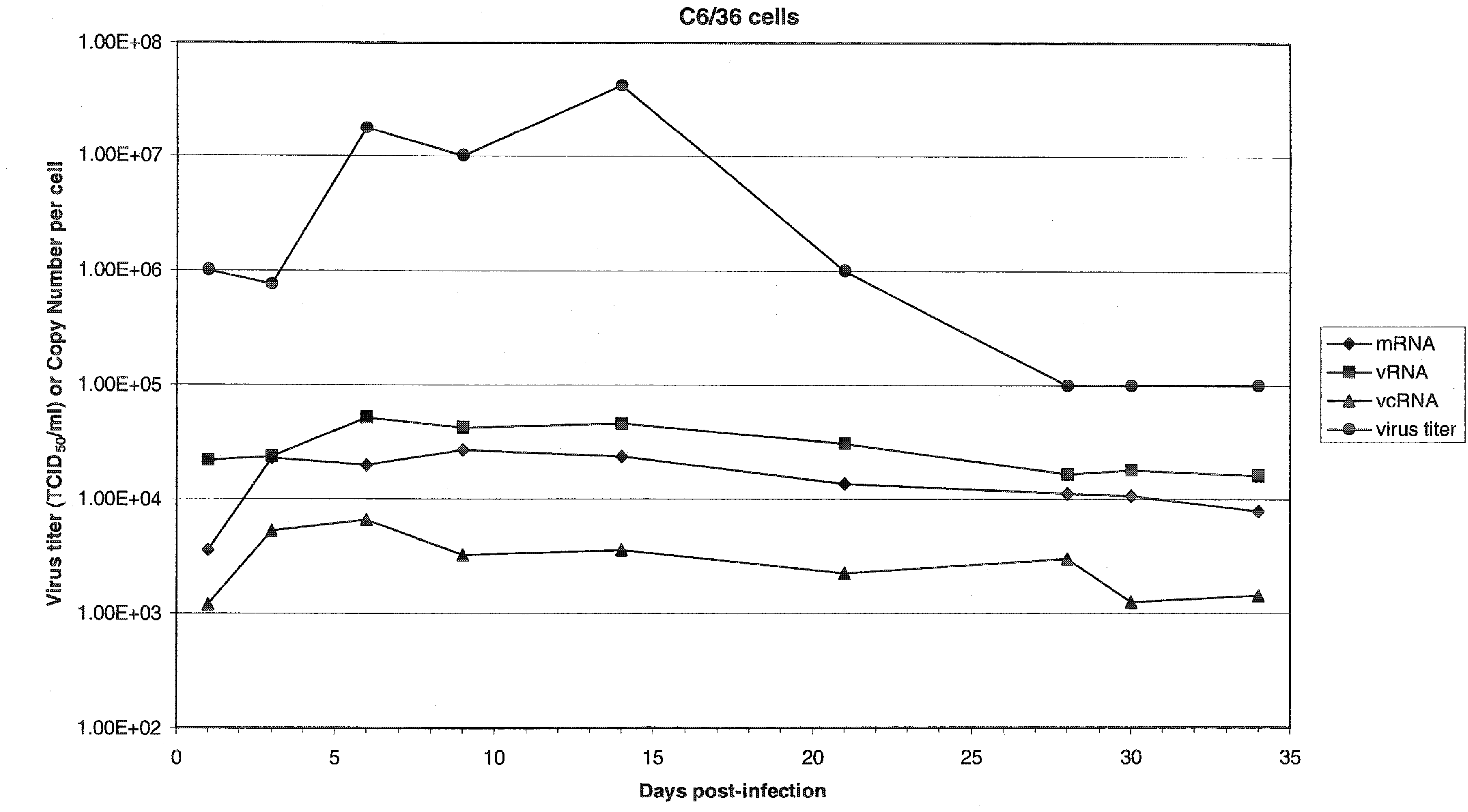

Figure 3.2 Q-PCR analysis of LAC virus RNA species in infected C6/36 cells and titration of LAC virus in the cell culture medium of infected C6/36 cells. Each copy number data point is from a single Q-PCR amplification. Actin values are not given. Each titration data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

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Table 3.5 Copy number of LAC S segment RNAs in infected cells. Each copy number reported is from a single Q-PCR amplification. Actin values are not given.

A. C6/36 cells

C6/36			
Days post-infection	**mRNA**	**vRNA**	**vcRNA**
Day 1	3.57E+03	2.18E+04	1.19E+03
Day 3	2.30E+04	2.38E+04	5.30E+03
Day 6	1.99E+04	5.12E+04	6.55E+03
Day 9	2.70E+04	4.22E+04	3.23E+03
Day 14	2.38E+04	4.61E+04	3.58E+03
Day 21	1.36E+04	3.08E+04	2.23E+03
Day 28	1.12E+04	1.67E+04	2.99E+03
Day 30	1.07E+04	1.81E+04	1.24E+03
Day 30+96h	7.88E+03	1.61E+04	1.43E+03

B. MAT cells

MAT			
Days post-infection	**mRNA**	**vRNA**	**vcRNA**
Day 1	6.94E+02	6.51E+03	4.94E+02
Day 3	3.79E+03	7.34E+04	1.01E+03
Day 6	1.03E+04	2.17E+05	2.21E+04
Day 9	1.13E+04	1.47E+05	1.05E+04
Day 14	9.47E+03	1.15E+05	5.38E+03
Day 21	5.41E+03	1.06E+05	5.25E+03
Day 28	1.08E+04	1.13E+05	2.16E+03
Day 30	6.20E+03	1.15E+05	6.44E+03
Day 30+96h	7.21E+03	1.63E+05	2.34E+03

C. BHK-21 cells

BHK-21			
Hours post-infection	**mRNA**	**vRNA**	**vcRNA**
4 hours	1.79E+02	8.02E+01	2.15E+01
8 hours	3.42E+04	4.07E+04	7.79E+02
12 hours	6.17E+04	1.13E+05	1.02E+04
16 hours	7.94E+04	1.77E+05	2.07E+04
20 hours	6.54E+04	1.53E+05	2.28E+04
24 hours	3.64E+04	1.55E+05	2.75E+04
28 hours	1.70E+04	1.47E+05	3.43E+04
32 hours	3.27E+04	1.26E+05	1.48E+04
36 hours	1.73E+04	1.32E+05	2.86E+04
40 hours	2.18E+04	1.30E+05	1.43E+04
44 hours	3.37E+04	9.35E+04	2.58E+04
48 hours	1.88E+04	1.07E+05	1.34E+04

day 1 post-infection and was detectable throughout the time course of infection. Virus titration by end point assay revealed that LAC virus titer in the cell culture medium increased to a peak of $10^{7.63}$ $TCID_{50}$/ml by day 14 and then decreased and reached a plateau of $10^{6.00}$ $TCID_{50}$/ml by day 34. Both the values for the LAC virus in the cell culture medium and copy numbers of vRNA and vcRNA species were higher than those observed in C6/36 cells. The copy number of LAC S segment mRNA increased to a peak of $1.1x10^4$ copies per cell on day 9 post-infection and then stabilized between 6.2 and $7.2x10^3$ copies per cell by day14 post-infection. The copy number of LAC S segment vRNA increased to a maximum of $2.2x10^5$ copies per cell on day 6 post-infection and then stabilized between 1.0 and $1.5x10^5$ copies per cell by day 9 post-infection. The copy number of the LAC S segment vcRNA increased to a maximum of $2.2x10^4$ copies per cell on day 6 post-infection and then stabilized between 2.0 and $2.5x10^4$ copies per cell by day 9 post-infection. At each time point throughout the course of infection, the quantity of LAC vRNA exceeded that of LAC mRNA, which exceeded the quantity of vcRNA, except at day 6 and day 30, when the copy number of LAC vcRNA was higher than LAC mRNA. As the copy number of each RNA species stabilized, the titer of LAC virus in the cell culture supernatant also stabilized (Figure 3.3 and Table 3.5 B).

3. Q-PCR analysis of LAC RNA species in infected BHK-21 cells. The copy numbers of LAC virus S segment vRNA, mRNA, and vcRNA in infected BHK-21 cells were determined at predetermined time points. Each RNA species could be detected as early as 4 hours post-infection and was detectable throughout the time course of infection. Virus titration by end point assay revealed that following infection, LAC virus titer in the cell culture medium increased to a peak of $10^{8.00}$ $TCID_{50}$/ml by 40 hours post infection and

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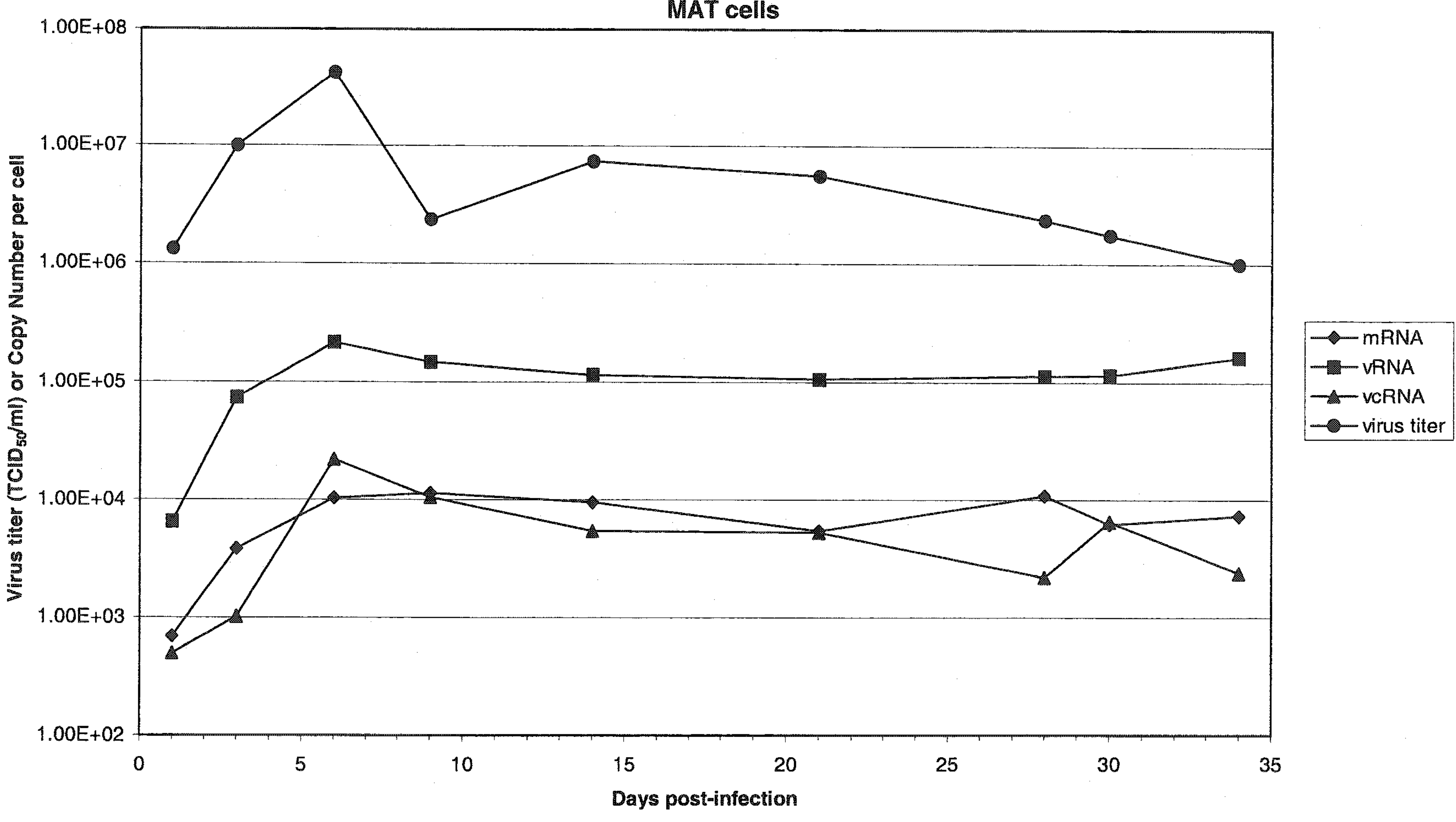

Figure 3.3 Q-PCR analysis of LAC virus RNA species in MAT cells and titration of LAC virus in the cell culture medium of infected MAT cells. Each copy number data point is from a single Q-PCR amplification. Actin values are not given. Each titration data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

remained between $10^{7.38}$ $TCID_{50}$/ml and $10^{8.00}$ $TCID_{50}$/ml from 16 hours to 48 hours post-infection. The titer of LAC virus in the supernatant was greater than that from both MAT and C6/36 cells. The copy numbers of the vRNA and vcRNA species were greater than those observed in C6/36 cells and similar levels of mRNA to those observed in C6/36 cells were seen. The copy numbers of mRNA and vcRNA were greater than those observed in MAT cells, and similar levels of vRNA were observed. The copy number of LAC S segment mRNA increased to a peak of 7.9×10^4 copies per cell at 16 hours post-infection and then stabilized between 1.7 and 2.5×10^4 copies per cell by 20 hours post-infection. The copy number of LAC S segment vRNA increased to a maximum of 1.8×10^5 copies per cell at 16 hours post-infection and then stabilized between 9.3×10^4 and 1.5×10^5 copies per cell by 20 hours post-infection. The copy number of the LAC S segment vcRNA increased to a maximum of 3.4×10^4 copies per cell at 28 hours post-infection and then stabilized between 1.3 and 2.8×10^4 copies per cell by 32 hours post-infection. At each time point throughout the course of infection, the quantity of LAC vRNA exceeded that of LAC mRNA, which exceeded the quantity of vcRNA except at 28 and 36 hours post-infection when the copy number of vcRNA exceeded) that of mRNA. As the copy number of each RNA species stabilized, the titer of LAC virus in the cell culture medium also stabilized (Figure 3.4 and Table 3.5 C).

4. Detection of LAC virus antigen in head tissues of infected female *Aedes triseriatus* mosquitoes by immunofluorescence assay (IFA). Each mosquito used in the Q-PCR analysis was assayed for virus infection by IFA prior to ovary and midgut dissection. Viral antigen was not detected in any head tissue prior to day 6 post-infectious blood meal. Viral antigen was detected in 20% (2/10) of mosquitoes on day 9 post-infectious blood meal,

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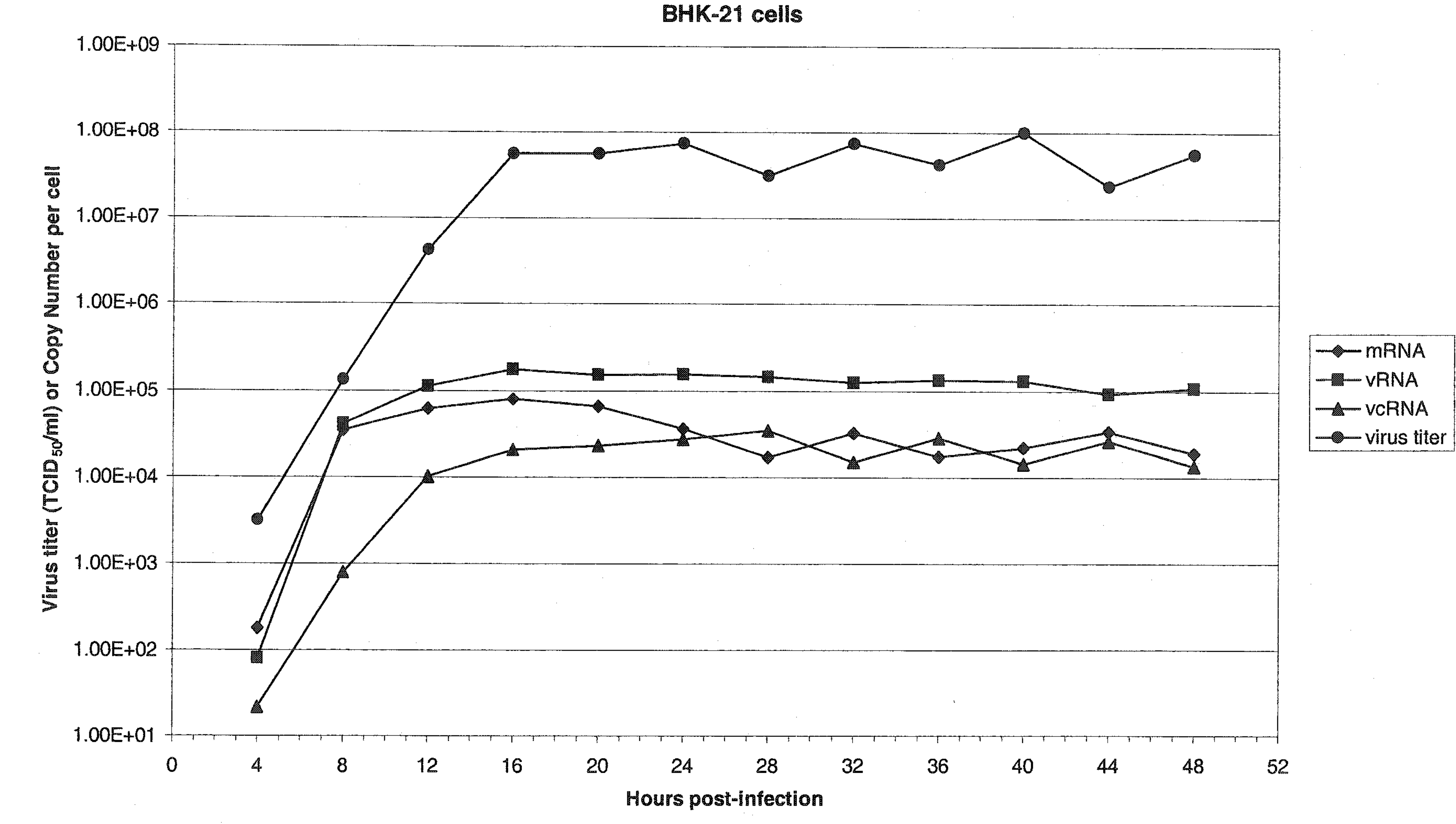

Figure 3.4 Q-PCR analysis of LAC virus RNA species in BHK-21 cells and titration of LAC virus in the cell culture medium of infected BHK-21 cells. Each copy number data point is from a single Q-PCR amplification. Actin values are not given. Each titration data point is the log $TCID_{50}$/ml/day from a single 96-well titration plate.

50% (5/10) of mosquitoes on day 14 post-infectious blood meal, and 80% (8/10) of mosquitoes on day 21 post-infectious blood meal. By day 28 post-infectious blood meal, 100% (10/10) of mosquitoes had detectable viral antigen in head tissue. Throughout the remainder of the experiments (through day 34 or day 30 plus 96 hours), viral antigen was detectable in head tissue from all mosquitoes. Five mosquitoes that ingested a non-infectious blood meal were also assayed at each time point. None of the uninfected mosquitoes had fluorescence detectable in head tissue at any time point (Table 3.6).

5. RT-PCR detection of LAC virus RNAs in mosquito tissues. To compare the sensitivity of Q-PCR analysis to standard RT-PCR protocols, LAC mRNA, vRNA, vcRNA, and actin mRNA cDNAs made from ovaries and midguts at each time point for each mosquito were PCR amplified individually. Amplified products were visualized after agarose gel electrophoresis followed by ethidium bromide staining and exposure to UV light. Previous studies using traditional RT-PCR analysis were limited in terms of sensitivity (Chandler et al., 1996). The lower detection limit was shown to be between $1.0x10^8$ and $1.0x10^7$ copies for each RNA species in a 25-cycle amplification (Figure 3.5).

a. RT-PCR detection of LAC virus RNAs in mosquito ovaries. LAC mRNA was not detectable at day 1 post-infectious blood meal in the ovaries and remained undetectable at day 3 as well. By day 6, mRNA was detectable in 40% of the ovaries. LAC vRNA was detectable in 20% of the ovaries at day 1, 40% of the ovaries at day 3, and in 50% of the ovaries at day 6. LAC vcRNA was not detectable in the ovaries at day 1, was detectable in 30% of the ovaries at day 3 and 50% of the ovaries at day 6. By day 9 post-infectious blood meal, all three LAC RNA species were detectable in 100% of the ovaries

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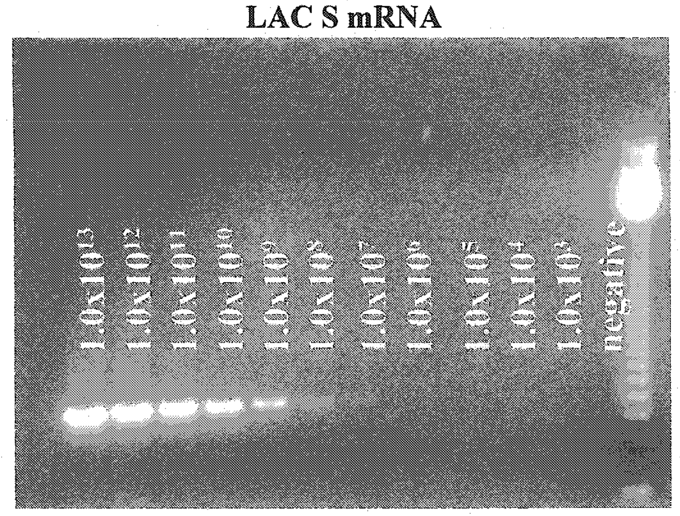

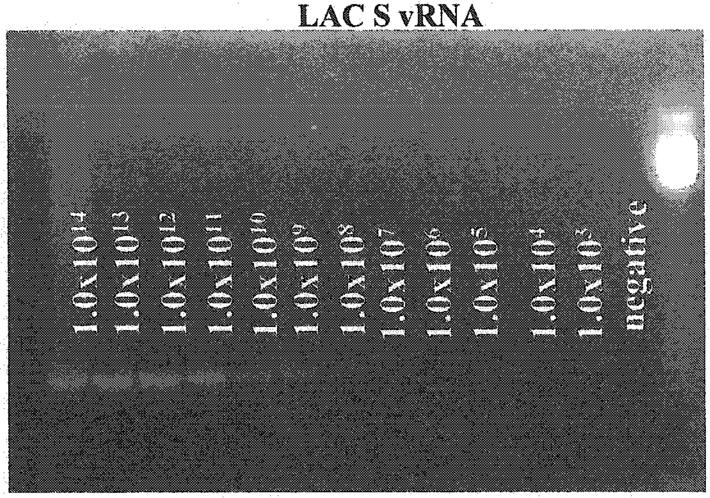

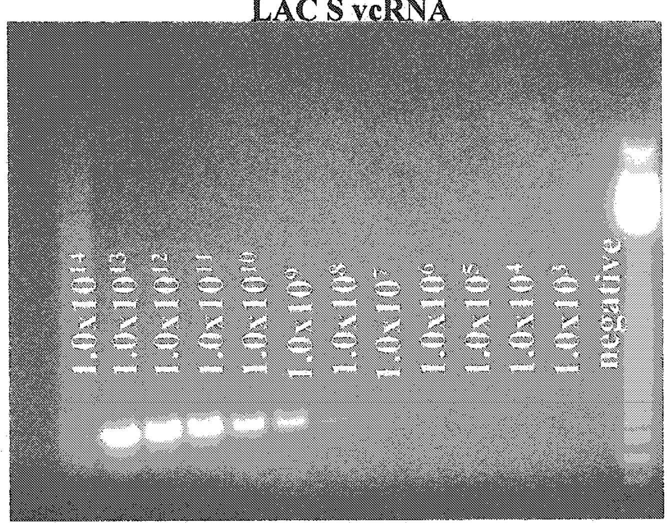

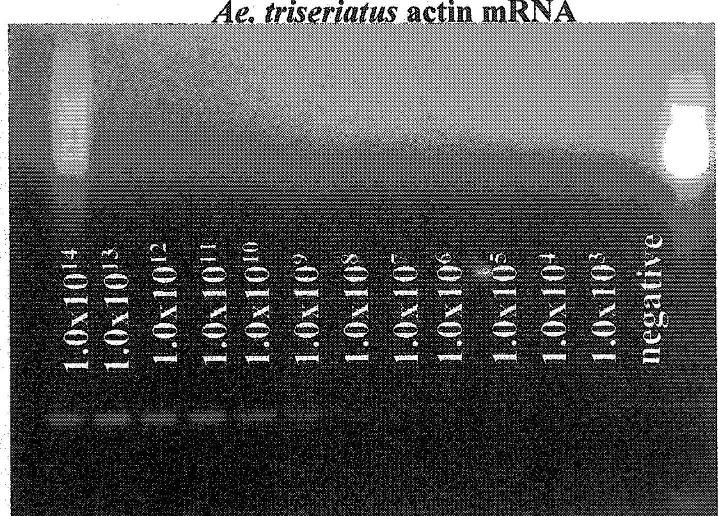

Figure 3.5 Detection limit of traditional RT-PCR. The copy numbers reported are from RT-PCR amplification products electrophoresed on 1.0% agarose 1X TAE gels following 25 cycles of amplification.

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Table 3.6 Detection of LAC virus antigen in head tissue of mosquitoes used for quantitative real-time PCR analysis.

Mosquitoes ingesting an infectious blood meal*			
Time Point	FA result (head squash)	Time Point	FA result (head squash)
Day 1	0% (0/10)	Day 30+24h blood	100% (10/10)
Day 3	0% (0/10)	Day 30+24h sugar	100% (10/10)
Day 6	0% (0/10)	Day 30+48h blood	100% (10/10)
Day 9	20% (2/10)	Day 30+48h sugar	100% (10/10)
Day 14	50% (5/10)	Day 30+72h blood	100% (10/10)
Day 21	80% (8/10)	Day 30+72h sugar	100% (10/10)
Day 28	100% (10/10)	Day 30+96h blood	100% (10/10)
Day 30	100% (10/10)	Day 30+96h sugar	100% (10/10)

Mosquitoes ingesting a noninfectious blood meal			
Time Point	FA result (head squash)	Time Point	FA result (head squash)
Day 1	0% (0/5)	Day 30+24h blood	0% (0/5)
Day 3	0% (0/5)	Day 30+24h sugar	0% (0/5)
Day 6	0% (0/5)	Day 30+48h blood	0% (0/5)
Day 9	0% (0/5)	Day 30+48h sugar	0% (0/5)
Day 14	0% (0/5)	Day 30+72h blood	0% (0/5)
Day 21	0% (0/5)	Day 30+72h sugar	0% (0/5)
Day 28	0% (0/5)	Day 30+96h blood	0% (0/5)
Day 30	0% (0/5)	Day 30+96h sugar	0% (0/5)

Percent (number of positive mosquitoes / number of mosquitoes examined x100)
*LAC virus titer in the infectious blood meal was $10^{8.4}$ $TCID_{50}$/ml.

and remained detectable in 100% of the ovaries at day 14. LAC mRNA detection in the ovaries decreased to 60% by day 21, 40% by day 28 and 20% by day 30. LAC vRNA was detectable in 100% of the ovaries from day 21 to day 30. LAC vcRNA detection in the ovaries decreased to 60% on day 21, 50% on day 28, and 30% on day 30 (Table 3.7).

Following a second blood meal at day 30, detection of LAC mRNA in ovaries increased to 100% at 24 hours, 48 hours, and 72 hours following the second blood meal. At 96 hours 90% of the ovaries had detectable levels of mRNA. LAC vRNA and vcRNA were detectable in 100% of the ovaries between 24 hours and 96 hours following the second blood meal. In mosquitoes ingesting a sugar meal at day 30, 100% had detectable LAC mRNA in their ovaries at 24 and 48 hours, 70% and 50% had detectable mRNA at 72 and 96 hours, respectively. LAC vRNA was detectable in 100% of the ovaries between 24 and 96 hours following the sugar meal at day 30. In mosquitoes ingesting a sugar meal at day 30, 100% had detectable LAC vcRNA in their ovaries at 24 and 48 hours, 60% and 50% had detectable levels at 72 and 96 hours, respectively (Table 3.7).

b. RT-PCR detection of LAC virus RNAs in mosquito midguts. LAC mRNA was detectable by standard RT-PCR at day 1 post-infectious blood meal in 20% of the midguts and increased to 80% at day 3. By day 6 mRNA was detectable in 100% of the midguts. LAC vRNA was detectable at day 1 post-infectious blood meal in 60% of the midguts and 100% by day 3. LAC vcRNA was detectable in 40% of the midguts at day 1 and 100% by day 3. By day 6 post-infectious blood meal, all three LAC RNA species were detectable in 100% of the midguts and remained detectable in 100% of the midguts until day 30 (Table 3.7).

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Table 3.7 RT-PCR detection of LAC virus RNAs from mosquito tissue assayed. The percentages reported are from RT-PCR amplification products electrophoresed on 1.0% agarose 1X TAE gels following 25 cycles of amplification.

Ovary			
Time	mRNA	vRNA	vcRNA
Day 1	0% (0/10)	20% (2/10)	0% (0/10)
Day 3	0% (0/10)	40% (4/10)	30% (3/10)
Day 6	40% (4/10)	50% (5/10)	50% (5/10)
Day 9	100% (10/10)	100% (10/10)	100% (10/10)
Day 14	100% (10/10)	100% (10/10)	100% (10/10)
Day 21	60% (6/10)	100% (10/10)	60% (6/10)
Day 28	40% (4/10)	100% (10/10)	50% (5/10)
Day 30	20% (2/10)	100% (10/10)	30% (3/10)
Day 31 blood-fed	100% (10/10)	100% (10/10)	100% (10/10)
Day 31 sugar-fed	100% (10/10)	100% (10/10)	100% (10/10)
Day 32 blood-fed	100% (10/10)	100% (10/10)	100% (10/10)
Day 32 sugar-fed	100% (10/10)	100% (10/10)	100% (10/10)
Day 33 blood-fed	100% (10/10)	100% (10/10)	100% (10/10)
Day 33 sugar-fed	70% (7/10)	100% (10/10)	60% (6/10)
Day 34 blood-fed	90% (9/10)	100% (10/10)	100% (10/10)
Day 34 sugar-fed	50% (5/10)	100% (10/10)	50% (5/10)

Percent (number of positive mosquitoes / number of mosquitoes tested)

Midgut			
Time	mRNA	vRNA	vcRNA
Day 1	40% (2/5)	60% (3/5)	40% (2/5)
Day 3	80% (4/5)	100% (5/5)	100% (5/5)
Day 6	100% (5/5)	100% (5/5)	100% (5/5)
Day 9	100% (5/5)	100% (5/5)	100% (5/5)
Day 14	100% (5/5)	100% (5/5)	100% (5/5)
Day 21	100% (5/5)	100% (5/5)	100% (5/5)
Day 28	100% (5/5)	100% (5/5)	100% (5/5)
Day 30	100% (5/5)	100% (5/5)	100% (5/5)
Day 31 blood-fed	60% (3/5)	100% (5/5)	80% (4/5)
Day 31 sugar-fed	100% (5/5)	100% (5/5)	100% (5/5)
Day 32 blood-fed	100% (5/5)	100% (5/5)	100% (5/5)
Day 32 sugar-fed	80% (4/5)	100% (5/5)	60% (3/5)
Day 33 blood-fed	100% (5/5)	100% (5/5)	40% (2/5)
Day 33 sugar-fed	100% (5/5)	100% (5/5)	100% (5/5)
Day 34 blood-fed	100% (5/5)	100% (5/5)	100% (5/5)
Day 34 sugar-fed	100% (5/5)	100% (5/5)	100% (5/5)

Percent (number of positive mosquitoes / number of mosquitoes tested x100)

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Following a second blood meal at day 30, LAC mRNA was detected in 60% of midguts at 24 hours and 100% between 48 and 96 hours. LAC vRNA was detectable in 100% of the midguts between 24 and 96 hours following the second blood meal. Following a second blood meal at day 30, detection of LAC vcRNA in midguts decreased to 80% at 24 hours, returned to 100% by 48 hours, decreased to 40% at 72 hours, before finally increasing to 100% at 96 hours. In mosquitoes ingesting a sugar meal at day 30, 100% had detectable LAC mRNA in their midguts at 24 hours, 80% at 48 hours, and 100% again at 72 and 96 hours. LAC vRNA was detectable in 100% of the midguts between 24 and 96 hours following the sugar meal at day 30. In mosquitoes ingesting a sugar meal at day 30, 100% had detectable LAC vcRNA in their ovaries at 24 hours, 60% at 48 hours, and 100% had detectable vcRNA at 72 and 96 hours (Table 3.7).

6. Q-PCR analysis of LAC infected *Aedes triseriatus* ovary tissues. The copy numbers of LAC virus S segment vRNA, mRNA, and vcRNA in ovaries from infected *Ae. triseriatus* female mosquitoes were calculated for each sample and average copy number for a pair of ovaries was also determined. Each RNA species could be detected as early as day 1 post-infectious blood meal, and each was detectable throughout the time course of infection.

a. mRNA detection. LAC virus S segment mRNA was detectable in every ovary sample at each time point throughout the time course of infection. Between day 1 and day 30 post-infectious blood meal, the lowest quantity of mRNA determined ($9.7x10^4$ copies) occurred in a pair of ovaries at day 14. Between day 1 and day 30 post-infectious blood meal, the highest quantity of mRNA ($1.3x10^{10}$ copies) occurred in a pair of ovaries at day 21. After the second meal of either blood or sugar at day 30, the lowest quantity of mRNA ($1.4x10^5$ copies) occurred in a pair of ovaries at 96 hours post-second blood meal. The

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highest quantity of mRNA (7.0×10^{10} copies) occurred in a pair of ovaries at 72 hours post-second blood meal (Table 3.8).

The average copy number of mRNA in ovaries from the ten mosquitoes tested each sample day was plotted to demonstrate trends. Quantities of mRNA copy number increased until day 6 post-infectious blood meal, then mRNA decreased approximately 3 logs. By day 9, the mRNA copy number returned to the amount observed prior to day 6 and it remained at that level until day 30. Upon ingestion of the second blood meal, the quantity of mRNA in ovaries increased approximately 1.5 logs within 48 hours before decreasing by 96 hours to the amounts observed prior to the second meal. In ovaries from mosquitoes fed a sugar meal, the levels of mRNA decreased slightly at 24 hours following the meal before increasing approximately 1.5 logs by 72 hours. By 96 hours the mRNA copy number returned to levels observed prior to the second meal (Figure 3.6).

b. vRNA detection. LAC virus S segment vRNA was detectable in every ovary sample at each time point after day 3 throughout the time course of infection. Seven of the ovary pairs tested at day 1 and four tested at day 3 did not have vRNA detectable by Q-PCR. Between day 1 and day 30 post-infectious blood meal, the lowest quantity of vRNA (1.3×10^5 copies), excluding those ovaries without detectable levels of vRNA, occurred in a pair of ovaries at day 9. Between day 1 and day 30 post-infectious blood meal, the highest quantity of vRNA (5.6×10^{11} copies) occurred in a pair of ovaries at day 14. After the second meal of either blood or sugar at day 30, the lowest quantity of vRNA (1.2×10^5 copies) occurred in a pair of ovaries 72 hours post-sugar meal. The highest quantity of vRNA (6.9×10^{11} copies) occurred in a pair of ovaries 24 hours post-second blood meal (Table 3.9).

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Table 3.8 Copy number of LAC S segment mRNA in ovaries from infected *Ae. triseriatus* female mosquitoes. Each copy number reported is from Q-PCR amplification of invidual tissue samples. Actin values are not given. Averages were plotted (See Figure 3.6).

Sample	Day 1	Day 3	Day 6	Day 9	Day 14	Day 21	Day 28	Day 30
1	3.57E+09	3.14E+09	9.17E+05	9.15E+05	4.67E+05	1.71E+09	2.93E+08	2.22E+07
2	2.33E+09	4.31E+09	3.26E+05	1.74E+06	5.10E+09	4.96E+06	5.97E+07	8.73E+08
3	1.36E+09	1.93E+08	1.04E+06	1.45E+06	4.47E+05	1.26E+10	2.02E+08	8.91E+08
4	5.92E+09	2.33E+09	7.85E+05	1.68E+06	1.52E+09	1.89E+09	3.27E+08	1.26E+08
5	3.80E+08	1.99E+09	4.77E+05	1.51E+09	2.95E+09	4.49E+08	1.32E+09	1.24E+09
6	1.84E+09	2.38E+09	5.78E+05	1.33E+06	1.45E+08	4.91E+06	7.09E+08	5.56E+08
7	8.58E+07	2.63E+09	7.80E+05	1.45E+06	4.50E+05	6.63E+06	1.46E+09	1.85E+08
8	2.82E+09	7.02E+07	2.56E+05	1.06E+10	4.06E+08	2.12E+06	3.50E+08	1.07E+09
9	1.38E+08	1.65E+09	7.13E+05	1.14E+06	2.30E+09	7.79E+09	8.36E+08	2.94E+08
10	7.62E+08	1.57E+09	2.16E+07	1.51E+06	9.66E+04	3.06E+09	1.43E+08	5.36E+08
Average	1.92E+09	2.03E+09	2.75E+06	1.22E+09	1.24E+09	2.75E+09	5.69E+08	5.79E+08

Sample	Day 31 Blood-fed	Day 31 Sugar-fed	Day 32 Blood-fed	Day 32 Sugar-fed	Day 33 Blood-fed	Day 33 Sugar-fed	Day 34 Blood-fed	Day 34 Sugar-fed
1	1.57E+09	5.43E+08	8.34E+09	6.20E+07	4.37E+09	1.21E+10	8.38E+09	1.58E+08
2	4.90E+08	2.22E+06	8.37E+09	8.39E+07	1.63E+07	4.59E+06	1.82E+06	4.57E+09
3	2.24E+09	5.83E+08	5.08E+10	7.28E+07	3.03E+07	3.15E+09	3.54E+06	5.34E+08
4	1.03E+08	9.15E+07	1.76E+08	1.93E+06	1.90E+10	9.50E+05	1.39E+05	1.68E+08
5	3.44E+09	1.47E+08	9.01E+09	1.51E+09	1.03E+08	2.87E+09	2.73E+06	3.56E+07
6	1.90E+10	2.40E+07	3.75E+09	5.73E+08	7.02E+10	8.66E+05	7.13E+06	2.60E+09
7	1.03E+09	1.70E+08	7.52E+10	8.03E+08	7.89E+07	1.51E+08	7.09E+09	1.53E+08
8	6.43E+08	1.24E+08	1.50E+08	1.06E+10	1.28E+07	7.57E+09	8.62E+04	2.31E+09
9	2.02E+08	4.92E+08	9.81E+08	2.66E+08	6.31E+06	4.30E+09	2.14E+06	1.93E+09
10	5.22E+06	9.08E+07	1.42E+09	3.41E+06	5.04E+08	1.52E+10	8.96E+05	2.70E+08
Average	2.87E+09	2.27E+08	1.58E+10	1.39E+09	9.43E+09	4.54E+09	1.55E+09	1.27E+09

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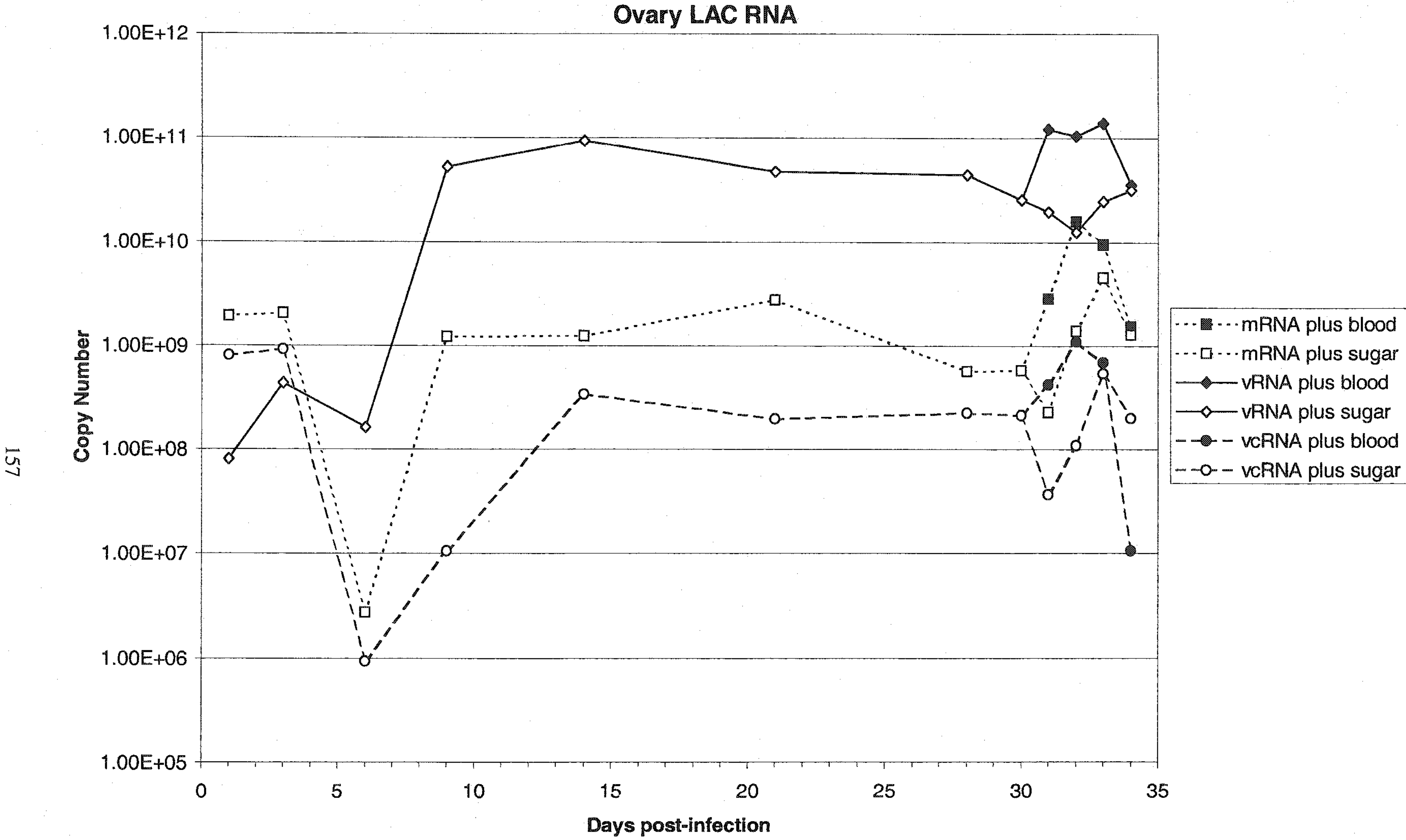

Figure 3.6 Q-PCR analysis of LAC RNA in ovary tissues from infected *Ae. triseriatus* female mosquitoes. Each copy number data point is the average from Q-PCR amplification of 10 individual ovaries. Actin values are not given. Error bars are excluded for simplicity.

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Table 3.9 Copy number of LAC S segment vRNA in ovaries from infected *Ae. triseriatus* female mosquitoes. Each copy number reported is from Q-PCR amplification of invidual tissue samples. Actin values are not given. Averages were plotted (See Figure 3.6).

Sample	Day 1	Day 3	Day 6	Day 9	Day 14	Day 21	Day 28	Day 30
1	ND	ND	1.72E+08	5.42E+06	1.49E+07	5.17E+10	5.98E+10	4.89E+08
2	ND	ND	3.37E+06	1.30E+06	1.18E+11	1.00E+10	4.71E+09	8.51E+10
3	ND	2.70E+07	1.47E+07	3.23E+05	2.62E+07	9.48E+10	7.12E+10	1.70E+10
4	ND	6.03E+08	9.40E+06	8.78E+05	5.62E+11	4.68E+10	7.65E+09	4.49E+09
5	ND	ND	1.05E+07	8.44E+10	1.22E+11	3.64E+10	5.94E+10	1.85E+10
6	ND	2.28E+06	1.13E+06	2.97E+05	3.60E+10	7.98E+08	2.69E+10	3.95E+10
7	ND	ND	2.72E+06	1.34E+05	5.45E+07	3.53E+08	3.93E+10	2.12E+10
8	2.59E+08	3.70E+09	1.40E+09	4.40E+11	4.68E+09	2.66E+08	7.29E+10	4.13E+10
9	2.10E+06	2.92E+07	6.45E+06	1.45E+06	1.05E+11	1.74E+11	2.30E+10	1.53E+10
10	5.50E+08	1.45E+06	4.36E+06	5.75E+05	1.45E+07	6.23E+10	7.91E+10	1.53E+10
Average	8.10E+07	4.37E+08	1.62E+08	5.24E+10	9.46E+10	4.77E+10	4.44E+10	2.58E+10
	Day 31	Day 31	Day 32	Day 32	Day 33	Day 33	Day 34	Day 34
Sample	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed
1	4.28E+10	1.25E+10	2.37E+11	7.22E+08	6.37E+10	5.40E+10	6.66E+10	2.74E+09
2	1.12E+11	1.47E+05	3.77E+11	1.58E+10	3.33E+11	2.33E+05	2.83E+11	2.25E+10
3	6.91E+11	9.19E+09	8.74E+09	5.58E+09	3.11E+09	6.36E+09	1.31E+09	7.39E+09
4	2.17E+10	7.99E+08	4.13E+05	2.73E+06	1.78E+09	4.19E+05	4.71E+07	2.26E+09
5	7.69E+10	1.59E+09	3.76E+10	2.51E+10	4.42E+11	1.31E+10	3.54E+08	1.03E+11
6	1.16E+11	4.69E+09	9.28E+10	5.54E+09	4.17E+11	1.24E+05	6.03E+09	6.19E+10
7	3.16E+10	1.57E+11	2.13E+11	1.98E+10	1.00E+11	6.80E+10	2.46E+09	5.30E+09
8	1.20E+11	1.45E+09	1.42E+06	3.73E+10	1.74E+06	3.16E+10	1.90E+06	1.16E+10
9	9.12E+09	8.74E+09	2.06E+10	8.86E+09	6.06E+07	2.16E+10	3.23E+07	9.84E+10
10	4.29E+08	8.78E+08	6.23E+10	7.14E+09	4.70E+10	5.24E+10	6.89E+05	3.26E+09
Average	1.22E+11	1.97E+10	1.05E+11	1.26E+10	1.41E+11	2.47E+10	3.59E+10	3.19E+10

ND = not detected

The average copy number of vRNA in ovaries from the ten mosquitoes tested each sample day was plotted to demonstrate trends. Quantities of vRNA increased until day 6 post-infectious blood meal, then vRNA copy number decreased approximately 0.3 log. By day 9, the vRNA copy number exceeded that observed prior to day 6 by approximately 2.5 logs and remained stable until day 30. Upon ingestion of the second blood meal, the copy number of vRNA in ovaries increased approximately 0.5 log within 48 hours before decreasing by 96 hours to amounts observed prior to the second meal. In ovaries from mosquitoes fed a sugar meal, the amounts of vRNA decreased 0.3 log by 48 hours following the meal. By 96 hours the vRNA copy number returned to amounts observed prior to the second meal (Figure 3.6).

c. vcRNA detection. LAC virus S segment vcRNA was detectable in every ovary sample at each time point throughout the time course of infection. Between day 1 and day 30 post-infectious blood meal, the lowest quantity of vcRNA (4.5×10^4 copies) occurred in a pair of ovaries at day 6. Between day 1 and day 30 post-infectious blood meal, the highest quantity of vcRNA (1.8×10^8 copies) occurred in a pair of ovaries at day 1. After the second meal of either blood or sugar at day 30, the lowest quantity of vcRNA (8.9×10^4 copies) occurred in a pair of ovaries 96 hours post-second blood meal. The highest quantity of vcRNA (3.4×10^9 copies) occurred in a pair of ovaries 48 hours post-second blood meal (Table 3.10).

The average copy number of vcRNA in ovaries from the ten mosquitoes tested each sample day was plotted to demonstrate trends. Copy number of vcRNA increased until day 6 post-infectious blood meal, then vcRNA decreased approximately three logs. By day 9, the vcRNA returned to amounts observed prior to day 6 and remained stable until day 30. Upon

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Table 3.10 Copy number of LAC S segment vcRNA in ovaries from infected *Ae. triseriatus* female mosquitoes. Each copy number reported is from Q-PCR amplification of invidual tissue samples. Actin values are not given. Averages were plotted (See Figure 3.6).

Sample	Day 1	Day 3	Day 6	Day 9	Day 14	Day 21	Day 28	Day 30
1	3.53E+08	1.31E+09	8.20E+04	5.38E+05	3.21E+05	1.63E+08	8.80E+07	8.09E+06
2	8.17E+08	2.24E+08	6.30E+05	6.43E+04	9.94E+08	1.11E+08	4.27E+07	1.27E+08
3	6.48E+08	1.51E+09	4.49E+04	2.11E+05	9.48E+05	4.14E+08	3.66E+08	2.30E+08
4	9.09E+08	6.76E+08	5.41E+04	1.85E+05	1.63E+09	3.80E+08	9.57E+07	1.95E+08
5	9.02E+08	1.47E+09	3.50E+05	1.60E+07	3.67E+08	6.22E+08	6.90E+08	2.17E+08
6	1.17E+09	1.91E+08	5.41E+04	1.16E+05	5.71E+07	4.96E+05	9.85E+07	1.63E+08
7	8.96E+08	2.37E+08	1.49E+05	1.05E+05	1.37E+06	1.37E+07	1.02E+08	2.16E+08
8	1.88E+08	1.55E+09	3.38E+06	8.80E+07	9.37E+06	1.58E+05	3.12E+08	6.65E+08
9	1.82E+09	6.32E+08	2.10E+05	2.95E+05	3.27E+08	2.13E+07	6.41E+06	1.17E+08
10	2.80E+08	1.34E+09	4.30E+06	5.88E+04	5.88E+05	2.27E+08	4.33E+08	1.67E+08
Average	7.98E+08	9.14E+08	9.26E+05	1.06E+07	3.39E+08	1.95E+08	2.24E+08	2.10E+08
	Day 31	Day 31	Day 32	Day 32	Day 33	Day 33	Day 34	Day 34
Sample	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed
1	2.71E+08	7.00E+07	2.48E+09	1.77E+07	1.81E+09	7.94E+08	8.38E+07	2.78E+08
2	2.51E+08	6.75E+05	2.70E+09	1.27E+08	1.27E+07	5.45E+05	1.47E+07	4.49E+08
3	1.20E+09	7.00E+07	1.78E+09	7.06E+07	1.44E+07	3.10E+08	1.72E+05	1.73E+08
4	8.22E+07	3.23E+06	8.52E+05	2.93E+05	1.54E+09	7.59E+05	8.86E+04	9.13E+07
5	8.95E+08	3.29E+07	5.84E+08	2.11E+08	1.99E+06	5.62E+08	6.87E+05	2.15E+07
6	1.11E+09	3.58E+07	3.37E+09	1.04E+08	2.87E+09	1.56E+05	5.74E+06	1.32E+08
7	6.16E+07	1.19E+07	6.43E+05	9.40E+07	3.70E+07	1.12E+09	1.15E+05	8.02E+07
8	1.48E+08	4.13E+07	1.01E+06	4.06E+08	3.37E+05	4.71E+08	1.42E+05	4.83E+08
9	1.83E+08	8.30E+07	6.98E+07	3.15E+07	3.57E+06	5.95E+08	1.79E+05	2.32E+08
10	4.52E+06	2.05E+07	8.38E+05	2.57E+07	6.21E+08	1.55E+09	1.14E+05	3.27E+07
Average	4.21E+08	3.69E+07	1.10E+09	1.09E+08	6.91E+08	5.41E+08	1.06E+07	1.97E+08

ingestion of the second blood meal, the amounts of vcRNA in ovaries increased approximately 0.7 log within 48 hours before decreasing by 96 hours to amounts observed prior to the second meal. In ovaries from mosquitoes fed a sugar meal, the copy number of vcRNA decreased 0.5 log at 24 hours following the meal before increasing approximately 1.2 logs by 72 hours. By 96 hours the vcRNA copy number decreased approximately 2.0 logs from the 72 hour time point and remained below the amounts observed prior to the second meal (Figure 3.6).

7. Q-PCR analysis of LAC infected *Aedes triseriatus* midgut tissues. The copy numbers of LAC virus S segment vRNA, mRNA, and vcRNA in midguts from infected *Ae. triseriatus* female mosquitoes were determined for each sample and average copy number for a midgut was also determined. Each RNA species could be detected as early as day 1 post-infectious blood meal and was detectable throughout the time course of infection. .

a. mRNA detection. LAC virus S segment mRNA was detectable in every midgut sample at each time point throughout the time course of infection. Between day 1 and day 30 post-infectious blood meal, the lowest quantity of mRNA (3.7×10^6 copies) occurred in a midgut at day 3. Between day 1 and day 30 post-infectious blood meal, the highest quantity of mRNA (2.4×10^{11} copies) occurred in a midgut at day 1. After the second meal of either blood or sugar at day 30, the lowest quantity of mRNA (1.4×10^7 copies) occurred in a midgut 24 hours post-second blood meal. The highest quantity of mRNA (6.1×10^9 copies) occurred in a midgut 24 hours post-sugar meal (Table 3.11).

The average copy number of mRNA in midguts from the five mosquitoes tested each sample day was plotted to demonstrate trends. Quantities of mRNA decreased until day 6 post-infectious blood meal, then mRNA increased slightly. By day 9, the mRNA decreased

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Table 3.11 Copy number of LAC S segment mRNA in midguts from infected *Ae. triseriatus* female mosquitoes. Each copy number reported is from Q-PCR amplification of invidual tissue samples. Actin values are not given. Averages were plotted (See Figure 3.7).

Sample	Day 1	Day 3	Day 6	Day 9	Day 14	Day 21	Day 28	Day 30
1	1.68E+09	8.56E+09	1.53E+09	1.42E+10	2.72E+08	1.52E+09	2.73E+08	1.02E+10
2	4.57E+07	4.43E+09	3.36E+08	1.18E+09	2.46E+08	2.18E+08	1.03E+09	1.15E+09
3	2.58E+09	1.71E+10	1.32E+10	4.14E+09	5.02E+08	2.44E+08	5.38E+08	1.52E+07
4	2.37E+11	9.06E+06	6.90E+07	8.31E+08	3.65E+08	5.77E+08	1.08E+09	6.00E+07
5	1.00E+08	3.69E+06	2.43E+09	1.07E+09	3.85E+08	1.31E+08	4.24E+08	9.20E+07
Average	4.84E+10	6.02E+09	3.52E+09	4.28E+09	3.54E+08	5.37E+08	6.68E+08	2.31E+09
	Day 31	Day 31	Day 32	Day 32	Day 33	Day 33	Day 34	Day 34
Sample	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed
1	1.52E+07	5.95E+09	3.82E+08	9.95E+08	1.50E+09	1.19E+08	8.62E+08	6.17E+08
2	1.35E+07	1.07E+09	1.08E+08	1.85E+09	3.93E+08	2.06E+08	5.56E+08	4.25E+08
3	1.12E+08	1.05E+08	2.10E+08	4.65E+08	3.62E+08	3.77E+07	6.42E+08	5.83E+08
4	8.80E+07	6.14E+09	1.83E+09	1.12E+09	3.57E+08	4.40E+08	3.81E+08	7.60E+08
5	3.11E+08	1.88E+09	1.22E+09	1.13E+08	3.78E+08	2.76E+08	3.03E+08	1.30E+09
Average	1.08E+08	3.03E+09	7.51E+08	9.09E+08	5.99E+08	2.16E+08	5.49E+08	7.37E+08

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1.0 log and remained stable until day 30 when it increased an additional 0.5 log. Upon ingestion of the second blood meal, the amounts of mRNA in midguts decreased approximately 1.5 logs within 24 hours before increasing by 96 hours to amounts observed prior to the second meal. In midguts from mosquitoes fed a sugar meal, the amounts of mRNA increased slightly at 24 hours following the meal before decreasing approximately 1.0 log by 72 hours. By 96 hours the mRNA copy number returned to amounts observed prior to the second meal (Figure 3.7).

b. vRNA detection. LAC virus S segment vRNA was detectable in every midgut sample at each time point throughout the time course of infection. Between day 1 and day 30 post-infectious blood meal, the lowest quantity of vRNA (3.6×10^5 copies) occurred in a midgut at day 1. Between day 1 and day 30 post-infectious blood meal, the highest quantity of vRNA (2.8×10^{11} copies) occurred in a midgut at day 9. After the second meal of either blood or sugar at day 30, the lowest quantity of vRNA (4.1×10^4 copies) occurred in a midgut 48 hours post-sugar meal. The highest quantity of vRNA (3.0×10^{11} copies) occurred in a midgut 24 hours post-sugar meal (Table 3.12).

The average copy number of vRNA in midguts from the five mosquitoes tested each sample day was plotted to demonstrate trends. Quantities of vRNA fluctuated over a range of approximately 1.0 log until day 9 post-infectious blood meal, then vRNA reached a stable level and remained stable until day 30. Upon ingestion of the second blood meal, the amounts of vRNA in midguts decreased approximately 2.0 logs within 72 hours before increasing by 96 hours to levels observed prior to the second meal. In midguts from mosquitoes fed a sugar meal, the amounts of vRNA increased slightly at 24 hours following the meal before decreasing approximately 1.0 log by 72 hours. By 96 hours the vRNA copy number returned to amounts just below that observed prior to the second meal (Figure 3.7).

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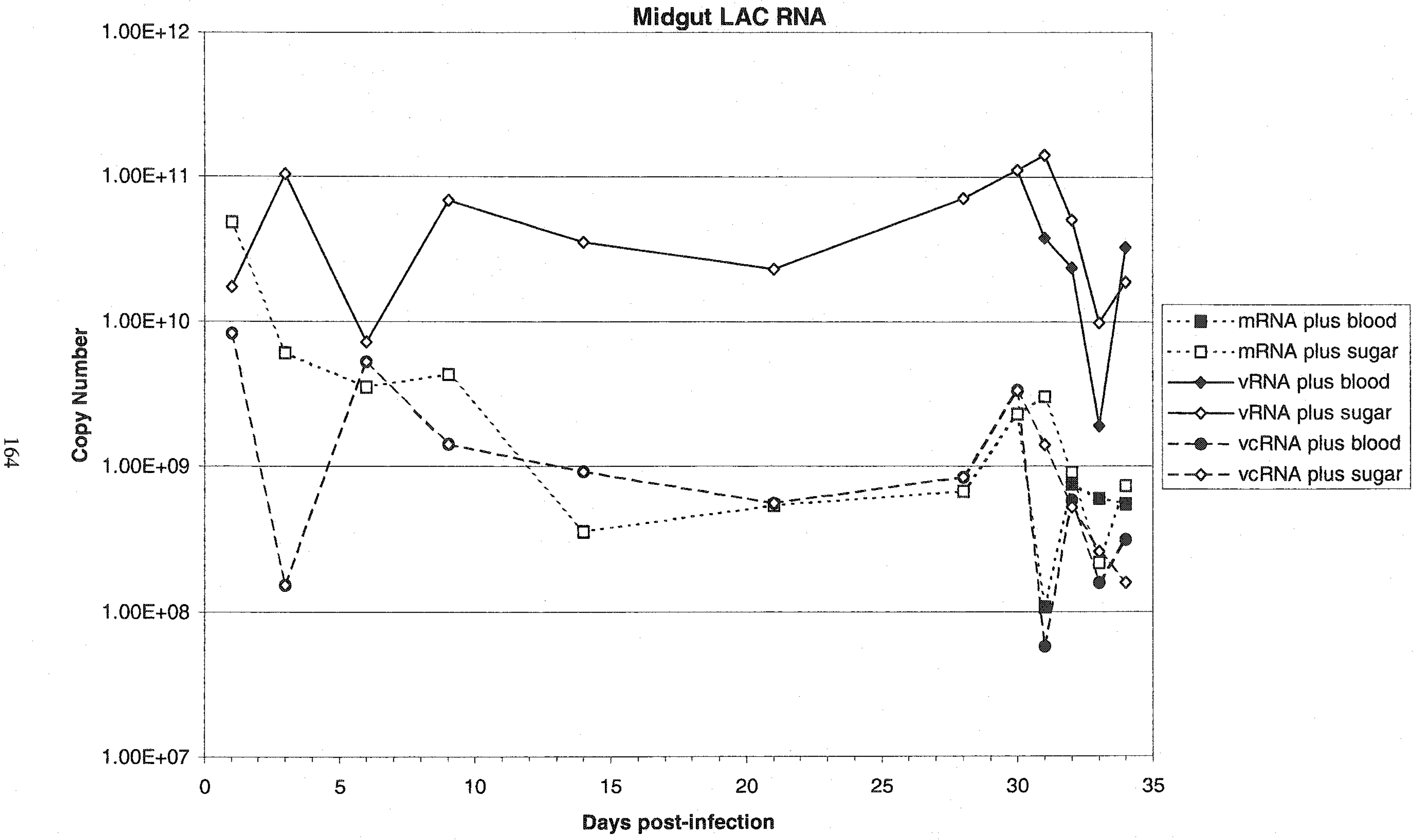

Figure 3.7 Q-PCR analysis of LAC RNA in midgut tissues from infected *Aedes triseriatus* female mosquitoes. Each copy number data point is the average from Q-PCR amplification of 10 individual ovaries. Actin values are not given. Error bars are excluded for simplicity.

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Table 3.12 Copy number of LAC S segment vRNA in midguts from infected *Ae. triseriatus* female mosquitoes. Each copy number reported is from Q-PCR amplification of invidual tissue samples. Actin values are not given. Averages were plotted (See Figure 3.7).

Sample	Day 1	Day 3	Day 6	Day 9	Day 14	Day 21	Day 28	Day 30
1	8.51E+10	5.55E+10	6.21E+09	7.41E+09	6.68E+10	3.65E+10	6.90E+10	1.66E+11
2	1.01E+09	1.88E+11	5.87E+07	6.11E+10	4.39E+10	3.89E+10	1.58E+11	1.60E+11
3	4.52E+05	2.75E+11	1.06E+10	2.77E+11	4.46E+10	3.20E+10	1.23E+11	2.35E+11
4	3.78E+07	1.03E+09	8.36E+07	5.06E+08	1.48E+10	3.68E+09	5.09E+08	8.74E+08
5	3.58E+05	8.83E+07	1.90E+10	4.78E+08	6.69E+09	4.80E+09	5.04E+09	7.87E+08
Average	1.72E+10	1.04E+11	7.19E+09	6.93E+10	3.53E+10	2.32E+10	7.13E+10	1.13E+11
	Day 31	Day 31	Day 32	Day 32	Day 33	Day 33	Day 34	Day 34
Sample	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed
1	2.39E+07	1.68E+11	4.15E+10	1.17E+11	6.27E+09	8.59E+09	3.92E+10	2.68E+10
2	1.51E+11	1.00E+10	3.08E+09	1.18E+11	1.90E+09	1.09E+10	6.06E+10	4.88E+09
3	1.57E+10	1.44E+11	2.70E+10	4.10E+04	1.59E+07	5.04E+09	2.56E+10	2.14E+10
4	1.94E+10	8.94E+10	3.62E+10	1.84E+10	6.38E+07	1.28E+10	3.47E+10	1.60E+10
5	4.80E+09	2.99E+11	1.02E+10	2.07E+09	1.26E+09	1.20E+10	3.68E+09	2.56E+10
Average	3.82E+10	1.42E+11	2.36E+10	5.11E+10	1.90E+09	9.86E+09	3.28E+10	1.89E+10

c. vcRNA detection. LAC virus S segment vcRNA was detectable in every midgut sample at each time point throughout the time course of infection. Between day 1 and day 30 post-infectious blood meal, the lowest quantity of vcRNA ($2.1x10^6$ copies) occurred in a midgut at day 9. Between day 1 and day 30 post-infectious blood meal, the highest quantity of vcRNA ($2.8x10^{10}$ copies) occurred in a midgut at day 1. After the second meal of either blood or sugar at day 30, the lowest quantity of vcRNA ($2.3x10^5$ copies) occurred in midguts 24 and 72 hours post-second blood meal. The highest quantity of vcRNA ($2.6x10^9$ copies) occurred in a midgut 24 hours post-sugar meal (Table 3.13).

The average copy number of vcRNA in midguts from the five mosquitoes tested each sample day was plotted to demonstrate trends. vcRNA copy number fluctuated over a range of approximately 1.5 logs until day 6 post-infectious blood meal, then vcRNA copy number had decreased an additional 0.5 log and then reached a stable amount and remained stable until day 30 when an increase of 0.5 log was observed. Upon ingestion of the second blood meal, the amounts of vcRNA in midguts decreased approximately 2.0 logs within 24 hours before increasing 1.0 log by 48 hours. An additional decrease of 0.5 log was observed by 72 hours following the second meal and by 96 hours the copy number of vcRNA remained below the amount observed prior to the second meal. In midguts from mosquitoes fed a sugar meal, the amount of vcRNA steadily decreased 1.5 logs until 96 hours. By 96 hours the vcRNA copy number remained below amounts observed prior to the second meal (Figure 3.7).

8. Statistical analysis of copy number data. Statistical analyses were performed using SAS statistical analysis software to determine the significance of differences in RNA copy numbers in ovaries and midguts for each mosquito between time points as well as

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Table 3.13 Copy number of LAC S segment vcRNA in midguts from infected *Ae. triseriatus* female mosquitoes. Each copy number reported is from Q-PCR amplification of invidual tissue samples. Actin values are not given. Averages were plotted (See Figure 3.7).

Sample	Day 1	Day 3	Day 6	Day 9	Day 14	Day 21	Day 28	Day 30
1	1.19E+10	2.00E+08	6.68E+08	2.51E+09	9.01E+08	6.25E+08	5.48E+08	1.43E+10
2	5.62E+07	4.89E+08	1.91E+08	3.57E+09	1.06E+09	1.39E+09	1.15E+09	2.13E+09
3	1.56E+09	3.16E+07	1.90E+10	6.10E+08	2.11E+09	6.20E+08	1.48E+09	1.18E+08
4	2.77E+10	1.98E+07	2.88E+08	2.13E+06	3.99E+08	9.90E+07	4.36E+08	6.78E+07
5	2.43E+06	2.29E+07	6.33E+09	4.32E+08	1.21E+08	3.88E+07	5.66E+08	1.19E+08
Average	8.26E+09	1.53E+08	5.29E+09	1.42E+09	9.18E+08	5.54E+08	8.36E+08	3.36E+09
	Day 31	Day 31	Day 32	Day 32	Day 33	Day 33	Day 34	Day 34
Sample	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed	Blood-fed	Sugar-fed
1	2.64E+05	1.53E+09	8.72E+08	1.20E+09	6.95E+08	2.83E+08	9.12E+08	2.58E+08
2	2.27E+05	5.22E+08	5.37E+07	1.32E+09	5.73E+06	1.30E+08	7.45E+07	7.03E+07
3	9.72E+07	2.61E+09	1.01E+09	2.69E+06	2.27E+05	1.17E+08	1.38E+08	5.29E+07
4	1.75E+08	2.09E+08	2.94E+08	6.74E+07	2.30E+06	4.75E+08	2.97E+08	3.06E+08
5	1.50E+07	2.16E+09	6.94E+08	1.97E+06	9.29E+07	2.92E+08	1.41E+08	1.05E+08
Average	5.76E+07	1.41E+09	5.86E+08	5.19E+08	1.59E+08	2.59E+08	3.13E+08	1.59E+08

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between blood-fed versus sugar-fed mosquitoes. Data obtained for each mosquito were compared in pairs using Tukey's t-test and Scheffe's t-test. Individual time points were compared sequentially and between mosquitoes that received blood and sugar meal treatments.

In the ovary samples between day 1 and day 30, the only significant difference between time points occurred between day 3 and day 6 (mRNA $P = 0.0002$, vcRNA $P < 0.0001$). In contrast, none of the differences between time points for vRNA were statistically significant. Following day 30, copy numbers did not differ significantly between any of the sequential time points for any of the RNA species. For mosquitoes fed a sugar meal at day 30, three time point comparisons differed significantly. Between day 30 and day 31 samples, the copy numbers of mRNA ($P = 0.0317$) and vcRNA ($P = 0.0057$) differed significantly. Between day 32 and day 33 the copy numbers of vcRNA differed significantly ($P = 0.0183$). None of the vRNA copy numbers differed statistically (Table 3.14).

Copy numbers of RNA species in ovaries were then compared between those mosquitoes fed a blood meal at day 30 to mosquitoes fed a sugar meal at day 30. Four time points were statistically different. At day 31, the copy numbers of vcRNA in ovaries from blood-fed mosquitoes differed statistically from those of sugar fed mosquitoes ($P = 0.0169$). At day 32, the copy numbers of vRNA and vcRNA in ovaries from blood-fed mosquitoes differed statistically from those of sugar fed mosquitoes ($P = 0.0360$ and 0.0322, respectively). At day 34, the copy numbers of vcRNA in ovaries from blood-fed mosquitoes differed statistically from those of sugar fed mosquitoes ($P = 0.0022$). In contrast, the copy numbers of mRNA in the ovaries of blood and sugar fed mosquitoes did not differ significantly (Table 3.14).

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Table 3.14 Statistical comparison between time points and second meal treatments of infected *Aedes triseriatus* mosquitoes used in quantitative real-time PCR analysis. Each statistical analysis is based on the average from Q-PCR amplification of 10 individual ovaries. Actin values are not given.

	Ovary			Midgut		
Day to Day	mRNA	vRNA	vcRNA	mRNA	VRNA	vcRNA
Day 1 to 3	NS	NS	NS	NS	NS	NS
Day 3 to 6	0.0002	NS	<0.0001	NS	NS	NS
Day 6 to 9	NS	NS	NS	NS	NS	NS
Day 9 to 14	NS	NS	NS	NS	NS	NS
Day 14 to 21	NS	NS	NS	NS	NS	NS
Day 21 to 28	NS	NS	NS	NS	NS	NS
Day 28 to 30	NS	NS	NS	NS	NS	NS
Day 30 to 31 blood-fed	NS	NS	NS	NS	NS	NS
Day 31 to 32 blood-fed	NS	NS	NS	NS	NS	0.0202
Day 32 to 33 blood-fed	NS	NS	NS	NS	0.0198	NS
Day 33 to 34 blood-fed	NS	NS	NS	NS	0.0108	NS
Day 30 to 31 sugar-fed	0.0317	NS	0.0057	NS	NS	NS
Day 31 to 32 sugar-fed	NS	NS	NS	NS	NS	NS
Day 32 to 33 sugar-fed	NS	NS	0.0183	NS	NS	NS
Day 33 to 34 sugar-fed	NS	NS	NS	0.0136	NS	NS
Blood-fed vs. sugar-fed						
Day 31	NS	NS	0.0169	0.0497	NS	0.0193
Day 32	NS	0.0360	0.0322	NS	NS	NS
Day 33	NS	NS	NS	NS	0.0023	NS
Day 34	NS	NS	0.0022	NS	NS	NS

Values given are calculated P values from Tukey's and Scheffe's t-test.
NS = Not significant

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In the midgut samples between day 1 and day 30, there were no significant differences between any of the time points for any of the RNA species. Following a blood meal at day 30, there were significant differences at three time points. Between day 31 and day 32, the vcRNA copy numbers in midguts from blood-fed mosquitoes differed statistically from those of sugar fed mosquitoes (P = 0.0202). Between day 32 and day 33 as well as between day 33 and day 34, the copy numbers of vRNA in midguts from blood-fed mosquitoes differed statistically from those of sugar fed mosquitoes (P = 0.0198 and 0.0108, respectively). In the mosquitoes fed a blood meal at day 30, there were no statistically significant differences in mRNA copy numbers in the midguts. For those mosquitoes fed a sugar meal at day 30, the copy numbers of mRNA differed significantly (P = 0.0136) between day 33 and day 34. None of the vRNA and vcRNA copy numbers were differed significantly.

Midgut copy number of viral RNA species were then compared in mosquitoes fed a blood meal or a sugar meal at day 30. Three time points were statistically different. At day 31, the copy number of mRNA and vcRNA in midguts from blood-fed mosquitoes differed statistically from sugar fed mosquitoes (P = 0.0497 and 0.0193, respectively). At day 33, the copy number of vRNA in midguts from blood-fed mosquitoes differed statistically from sugar fed mosquitoes (P = 0.0023) (Table 3.14).

D. DISCUSSION

The remarkable ability of LAC virus to be transovarially transmitted in *Ae. triseriatus* mosquitoes and to survive adverse environmental conditions within the diapausing embryos suggests co-regulation of viral transcription and replication with that of host metabolic activity. The studies described in this research were conducted to investigate the possible co-

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regulation of viral transcription with different host physiological states. Specific attention was given to four main questions: when does virus enter the ovaries following an infectious blood meal; how does the level of virus activity in mosquito tissues change as metabolic activity changes during ovary quiescence and ovary reactivation; how does the establishment of a persistent infection in mosquito tissues and cell cultures correlate to virus activity; and could the potential co-regulation of viral and host transcription act as a determinant of efficient transovarial transmission? A persistent infection results in a noncytopathic infection for the life of the mosquito or mosquito cells and can occur within 24 hours post infection, at which time mRNA levels remained high while genome and N protein synthesis were severely reduced (Rossier et al., 1988). The correlation between the copy numbers of viral RNA species and virus titer was also explored in this study.

Previous studies utilized either immunofluorescence microscopy (Beaty and Thompson, 1978; Miller et al., 1979; Chandler et al., 1998) or reverse transcription-polymerase chain reaction (RT-PCR) (Chandler et al., 1996) to examine virus infection and replication after a blood meal. The studies described here were designed to use a new technique, quantitative real-time polymerase chain reaction (Q-PCR), which is more sensitive than traditional detection methods for determining levels of vRNA, mRNA, and vcRNA (Freeman et al., 1999). The use of fluorogenic probes and continuous monitoring during real-time analysis also allows for accurate quantification and discrimination between target molecules.

Comparison of the sensitivity of traditional immunofluorescence, RT-PCR, and Q-PCR revealed that by day 9, disseminated infection could be detected in head tissue in mosquitoes by immunofluorescence, and infection of ovaries and midguts could be detected

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by RT-PCR and Q-PCR by day 1. Immunofluorescence detected the smallest proportion of infected individuals overall. This was due to the length of time necessary for infection to disseminate from the midgut to head tissue. RT-PCR detected infection in more mosquitoes than immunofluorescence, but not in 100% of mosquitoes at all time points. The lower limit of detection of RT-PCR was demonstrated to be between $1x10^7$ and $1x10^8$ copies for each RNA species in a 25-cycle amplification (Figure 3.5). Q-PCR detected LAC RNA species in every mosquito at all time points, except for vRNA in 7 mosquitoes at day 1 and 4 mosquitoes at day 3. The lower detection limit for Q-PCR was approximately $4.1x10^4$ copies (Table 3.12). The ability to detect each LAC RNA species by Q-PCR was approximately 244 to 2,440 times greater than traditional RT-PCR.

The results from Q-PCR analysis from infected cell cultures demonstrated that each LAC RNA species could be detected at each time point and could be quantitated per cell. Increases in virus titer immediately following infection corresponded to increases in copy number of each RNA species. As virus titers in the supernatant decreased or plateaued, copy numbers of each RNA species also plateaued.

In persistently infected C6/36 cells, following an initial increase of 0.7 $\log_{10}$ (6-fold) for mRNA, 0.4 $\log_{10}$ (2-fold) for vRNA, and 0.7 $\log_{10}$ (6-fold) for vcRNA between days 1 and 6 (Section C.1 and Figure 3.2), the copy number of LAC mRNA, vcRNA, and vRNA remained relatively stable through day 34 post-infection. Similar results were observed in persistently infected MAT cells, with initial increases of 1.2 $\log_{10}$ (15-fold) for mRNA, 1.5 $\log_{10}$ (33-fold) for vRNA, and 1.7 $\log_{10}$ (45-fold) for vcRNA between days 1 and 6 (Section C.2 and Figure 3.3), followed by stable copy numbers of LAC mRNA, vcRNA, and vRNA through day 34 post-infection. In cytolytic infection of BHK-21 cells, increase in copy

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numbers of each LAC RNA species occurred from 4 to 16 hours post-infection [2.6 $\log_{10}$ (444-fold) for mRNA, 3.3 $\log_{10}$ (2,207-fold) for vRNA, and 3.0 $\log_{10}$ (963-fold) for vcRNA] (Section C.3 and Figure 3.4), followed by stable copy numbers of LAC mRNA, vcRNA, and vRNA through 48 hours post-infection. The titer of infectious virus in cell culture medium of each cell type was correlated with the amount of vRNA calculated from Q-PCR. The r values for C6/36 cells, MAT cells, and BHK-21 cells were $r = 0.7724$, 0.5925, and 0.6699, respectively.

The results from experiments with LAC infected mosquito tissues demonstrated that LAC virus was detectable in ovaries prior to dissemination from the midgut to the head. LAC S vRNA was detectable by RT-PCR by day 1 post-infectious blood meal in 2 of 10 ovaries (Table 3.7) and in 3 of 10 ovaries by Q-PCR (Table 3.9). Neither LAC S segment mRNA or vcRNA were detectable by RT-PCR in any of the ovaries by day 1 post-infectious blood meal (Table 3.7). Both RNA species were detectable in all of the ovaries by day 1 post-infectious blood meal by Q-PCR (Table 3.8 and 3.10) suggesting that Q-PCR is more sensitive than traditional RT-PCR. Because it has been thought that dissemination of the virus from the midgut must occur before infection of the ovaries, these results in combination with those of a previous study showing virus in ovaries by day 2 post-infectious blood meal (Chandler et al., 1998) suggest an alternate mechanism of virus entry into the ovary. This observation is probably a laboratory artifact resulting from feeding on a high-titered artificial blood meal, which in this study was in excess of 8.00 $\log_{10}$ $TCID_{50}$/ml. This does not accurately reflect the titer of virus a mosquito would ingest from a natural vertebrate host; chipmunks and squirrels develop viremia titers between 1.5 to 4.5 $\log_{10}$ $SMICLD_{50}$/0.02 ml (Pantuwatana et al., 1972). In addition, dissection of the ovaries did not remove the

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tracheoles, common oviduct, or ampulla, which may have resulted in additional virus or virus-infected cells inflating actual quantities of LAC RNAs present.

Virus could infect these tissues by spreading from the bursa or via direct spread from the midgut to the ovaries through the tracheoles (Chandler et al., 1998). Baculoviruses are able to infect tracheoles of lepidopteran insects shortly after oral infection (Kirkpatrick et al., 1994) and that Sindbis virus (family Togaviridae, genus *Alphavirus*) can infect and replicate in tracheole cells after a blood meal (Bowers, 1991; K. Myles, in preparation). Another potential mechanism allowing for virus infection of ovaries prior to dissemination from the midgut include a "leaky" midgut phenomenon and permeabilization of the ovaries. It has been demonstrated that feeding *Culiseta melanura* mosquitoes artificial blood meals can result in "leaky" midguts. This leaky midgut would allow for rapid systemic infection prior to normal routes of dissemination (Weaver and Scott, 1990; Weaver et al., 1991). Shortly after ingesting a blood meal the ovaries of the mosquito become permeable. The intercellular spaces of the follicular epithelium and the ovarian sheath become larger in preparation for the transport of vitellogenin from fat body and other nutrients from the blood meal into the developing oocyte (Clement and Boocock, 1984). This permeabilization allows for rapid uptake of large amounts of nutrients into the ovary and developing oocyte (Anderson and Spielman, 1972; Koller et al., 1989). Some arboviruses have been shown to escape via a "leaky" midgut prior to infection of and dissemination from the midgut (Hardy, 1988; Woodring et al., 1996). A "leaky" midgut could permit virus to infect the ovaries during import of nutrients into the highly permeabilized ovary. Fat body synthesis of vitellogenin occurs following a blood meal. Infection of fat body by the virus occurs following dissemination or escape from the midgut and FA analysis has shown large amounts of viral

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antigen in fat body tissue (Beaty and Thompson, 1978). Infection of ovaries may also occur during the transport of vitellogenin from the infected fat body to the ovary.

The copy numbers of each LAC virus RNA species in mosquito ovaries were determined following an infectious blood meal. LAC RNA was detected at all time points in all mosquitoes following the infectious blood meal. LAC mRNA (Table 3.8) and vcRNA (Table 3.10) copy numbers remained stable at days 1 and 3 post-infectious blood meal while vRNA increased 0.7 $\log_{10}$ (5-fold) between days 1 and 3 post-infectious blood meal (Table 3.9). Decreases of approximately 2.8 $\log_{10}$ (738-fold) in mRNA copy number (Table 3.8), 3.0 $\log_{10}$ (987-fold) in vcRNA copy number (Table 3.10), and 0.4 $\log_{10}$ (3-fold) in vRNA (Table 3.9) copy number were observed between day 3 and day 6 post-infectious blood meal. This most likely correlated with oviposition, and the dramatic decrease in amount of tissue visually observed in dissected material resulting from the oviposition of eggs with the LAC RNAs. By day 9 post-infectious blood meal, the copy number of mRNA increased by 2.6 $\log_{10}$ (444-fold) (Table 3.8) and the copy number of vRNA increased 2.5 $\log_{10}$ (323-fold) (Table 3.9), and by day 14, the copy number of vcRNA increased 2.6 $\log_{10}$ (366-fold) (Table 3.10). Each RNA species remained relatively stable at those amounts through day 30 post-infectious blood meal (Figure 3.6). Presence of LAC RNAs remained high throughout the time course of infection in the ovaries with a transient decrease only at day 6.

Following a second meal of either noninfectious blood or sugar at day 30, LAC RNA synthesis was again monitored. Within 48 hours following a second blood meal, the copy numbers increased 1.4 $\log_{10}$ (27-fold) for mRNA (Table 3.8), 0.7 $\log_{10}$ (5-fold) for vcRNA (Table 3.10), and 0.7 $\log_{10}$ (5-fold) for vRNA (Table 3.9), and by 96 hours synthesis had declined to levels observed prior to the second feed (Figure 3.6). The decrease in LAC

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RNAs probably correlated to the female beginning to oviposit her batch of eggs. Following a sugar meal, a 24-hour lag in mRNA and vcRNA synthesis was observed, but within 72 hours after the sugar meal the copy numbers of mRNA had increased by 1.3 $\log_{10}$ (20-fold) (Table 3.8) and vcRNA had increased by 1.2 $\log_{10}$ (15-fold) (Table 3.10). By 96 hours, mRNA and vcRNA were reduced to amounts observed prior to the second feed (Figure 3.6). Following a sugar meal, a decrease of 0.3 $\log_{10}$ (2-fold) was observed within 48 hours, but within 96 hours after the sugar meal the copy number of vRNA increased 0.4 $\log_{10}$ (2.5-fold) (Table 3.9) and had returned to amounts observed prior to the second feed (Figure 3.6). Throughout the course of time following the second feed, the copy numbers of LAC RNAs from mosquitoes ingesting a second blood meal were higher than in mosquitoes ingesting a sugar meal except for the 96 hour time point for vcRNA (Figure 3.6). Also, the copy number of vRNA remained higher than that of either mRNA or vRNA, suggesting that the most stable LAC RNA species in infected tissues was the vRNA. vRNA may potentially be protected in virions resulting in the stability observed.

Interestingly, the sugar meal did result in an increase of LAC virus mRNA and vcRNA synthesis, but not vRNA synthesis, in the mosquito ovaries. The sugar meal contained green food coloring and was administered through a parafilm membrane that had been "scented" with human skin. Dissection of the sugar fed mosquitoes revealed that at the 24 hour and 48 hour time points, approximately 50% of the mosquitoes had the green-colored sugar meal in their midguts rather than the crop. The scent of the "host" apparently played a role in digestion as well as ovary metabolic activity, suggesting that hormonal regulation of blood feeding and egg production may also regulate LAC virus transcriptional activity (mRNA and vcRNA) but does not regulate LAC virus replication activity (vRNA).

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LAC virus transcription and replication in the midgut of infected mosquitoes fluctuated following ingestion of an infectious blood meal until a persistent infection was established. Synthesis of LAC RNAs was detectable in midguts by day 1 post-infectious blood meal. Copy number of mRNA decreased 2.1 $\log_{10}$ (137-fold) by day 14, and remained stable at that amount through day 30 post-infectious blood meal (Table 3.11 and Figure 3.7). Copy number of vcRNA decreased 1.7 $\log_{10}$ (54-fold) by day 3, increased 1.5 $\log_{10}$ (35-fold) by day 6, and remained relatively stable at that amount through day 30 post-infectious blood meal (Table 3.13 and Figure 3.7). Copy number of vRNA increased 0.8 $\log_{10}$ (6-fold) by day 3, decreased 1.2 $\log_{10}$ (14-fold) by day 6, increased 1.0 $\log_{10}$ (10-fold) by day 9, and remained relatively stable at that amount through day 30 post-infectious blood meal (Table 3.12 and Figure 3.7).

Following a second noninfectious blood meal at day 30, the copy numbers of both LAC mRNA and vcRNA decreased within 24 hours (mRNA decreased 1.3 $\log_{10}$ (21-fold), vcRNA decreased 1.8 $\log_{10}$ (58-fold)) (Tables 3.11 and 3.13). Between 48 hours and 96 hours after the second feed, the copy numbers of mRNA and vcRNA remained below that observed prior to the second feed (Figure 3.6). The copy number of LAC vRNA decreased 1.8 $\log_{10}$ (59-fold) within 72 hours after the feed (Table 3.12) and by 96 hours remained below the quantities observed prior to the second feed (Figure 3.7). Following a sugar meal at day 30, the copy numbers of both LAC mRNA and vcRNA decreased within 72 hours after the feed (mRNA decreased 1.0 $\log_{10}$ (11-fold), vcRNA decreased 1.1 $\log_{10}$ (13-fold)) (Tables 3.11 and 3.13) and by 96 hours after the second feed remained below the quantities observed prior to the second feed (Figure 3.7). Following a sugar meal at day 30, the copy number of LAC vRNA increased slightly within 24 hours after the feed. By 72 hours after

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the sugar meal vRNA decreased 1.2 $\log_{10}$ (14-fold) (Table 3.12) and by 96 hours after the sugar feed remained below the quantities observed prior to the second feed (Figure 3.7). Together this demonstrated LAC RNA synthesis had returned to amounts equivalent to or just below that observed prior to the second feed. Throughout the time course of infection, the quantity of vRNA remained higher than that of either LAC mRNA or vcRNA, suggesting that the most stable form of LAC RNA species was the vRNA.

The most likely explanation for the decrease in amounts of LAC RNA observed in the midguts following a second feed was the dramatic physiologic changes occurring in the midgut to allow for digestion of the blood or sugar meal (Clements, 1992; Jacobs-Lorena and Oo, 1996; Rosomer, 1996). The increase in transcription and translation of digestive enzymes (trypsins, etc.) and proteins (peritrophic matrix) necessary for digestion probably removed substrates for macromolecular synthesis (amino acids and ribonucleotides) from the pool necessary for LAC virus RNA synthesis. Also, the decreased quantities of LAC RNA species in midgut following a second feed could result from the collapse and elongation of midgut cells after a blood meal, pushing virus out of the cells into the lumen of the gut or into the hemolymph. The number of LAC virions and the quantity of vRNA in the midgut would decrease, reducing the quantity of template for transcription of mRNA and vcRNA. Decline in RNA copy number could also be due to degradation rather than a lack of synthesis.

The ability of LAC virus to rapidly infect and replicate in ovaries following an infectious blood meal may allow efficient transovarial transmission. The observation of a decrease in the quantities of LAC mRNA, vcRNA, and vRNA following oviposition of eggs suggests that the potential amount of each RNA species present in the newly-deposited eggs is approximately 1,000 copies of LAC mRNA, less than 10 copies of LAC vRNA, and

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1,000 copies of LAC vcRNA. Thus, there is a potential for eggs from the first egg batch after an infectious blood meal to be transovarially infected. This observation may be due to laboratory artifact; however, high titered blood meals are generally not obtained in nature and the LAC vRNA was not detected in some ovaries until day 6, by which time most of the mosquitoes had already oviposited. Only 30% of the mosquito ovaries on day 1 and 50% of the mosquito ovaries on day 3 had detectable levels of LAC vRNA. The detection of high copy numbers of LAC mRNA and vcRNA at the same time points suggests that transcription occurred at high rates but replication of the genome is slower, thus-infectious LAC virus would not likely be present in the first batch of eggs. This would most likely be the result of a lag between primary transcription and switching to full-length genome replication. When the ovaries became quiescent following oviposition and prior to a second blood meal, the copy numbers of the three LAC RNA species stabilized. Thus it appears that as the mosquito ovary regulates metabolic activity in response to the presence or absence of a blood meal, LAC RNA transcription and replication are co-regulated. As the mosquito ovary becomes metabolically active following a second blood meal, LAC RNA transcription and replication are upregulated (Figure 3.6). This upregulation following a meal could result in increased titers of LAC virus and enhanced transovarial transmission. The remarkable relationship between host metabolic activity and LAC transcription and replication would allow for persistence and efficient transovarial transmission and cause few adverse effects to the mosquito host.

Comparison of the second blood meal to a sugar meal at day 30 in ovaries revealed that the quantities of each LAC RNA species were lower in the sugar-fed mosquitoes. It appeared that stimulation of LAC mRNA and vcRNA synthesis in sugar-fed mosquitoes

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(after a 24-hour lag) was comparable to that of blood-fed, but vRNA synthesis was not stimulated. Peaks in LAC RNA quantities at 48 and 72 hours post-blood meal (Figure 3.6) correlate to the peak RNA and protein levels observed at 36-48 hours and 72 hours in *Aedes aegypti* following a blood meal (Banks et al., 1994). This could be the result of large amounts of RNA present in ovaries, thus providing targets for scavenging of 5' nonviral sequences to prime LAC mRNA transcription. In midguts, the decrease in LAC RNA occurred slower in sugar-fed mosquitoes than in blood-fed mosquitoes (Figure 3.7). This would most likely be the result of the midguts not being as metabolically active following a sugar meal. Fewer digestive enzymes would be necessary to digest a sugar meal. This would allow macromolecules normally diverted for the synthesis of digestive enzymes to be used by LAC virus in transcription and replication. A decrease in LAC RNA was still observed and may be the result of morphological changes in midgut cells pushing virus out of cells or deceases in macromolecules necessary for LAC virus transcription and replication during the gradual digestion of a sugar meal by the midgut. Comparison of the copy numbers of each LAC S segment RNA species between ovaries and midguts of infected mosquitoes provided a second blood meal at day 30 revealed that the quantities of each RNA species in the ovaries exceeded that of the midguts, with the exception of vcRNA at day 34 (96 hours post-second blood meal). The increase in metabolic activity of ovaries following a blood meal allowed LAC virus transcription and replication to exceed that of midguts, even though in tissue of much smaller size.

Comparison of this study with the previous study (Chandler et al., 1996) revealed several similarities. Examination of the copy numbers of each LAC RNA species in individual ovaries (Tables 3.8-3.10) revealed that several individual ovaries contained

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quantities of RNAs that would have been undetectable, or only slightly detectable, by traditional RT-PCR (detection limit of $1x10^7$ to $1x10^8$). Indeed, the majority of individual ovaries between day 1 and day 9 contained vRNA quantities below detection limits of RT-PCR (Table 3.9). Infection of ovaries did result in a stabilization of LAC RNA during ovary quiescence and upregulation following a second blood meal. Additionally, several individual ovaries from sugar-fed mosquitoes contained quantities of LAC RNAs above RT-PCR detection limits, similar to mosquito controls not refed, but exposed to a vertebrate host, in the previous study (Chandler et al., 1996). In midguts, the quantities of mRNA and vcRNA gradually decreased following infection, while vRNA remained stable. This result was observed in the previous study where vRNA was detectable in midguts throughout infection while detectable amounts of mRNA and vcRNA decreased. During ovary quiescence, LAC mRNA and vcRNA can be detected with the sensitivity of Q-PCR.

One potential drawback to the Q-PCR assay to quantitate LAC virus RNAs is the unknown turnover rate and half-life of the RNA species. Analysis at any given time point following infection may not provide an accurate representation of the actual copy number depending on the turnover of LAC virus RNAs in the tissues or cells. The increases or decreases observed may reflect only transient fluctuations in LAC RNA copy number caused by environmental condition, intrinsic factors of the mosquito, or human manipulation.

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CHAPTER 4: SUMMARY AND CONCLUSIONS

Arthropod-borne viruses are major causes of morbidity and mortality in both human and animal populations. Recent emergence and re-emergence of arboviruses worldwide have increased the global public and veterinary health threats to human and animal populations. Understanding the molecular biology of an arbovirus, as well as the mechanisms that allow interactions between an arbovirus and its arthropod vector, are critical for creating new surveillance strategies to predict disease emergence or reemergence, for developing novel control strategies to block transmission, and for reducing overall disease incidence. In this dissertation, the molecular determinants of LAC virus infection and replication in *Ae. triseriatus* mosquitoes and mosquito and mammalian cells were investigated.

LAC virus infection of and replication in mosquitoes and mosquito cells results in persistent infection, constant virus shedding, and no detectable adverse effects on the cells. In contrast, LAC virus infection of and replication in mammalian cells results in cytopathic effects and cell death. Several mechanisms have been proposed to account for the different outcomes of infection in the two disparate systems. Encapsidation of viral mRNA by the N protein in C6/36 mosquito cells (Kolakofsky and Hacker, 1990), and differential cap scavenging specificity in mosquito and mammalian cells (Dobie et al., 1997) have been hypothesized as mechanisms for the establishment of persistence in mosquitoes and mosquito cells.

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The first goal of this research was to study the molecular basis and determinants of cap scavenging by LAC virus *in vitro* and *in vivo*. Initial analysis of cap scavenging in C6/36 *Ae. albopictus* cell cultures revealed that LAC virus mRNA was present in cells and infectious LAC virus was present in cell culture medium through 42 days post-infection (Chapter 2, Section C.1), demonstrating persistent infection with ongoing transcription and replication. LAC mRNA species were assayed to determine the identity of the 5' nonviral sequences. The 5' nonviral sequence 5'-CAGTTACGTT-3' (designated cap A) predominated throughout infection (52.8%). A second 5' nonviral sequence, 5'-TTGGTCGTCATCG-3', (designated cap B) comprised 4.0% of the scavenged sequences, and 27.8% were heterogeneous.

Similar results were obtained in MAT *Ae. triseriatus* cell culture. LAC virus transcription and replication were detected throughout infection (Chapter 2, Section C.2). As was observed in C6/36 cell culture, cap A was the preferred 5' nonviral sequence, comprising 78.7% of the scavenged sequences. Cap B comprised 4.5% of the scavenged sequences, and only 4.5% were heterogeneous

In BHK-21 baby hamster kidney cells, LAC virus transcription and replication were detected throughout infection (Chapter 2, Section C.3). Cap A was the predominant 5' nonviral sequence scavenged, comprising 73.5% of the scavenged sequences. Cap B was detected only two times, once at 12 hours and once at 36 hours post-infection, and only 17.4% of the remaining scavenged 5' nonviral sequences were heterogeneous.

Several 5' nonviral sequences were observed in more than one cell type (Chapter 2, Table 2.12). Caps A and B were observed to prime viral transcription in each cell type. Five additional 5' nonviral sequences were observed to prime transcription in both C6/36 and

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MAT cells and four other 5' nonviral sequences were observed to prime transcription in both C6/36 and BHK-21 cells. All other 5' nonviral sequences differed between MAT and BHK-21 cells.

The predominant 5' nonviral sequence obtained and utilized by LAC virus in mosquito cell culture is identical to that in mammalian cell culture. These results, along with the observation of several other 5' nonviral sequences in common between mosquito cells and mammalian cells, suggest that cap scavenging targets a specific or abundant message, group of messages, or a capped mRNA motif that is conserved between mosquitoes and mammals. Thus, cap scavenging is probably not the principal mechanism responsible for the different outcomes observed during infection in the two disparate systems. However, the unique caps observed during mammalian cell infection but not in mosquito cell infection may be involved in the different outcomes and the donor mRNAs should be identified. Identification of the unique mRNA species scavenged for 5' nonviral sequences in BHK-21 cells may provide insight into the mechanism of cytolytic infection in mammalian cells.

Previous studies of cap scavenging revealed a preference for the 5' nonviral sequence 5'-CCACTCGCCACT-3' in mosquito cell culture, in *Ae. triseriatus* embryos during embryogenesis, and during early diapause and post-diapause (Dobie et al., 1997). An alternate 5' nonviral sequence, 5'-AGGAAAAGTAGGT-3', was observed during late embryogenesis and during early and deep diapause. Dobie et al. (1997) also observed that the GC content of 5' nonviral sequences was 64%, and that the majority of 5' nonviral sequences ended in 3' terminal T residues. In addition, the majority of associated viral mRNA sequences contained a T to C transition at position 11. In the current study, a preference for a specific 5' nonviral sequence, in this case 5'-CAGTTACGTT-3' with

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secondary preference for 5'-TTGGTCGTCATCG-3', was observed, the majority (85.7%) of 5' nonviral sequences also ended in a 3' terminal T residue and the associated viral mRNA contained a T to C transition at position 11 (86.7%). However, in contrast to the previous study, the GC content was only 42.5% and the predominant cap, 5'-CCACTCGCCACT-3', was not observed. A different 5' nonviral sequence predominated throughout infection. The reasons for the different 5' nonviral sequence predominance between the two studies remains to be determined. Passage history of the virus used in this study and the previous study may have resulted in the different outcomes due to mutations in the RNA-dependent RNA polymerase. Alternatively, differences in RNA extraction (RNAMATRIX versus isopropanol and ethanol precipitations) or cDNA purification (GlassMax versus QIAquick) protocols may have contributed to the differences observed. It is possible that the use of a binding matrix in RNA extraction may have selected for RNAs with higher GC content than a total RNA extraction protocol. Slight differences in cell culture passage history may have also contributed to the differences between the two studies. The large number of 5' nonviral sequences determined in this study, along with the observations that similar 5' nonviral sequences were obtained from cell cultures and mosquito tissues, suggest that these results are an accurate representation of cap scavenging by LAC virus.

Cap scavenging preferences were also observed in mosquito and mammalian cell cultures infected with other viruses in the CAL serogroup. Mosquito and mammalian cells infected with either SSH or TAH virus were examined for capped primer sequences at either 33 days (mosquito cells) or 24 hours (mammalian cells) post-infection. Limited analysis revealed that, similar to LAC virus infection, cap A predominated during SSH and TAH virus infection (70.0% to 96.0% depending on cell type for SSH and 30.0% to 75.4%

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depending on cell type for TAH) (Chapter 2, Section C.5). Interestingly, cap B was not observed on mRNA of either SSH or TAH virus; however, a different 5' nonviral sequence, cap C 5'-TCACTCCC-3', was observed to prime viral transcription of SSH virus in C6/36, MAT, and BHK-21 cells, although with much less frequency than cap A. Cap C primed TAH virus transcription more frequently than SSH virus transcription in each cell type. The 5' nonviral sequence characteristics (GC content, 3' terminal T, and T to C at position 11) were similar to those associated with LAC virus transcription. There appeared to be no difference in 5' nonviral sequence preference for cap A between LAC virus and other viruses of the CAL serogroup. However, there were differences in the lack of scavenging of cap B and a preference for cap C by SSH and TAH viruses.

To determine if passage history altered cap scavenging specificity, several LAC virus isolates were characterized for cap scavenging in mosquito ovary and midgut tissue (Chapter 2, Section C.6). Two transovarially passed viruses, two low passage human isolates, and one high passage field isolate were compared to LAC virus wt10, the virus isolate used during cell culture infection. Cap A comprised 89.2% of all 5' nonviral sequences scavenged by the different LAC virus isolates, 91.4% of those priming viral transcription in ovaries, and 87.2% of those priming viral transcription in midguts. Cap B was also observed at low frequency (2.7%), but only on viral mRNAs from midguts of mosquitoes infected with the low passage human isolate (LAC H78mp1) or the high passage field isolate (LAC TCP6). Cap C was also observed at low frequency (2.7%), but only on viral mRNAs from ovaries of mosquitoes infected with the LAC wt10. The 5' nonviral sequence heterogeneity was also low in mosquito tissues (4.1% overall, 2.9% in ovaries, and 5.1% in midguts). The 5' nonviral sequence characteristics observed in the cell culture experiments were also observed in

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mosquito tissue. The 5' nonviral sequences had GC content of 41.6% (41.6% in ovaries, 41.7% in midguts), the majority (97.3%) ended in a 3' terminal T residue (100% in ovaries, 94.9% in midguts), and all 5' nonviral sequences were associated with the T to C transition at position 11 of the viral mRNA, although the transition observed is most likely the actual sequence of the viral mRNA and not a specific mutation repeatedly observed. From these results, it appears that different LAC isolates have similar cap scavenging preferences independent of passage history or isolation, and that cap scavenging in mosquito tissues is more targeted to a specific message or capped mRNA motif. Dobie et al., (1997) demonstrated that during embryogenesis and diapause of *Ae. triseriatus* embryos, 5' nonviral sequence homogeneity was more pronounced than in cell culture, as observed in mosquito tissues in this study, although embryo tissue was not examined.

The results from the infected cell culture and mosquito experiments suggest that in *Ae triseriatus* ovaries, midguts, and cell culture, LAC virus cap scavenging is restricted, with a specific 5' nonviral sequence predominating. This restricted approach to cap scavenging may allow for efficient infection of tissues and cells, and in ovaries, may aid in efficient transovarial transmission of LAC virus. By targeting specific mRNA species that are abundant or for which the loss of the 5' 7-methyl guanosine cap does not affect expression, LAC virus would not reduce fitness of mosquito cells, mosquito tissues, or embryos. However, the weak signal observed by northern analysis and abundance of SIN caps in co-infection suggests that cap scavenging is not based upon abundance of messages, but rather specificity of the polymerase for particular sequences or secondary structures. If fitness of infected ovary tissue and embryos is not reduced, efficient transovarial transmission could occur without any adverse effects to the mosquito.

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RT-PCR analysis of LAC S segment mRNAs from infected cells revealed that each predominant 5' nonviral sequence observed in cell culture and mosquito tissues (caps A, B, and C), as well as the two predominant 5' nonviral sequences (caps 1 and 2) from the previous study (Dobie et al., 1997), were used to prime transcription of LAC virus mRNA (Chapter 2, Section C.7). Visual comparison of amplification products after agarose gel electrophoresis confirmed that use of the 5' nonviral sequences observed in this study as forward primers resulted in amplification products with much stronger intensity than those from the previous study (Chapter 2, Figure 2.4). Although not quantitative in nature, the results do suggest that in the present study, caps A, B, and C were more prevalent than caps 1 and 2 based on intensity of amplification products.

Northern blot analysis of total RNA and mRNA from mosquito and mammalian cell culture with probes specific for cap A, B, and C was not very productive. A faint band was detected in BHK-21 cell total RNA using a probe complementary to cap B (Chapter 2, Section C.10). Sequence analysis of host RNA amplified by RT-PCR with primers derived from predominant viral caps resulted in detection of several mitochondrial and ribosomal RNA genes (Chapter 2, Section C.11). The low GC content and short length of the 5' nonviral sequences used to design radiolabeled probes and PCR primers complicated these efforts, thus RNAs identified may or may not be the actual host donors involved. The short length of the 5' nonviral sequences also complicated direct BLAST searches of the *Anopheles gambiae* and *Drosophila melanogaster* genomes. Numerous genes were identified, but with mismatch errors and limited targeting of the input query to the 5' end of mRNA sequences, the results were not precise. Future analyses using improved detection

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and amplification methods may reveal the host messages targeted by the viral RNA-dependent RNA polymerase to prime viral transcription.

It has yet to be determined if the messages scavenged by LAC virus are in response to infection, if they are abundant messages or specific targets, or if secondary structure of the 5' terminus of the message plays a role in the specificity of the polymerase and the targeting observed in cap scavenging experiments. It is possible that the endonuclease activity cleaves host mRNAs in proximity (prior to or after) secondary structures near the 5' ends. The relative abundance and function of host mRNAs targeted by cap scavenging was not determined and requires further study. Additionally, it has yet to be determined if the 5' nonviral sequences observed to prime transcription of S segment mRNA also prime transcription of M and L segment mRNAs. Clearly, targeted cap scavenging reflects a highly evolved host-virus association. Specific residue(s) were targeted as the 3' terminus of 5' nonviral sequences and these residues have considerable complementarity to the first 10 to 15 nucleotides of the viral sequence.

Co-infection of tomato spotted wilt virus and alfalfa mosaic virus by Duijsings et al. (1999; 2001) revealed that a bunyavirus could scavenge 5' sequences to prime viral transcription from an unrelated virus during co-infection, and served as a model for LAC SIN co-infection experiments. In these studies, the hypothesis was tested that LAC virus would scavenge abundant 5' sequences to prime viral transcription from the mRNA of an unrelated virus during co-infection (Chapter 2, Section C.9). Sindbis virus and LAC virus co-infection studies revealed that scavenged 5' sequences were more heterogeneous in C6/36 cells (59.2%) than in MAT cells (7.4%) and that cap A sequence still predominated, but was scavenged less frequently in C6/36 (38.0%) cells than in MAT cells (82.4%). A SIN virus

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subgenomic mRNA sequence was found on a single LAC mRNA (1/71, 1.4%) at day 3 following simultaneous co-infection in C6/36 cells; the sequence was scavenged from either the first or second subgenomic mRNA of double subgenomic SIN virus. Selection for and specific targeting for LAC mRNAs containing SIN capped primer sequences was examined by RT-PCR analysis. In MAT cells, LAC virus did not appear to scavenge 5' sequences from SIN virus with much regularity. In C6/36 cells, however, SIN 5' sequences are scavenged at nearly every time point following simultaneous co-infection and at all time points following co-infection after the establishment of LAC virus persistence (Chapter 2, Figure 2.9). Overall GC content remained at approximately 40% and a large proportion of the scavenged 5' sequences contained a 3' terminal T residue and were associated with viral mRNA sequences containing the T to C transition at position 11. The results suggest that in MAT cells, LAC virus infection is more stable and specific than in C6/36 cells, and in MAT cells is not affected by SIN virus infection to the same extent as in C6/36 cells. This could be the result of MAT cells being derived from the natural vector of LAC virus, *Ae. triseriatus* mosquitoes.

Interestingly, titration of LAC and SIN viruses in the cell culture medium following infection revealed that when co-infected simultaneously, SIN virus transiently inhibited the ability of LAC virus to establish a persistent infection (Chapter 2, Figures 2.5 and 2.7). When LAC virus established a persistent infection prior to SIN virus infection, SIN virus rapidly inhibited LAC virus replication. LAC virus was able to rapidly recover following co-infection and reestablish virus production (Chapter 2, Figures 2.6 and 2.8).

The second goal of this research was to characterize interactions between LAC virus and persistently infected *Ae. triseriatus* mosquitoes. The potential for the co-regulation of

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LAC virus activity with the host cell at different physiologic and metabolic states following an infectious blood meal was investigated.

A real-time quantitative PCR (Q-PCR) assay was designed to detect and quantitate the three LAC virus S segment RNAs (mRNA, vRNA, and vcRNA) in infected mosquito and mammalian cell cultures and in infected *Ae. triseriatus* midgut and ovary tissues. Initial analysis of LAC RNAs in cell cultures revealed that each RNA species could be detected within 1 day post-infection. Following an initial increase in quantities within 3 days post-infection (mosquito cells) or 8 hours post-infection (mammalian cells), the quantities of LAC RNAs remained stable throughout the remainder of infection. In general, the vRNA species were more abundant than mRNA, which was more abundant than vcRNA. Increased virus titers in cell culture medium closely followed increased abundance of RNA species within the cells, and virus titers were correlated with the quantity of vRNA detected. The Q-PCR assay permitted estimation of the copy number of each RNA species per cell (Chapter 3, Sections C.1-C.3).

The Q-PCR approach was used to quantitate RNA extracted from *Ae. triseriatus* ovaries and midguts dissected at predetermined time points post-infectious blood meal (Chapter 3, Section C.6-C.7). Each LAC RNA species was detected in all ovaries as early as day 1 post-infectious blood meal, with the exception of vRNA, which was detected in only 3 of 10 ovaries on day 1 and 6 of 10 ovaries on day 3 (Chapter 3, Section C.6). Between day 6 and day 30 post-infectious blood meal, the abundance of viral RNA species in ovary tissues was: vRNA > mRNA > vcRNA. This relationship was also observed in LAC virus infected cell cultures. Between days 3 and 6 post-infectious blood meal, the quantities of each LAC RNA species decreased (Chapter 3, Figure 3.6). This decrease was most likely due to the

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oviposition of eggs containing LAC RNAs. After oviposition and prior to a second blood meal, the quantities of each RNA species remained stable. Following a second blood meal, quantities of each LAC RNA increased within 48 hours after the feed and then decreased by 96 hours (Chapter 3, Figure 3.6), again most likely correlating to ovipositing of eggs. Following a sugar meal, rather than a blood meal, mRNA and vcRNA increased by 72 hours (after a 24-hour lag period) then decreased to quantities similar to those observed at day 30 following the initial blood meal (Chapter 3, Figure 3.6). The upregulation of virus transcription could have resulted from many factors, including hormonal activation of ovarian macromolecular synthesis (triggered through the scented membrane), distension of the abdomen due to quantity of sugar ingested, or from uptake of the sugar meal into the midgut.

The apparent lack of detectable genomic RNA in ovaries at days 1 and 3 post-infectious blood meal (Chapter 3, Table 3.9) was most likely due to quantities of vRNA in the tissues below detection limits. Synthesis of vRNA would lag behind that of mRNA and vcRNA because these RNA species or their products are necessary for genome replication. Although vRNA was not detectable at the early time points, transcription was occurring in the tissues. Large quantities of mRNA and vcRNA were detectable, reflecting transcription and replication in the ovaries. Similar to previous studies which found virus in the calyx but not in follicles (Chandler et al., 1998), the presence of LAC virus RNA species in ovary tissues prior to dissemination from the midgut may be an artifact from ingestion of a high-titered blood meal ($10^{8.4}$ $TCID_{50}$/ml) and possible infection of accessory sex tissues. Virus could infect these tissues by spreading from the bursa or via direct spread from the midgut to the ovaries through the tracheoles, as previously hypothesized (Chandler et al., 1998). A

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"leaky" midgut (Hardy, 1988; Woodring et al., 1996) may also have allowed the virus to escape prior to infection of and dissemination from the midgut. Contamination of the tissue during dissection is possible, although precautions were taken to prevent such contamination (rinsing twice in sterile PBS, dissection of ovaries prior to midgut dissection, clean slide for each dissection).

As the ovaries entered quiescence prior to a second blood meal (days 9 to 30 post-infectious blood meal), the quantities of LAC RNA stabilized (Chapter 3, Figure 3.6). This stabilization could reflect co-regulation of LAC virus RNA synthesis with host metabolic activity. Following a second blood meal, the upregulation of LAC RNA synthesis in the ovaries produced an increased number of virions that could enhance transovarial transmission, thereby increasing the filial infection rate to the mosquito progeny. The results suggest that the co-regulation of viral transcription and host metabolic activity do condition efficient transovarial transmission.

Each LAC RNA species was detected in all midguts as early as day 1 post-infectious blood meal (Chapter 3, Section C.7). Between day 3 and day 30 post-infectious blood meal, the abundance of viral RNA species in midgut tissues was: vRNA > mRNA > vcRNA. This relationship was also observed in LAC virus infected cell cultures and *Ae. triseriatus* ovaries. Between days 3 and 6 post-infectious blood meal, the quantities of mRNA and vRNA species decreased, while vcRNA increased. After day 9 post-infectious blood meal and prior to a second blood meal, the quantities of each RNA species remained stable (Chapter 3, Figure 3.7). The mRNA and vcRNA remained closely associated in quantity and fluctuation throughout infection. Following a second blood meal or a sugar meal, quantities of each

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LAC RNA species decreased within 48 hours after the feed and remained low through 96 hours (Chapter 3, Figure 3.7).

The most likely explanation for the decrease in amounts of LAC RNA following the second feed was the dramatic physiologic changes occurring in the midgut to allow for digestion of the blood or sugar meal (Clements, 1992; Jacobs-Lorena and Oo, 1996; Rosomer, 1996). Substrates for macromolecular synthesis (amino acids and ribonucleotides) would likely be diverted to transcription and translation of digestive enzymes (trypsins, etc.) and proteins (peritrophic matrix) necessary for digestion, thereby diminishing the pool of macromolecules necessary for LAC virus RNA synthesis. Another potential explanation for the decreased quantities of LAC RNA species in midgut following a second feed would be that as the midgut cells collapse and elongate after a blood meal, virus may be released from the cells into the lumen of the gut or into the hemolymph. This would reduce the number of LAC virions and the quantity of vRNA in the midgut, and would decrease the template for transcription of mRNA and vcRNA.

Several similarities were observed during comparison of this study with the previous study (Chandler et al., 1996). Individual ovaries with low copy numbers of LAC RNA species would have been undetectable, or only slightly detectable, by traditional RT-PCR (detection limit of $1x10^7$ to $1x10^8$ copies, Q-PCR detection limit between $1.0x10^4$ to $1.0x10^5$ copies), including vRNA in the majority of individual ovaries between day 1 and day 9. Stabilization of LAC RNA synthesis in infected ovaries occurred during ovary quiescence and upregulation following a second blood meal, although, LAC mRNA and vcRNA continue to be synthesized and can be detected with more sensitive Q-PCR. LAC RNA species copy numbers in ovaries did increase rapidly following a second blood meal, and

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LAC RNAs in ovaries from several sugar-fed mosquitoes increased above RT-PCR detection limits, similar to mosquito controls exposed to, but not refed on, a vertebrate host, in the previous study (Chandler et al., 1996). Quantities of mRNA and vcRNA in midguts gradually decreased following infection, while vRNA remained stable, similar to observations in the previous study (Chandler et al., 1996).

The Q-PCR analysis is a powerful tool for quantitating copy number of LAC virus RNAs in specific tissues and under varying experimental conditions. Potential co-regulation of LAC virus RNA synthesis with host metabolic activity and with other factors influencing efficient transovarial transmission were investigated quantitatively.

The cap scavenging results demonstrated a specific interaction between LAC virus and host cells. The quantitative analysis of LAC virus copy numbers in mosquito tissues at different physiologic states demonstrated the close relationship between the virus and the vector. These results provide insight into the molecular basis of a remarkable arbovirus vector relationship and provide knowledge and tools to further explore these interactions.

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APPENDIX

I. CELL CULTURE REAGENTS

Leibovitz L-15 10% Growth Medium, 500 ml

450 ml L-15 medium (Sigma)
50 ml fetal bovine serum (heat inactivated)
100 units/ml penicillin
100 μg/ml streptomycin

Leibovitz L-15 20% Growth Medium, 500 ml

400 ml L-15 medium (Sigma)
100 ml fetal bovine serum (heat inactivated)
100 units/ml penicillin
100 μg/ml streptomycin

Leibovitz L-15 Maintenance Medium, 500 ml

495 ml L-15 medium (Sigma)
5 ml fetal bovine serum (heat inactivated)
100 units/ml penicillin
100 μg/ml streptomycin

II. BACTERIAL CULTURE REAGENTS

Luria Bertani (LB) medium, 1 liter

10 g bacto-tryptone (Difco)
5 g yeast extract (Sigma)
10 g NaCl
Adjust pH to 7.5 with NaOH
Bring volume to 1000 ml with distilled water
Autoclave to sterilize

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Luria Bertani (LB) agar, 1 liter

10 g bacto-tryptone (Difco)
5 g yeast extract (Sigma)
10 g NaCl
15 g bacto-agar (Difco)
Adjust pH to 7.5 with NaOH
Bring volume to 1000 ml with distilled water
Autoclave to sterilize

SOC medium, 1 liter

950 ml distilled water
20 g bacto-tryptone (Difco)
5 g yeast extract (Sigma)
0.5 g NaCl
10 ml 250 mM KCl
10 ml filter-sterilized 2 M glucose
10 ml 1 M $MgCl_2$
Autoclave to sterilize

III. GENERAL BUFFERS AND REAGENTS

1X Tris-EDTA (TE), pH 8.0

10 mM Tris-Cl, pH 8.0
1 mM EDTA, pH 8.0

20X SSC, 1 liter

3.0 M NaCl
0.3 M sodium citrate
Adjust pH to 7.0
Bring volume to 1000 ml with distilled water
Autoclave to sterilize

10X MOPS-EDTA Buffer

0.5 M MOPS ph 7.0 (3-[N-Morpholino]propane-sulfonic acid)
0.01 M EDTA, pH 7.5

DEPC-treated water (DEPC-H_2O)

To 1 liter of sterile filtered, distilled and deionized water, add 1 ml of diethylpyrocarbonate (DEPC, Sigma). Shake vigorously, let sit at room temperature for 24 hours. Autoclave to destroy the DEPC.

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RNA Lysis buffer (4 M GTC)

117 ml DEPC treated water
100 g guanidinium thiocyanate (final concentration 4 M)
7 ml 0.75 M sodium citrate solution pH 7.0 (final concentration 25 mM)
10.6 ml 10% sarcosyl (final concentration 0.5%)
1.44 ml 2-mercaptoethanol (final concentration 0.1 M)
Aliquot and store at –20°C

IV. ELECTROPHORESIS REAGENTS

1X TAE buffer

40 mM Tris-acetate, pH 8.0
2 mM EDTA, pH 8.0

Formaldehyde-agarose gel for RNA electrophoresis (50 ml gel)

0.5 g agarose
5 ml 10x MOPS-EDTA buffer
36 ml DEPC-treated water
Dissolve agarose, then cool to 60°C
Add 9 ml 37% formaldehyde

V. MATHEMATICAL AND STATISTICAL FORMULAS.

The equation for the correlation coefficient was:

$$\rho_{x,y} = \frac{Cov\ (X,Y)}{\sigma_x, \sigma_y}$$

where: $-1 \leq \rho_{xy} \leq 1$

and: $Cov\ (X,Y) = \frac{1}{n} \sum_{i=1}^{n} (x_i - \mu_x)(y_i - \mu_y)$

The equation for calculating E scores for BLAST searches was:

$$E = K\, m\, n\, e^{-\lambda S}$$

Where m and n are the length of the respective sequences, K and λ are simply natural scales for the search space size and the scoring system, respectively, and S is the score.

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The SAS code for statistical analysis was:

```
options center ls=80 ps=60;
data USER INPUT;
input time $ sample $ copynumber;
datalines;
USER INPUT
;
proc sort data= USER INPUT;
by time;
proc univariate data= USER INPUT;
var copynumber;
by time;
proc ttest alpha=0.05 h0=0 data= USER INPUT;
class time;
var copynumber;
proc glm alpha=0.05 data= USER INPUT;
class time;
model copynumber=time;
lsmeans time /adjust=tukey;
lsmeans time /adjust=scheffe;
run;
```

VI. COLLABORATIVE RESEARCH WITH DR. MONICA BORUCKI, PUBLISHED PAPERS

"The effect of mosquito passage on the La Crosse virus genotype.:
Monica K. Borucki, Brian J. Kempf, Carol D. Blair, and Barry J. Beaty
Journal of General Virology 2001 Volume 82, pages 2919-2926.

"La Crosse virus: replication in vertebrate and invertebrate hosts.:
Monica K. Borucki, Brian J. Kempf, Bradley J. Blitvich, Carol D. Blair, and Barry J. Beaty
Microbes and Infection 2002 Volume 4, pages 341-350.

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Journal of General Virology (2001), **82**, 2919–2926. Printed in Great Britain

The effect of mosquito passage on the La Crosse virus genotype

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The genetic consequences of passing three different strains of La Crosse (LAC) virus orally and transovarially in *Aedes triseriatus* mosquitoes were examined. Two of the LAC strains (WT LAC and LAC ORI) had been passaged numerous times in cell culture; the third strain (SM1-78) had been passaged only once in suckling mice. Genetic changes were monitored in three regions of the LAC genome after oral infection and dissemination in the mosquito, and transovarial transmission (TOT) of the virus to progeny. Sequence analyses were used to characterize the genetic changes occurring in regions of G1, G2 and N open reading frames (ORFs) during passage. Only one mutation was detected in the G1 ORF of SM1-78 virus after mosquito passage; however, numerous nucleotide and amino acid substitutions were detected in the G1 ORF of WT LAC and LAC ORI (cell culture-adapted viruses). In contrast to G1, the N and G2 ORF sequences examined were stable. Mutations introduced into viral genomes during replication in parental mosquitoes were expressed in progeny mosquitoes following TOT. Genetic diversity of virus populations from a single mosquito was examined by single-strand conformation polymorphisms analysis of the variable region of glycoprotein G1. LAC virus RNA genotype diversity was greatest in virus that infected and replicated in the midgut, and declined as virus disseminated from the midgut and infected ovaries and salivary glands.

Introduction

Arthropod-borne viruses are maintained in nature principally through biological transmission between susceptible vertebrate hosts and haematophagous arthropod vectors (Beaty *et al.*, 1997). Therefore, these viruses must be able to infect and replicate in cells of both vertebrates and invertebrates. The ability of arboviruses to bridge the gap between vertebrate and invertebrate hosts is likely to be a function of their RNA genomes. RNA viruses have extremely high mutation rates (because of the poor fidelity of RNA-dependent RNA polymerases and lack of proofreading enzymes) and are proposed to exist as quasispecies populations (Holland *et al.*, 1982). The quasispecies represents a balance between the expansive influence of mutation and the conservative forces of selection and is envisaged to contain a cloud of genotypes that provide the genetic variability that enables RNA viruses rapidly to exploit new niches (Holland *et al.*, 1982; Eigen & Biebricher, 1988). The quasispecies is an excellent model to explain the remarkable ability of arboviruses to pass rapidly and continuously between vectors and vertebrate hosts. Certainly, passage of arboviruses in laboratory systems rapidly selects for specific virus subpopulations that are more fit in the respective system (de la Torre *et al.*, 1988; Barrett *et al.*, 1990; Novella *et al.*, 1995).

La Crosse (LAC) virus, a member of the family *Bunyaviridae*, genus *Bunyavirus*, is the causative agent of La Crosse encephalitis, the most commonly reported form of paediatric arboviral encephalitis in the United States (Rust *et al.*, 1999; McJunkin *et al.*, 2001). LAC virus is transmitted horizontally and vertically by *Aedes triseriatus* mosquitoes, with infected female mosquitoes transmitting the virus transovarially to their progeny. LAC virus overwinters in the infected eggs, and infected progeny emerge in the spring to

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The GenBank accession numbers for the sequences of the WT LAC, LAC ORI and SM1-78 strains are as follows: nucleocapsid (AF417246–AF417248), G1 (AF417249–AF417251) and G2 (AF417252–AF417254).

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resume the transmission cycle (Grimstad, 1988; Beaty & Calisher, 1991; Beaty *et al.*, 2000).

LAC virus replication in its vector and vertebrate hosts has been the subject of extensive investigation, and thus presents a unique opportunity to determine if the quasispecies model enables effective LAC virus passage between the two disparate systems. The genomic coding strategies of LAC virus are known: the large (L) RNA segment (6980 nt) encodes the polymerase, the middle-sized (M) RNA segment (4526 nt) encodes the envelope glycoproteins (G1 and G2) and nonstructural protein NS_M, and the small (S) RNA segment (984 nt) encodes the nucleocapsid (N) protein and nonstructural protein NSs in overlapping reading frames (Schmaljohn, 1996; Gonzalez-Scarano & Nathanson, 1996; Elliott *et al.*, 1991). Gene structure–function studies revealed that many important biological functions co-segregate with the M RNA segment. In vertebrates, G1 is a major determinant of tissue tropisms, membrane fusion, neuroinvasiveness and elicitation of neutralizing antibody (Gonzalez-Scarano, 1985; Gonzalez-Scarano *et al.*, 1992). In the vector, midgut infection and transmission also co-segregate with the M RNA segment (Beaty *et al.*, 1981, 1982). Importantly, it seems that G1 must be proteolytically cleaved in the lumen of the midgut for efficient vector infection to occur. This cleavage exposes either a receptor ligand or a hydrophobic region on G2 that conditions vector infection (Ludwig *et al.*, 1989). Proteolytic processing of G1 is apparently not necessary for virus disseminating from the midgut to infect secondary target organs (Ludwig *et al.*, 1991). G1 and G2 would thus seem to be good candidates to monitor for selection during passage. The S RNA segment and its gene products do not co-segregate with vector infection, and thus would presumably not be subject to selection during mosquito passage. Little is known about the potential variability in the sequence or differential function of the L protein in invertebrate compared to vertebrate hosts.

In these studies, three different strains of LAC virus with dramatically different laboratory passage histories were characterized genetically after *Ae. triseriatus* midgut infection, dissemination to salivary glands and ovaries, and transovarial transmission (TOT). At each stage of vector infection, portions of the G1, G2 and N open reading frames (ORFs) were sequenced to determine if selection occurred during virus passage in the vector. L segment sequences were not compared due to lack of knowledge about potential variability between hosts. Virus populations at each stage were also characterized for genetic diversity using single-strand conformation polymorphism (SSCP) analysis. The central hypothesis was that quasispecies populations of LAC viruses would respond genetically to the selective pressures posed by infection and replication in different mosquito tissues and organs. Further it was hypothesized that the most dramatic changes would occur in ORFs encoding the G1 and G2 proteins, which condition vector tropisms.

Methods

Viruses. Three strains of LAC virus were used, two of which had undergone moderate to extensive passage exclusively in vertebrate cells, and one with minimal vertebrate passage. Prototype LAC virus (WT LAC) originally came from the Yale Arbovirus Research Unit, New Haven, CT, USA. The virus had been passaged three times in suckling mice and six times in BHK-21 cells. LAC ORI was derived from WT LAC virus. It was plaque-purified and used to generate reassortant viruses for gene structure–biological function studies in vertebrates (Gonzalez-Scarano *et al.*, 1992). The virus was kindly provided by Dr Neal Nathanson (U. Pennsylvania) in 1985. It had been passaged many times in BHK-21 cells after plaque purification. LAC ORI is phenotypically distinguishable from WT LAC virus due to its low infection rate in *Ae. triseriatus* mosquitoes (unpublished data; see Table 2). The SM1-78 strain of LAC virus was isolated directly from the brain of a patient who died of LAC encephalitis in 1978 via intracranial inoculation into suckling mice of homogenized tissue from the brain cortex (Chandler *et al.*, 1998). Virus isolated in the first suckling mouse passage (SM1-78) was used in this study; the virus was not passaged in cell culture.

Mosquito feeding and assay. The *Ae. triseriatus* mosquitoes used in this study originated from field material collected near La Crosse, Wisconsin, in 1981, and have been colonized continuously at Colorado State University (Wasieloski *et al.*, 1994). Three groups of 50–100 female *Ae. triseriatus* mosquitoes were orally infected with an artificial blood meal containing either WT LAC, LAC ORI or SM1-78. Medium from BHK-21 cells infected with LAC virus (m.o.i. of 0·01 or 0·001) was removed 24–48 h after infection, clarified and used in blood meal preparation. The blood meal consisted of equal volumes of infected cell culture medium, washed fresh sheep red blood cells and foetal bovine serum containing 10% sucrose. The blood meal was prepared immediately before feeding and warmed to 37 °C. Mosquitoes were fed by placing drops of the blood meal onto the netting of their cartons for 1 h. Virus titre of the blood meal at the end of feeding was determined by end-point assay in Vero cells.

Mosquitoes that fed to repletion were maintained for 2 weeks extrinsic incubation, then assayed for LAC virus infection by fluorescent antibody assay of leg tissue using a FITC-conjugated anti-LAC polyclonal mouse antibody (Beaty *et al.*, 1981). Slides were examined at 200 × using an Olympus BH2 epifluorescence microscope.

RNA isolation and preparation. The midguts, ovaries and salivary glands were dissected from live, anaesthetized, infected mosquitoes. Each organ was placed immediately in 200 μl of RNAgents (Promega) denaturation solution (guanidine thiocyanate and 2-mercaptoethanol). The organs were stored at −70 °C until processed. RNA was extracted with acid phenol–chloroform–isoamyl alcohol and finally dissolved in 17 μl RNase-free water.

LAC virus RNA sequence analysis. Portions of the LAC M and S RNA segments were amplified using the Access RT–PCR kit (Promega). Four μl of total cellular RNA solution was added to 46 μl of reaction mixture containing 1 × AMV/*Tfl* reaction buffer, 0·2 mM dNTP mix, 1 μM forward and reverse primers, 1 mM $MgSO_4$, 5 units AMV reverse transcriptase and 5 units *Tfl* DNA polymerase. Reactions were incubated at 48 °C for 45 min for reverse transcription. After 2 min at 94 °C for RT inactivation and template denaturation, the samples were thermocycled as follows: 94 °C for 30 s, 55 °C for 1 min, 68 °C for 2 min for 35–40 cycles, final extension at 68 °C for 7 min. Primers were designed using OLIGO 4.0 (National Biosciences) for amplification of a conserved region of the nucleocapsid ORF, a variable region of the G1 ORF and a variable

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Table 1. RT–PCR primers used to amplify regions of the LAC virus M and S segments

Three pairs of primers were designed to amplify portions of the N, G1 and G2 ORFs from LAC virus RNA for direct sequence analysis. The fourth primer pair was designed to amplify a smaller region at the 3′ end of the G1 amplicon for SSCP and sequence analysis.

Genome segment (ORF)	Primer pair	Primer sequence (5′ to 3′)	Primer position	Product size (bp)
S (N)	LSF	GTCGGATTTGGTGTTTTATGA	84–104	266
	LSR	GACCCATCTGGCTAAATACC	371–390	
M (G1)	LMF2	CCAAAAGCAACAAAAGAAAGA	1564–1584	410
	LMR2	CTGAAGGCATGATGCAAAG	1995–2013	
M (G2)	G2F	GCCAGGTATGTAAGAAATGC	215–234	599
	G2R	ACACTCTGTGAATGGGTGAT	834–853	
M (G1)	LMF2I	CTATATCCAAATCAGCACT	1850–1868	126
	LMR2	CTGAAGGCATGATGCAAAG	1995–2013	

region of the G2 ORF. The sequences and positions of the primers are listed in Table 1.

RT–PCR products were purified using a Wizard PCR Prep kit (Promega) and sequenced directly by Macromolecular Resources (Colorado State University) using an ABI Prism automated sequencing apparatus. The primers used for RT–PCR amplification were also used as sequencing primers. Nucleotide sequences were aligned using SEQMAN II (DNASTAR) and CLUSTAL W version 1.6 (Thompson *et al.*, 1994).

■ **LAC virus RNA SSCP analysis.** A 126 nt region of the G1 ORF of WT LAC virus was amplified by RT–PCR from midgut, ovaries and salivary glands of a single infected mosquito as described above using primers LMF2I and LMR2 (Table 1). PCR products were cloned into pCR2.1 using a TA cloning kit (Invitrogen). Individual clones were then amplified by PCR and analysed for SSCP (Farfan *et al.*, 1997). Nucleotide sequences of selected clones were determined as described above.

Results

Oral infection rates

The oral and filial infection rates (FIR) for the three virus strains are presented in Table 2. Oral infection rates ranged from approximately 50% for WT LAC and SM1-78 to 28% for LAC ORI. Infected mosquitoes were then provided with an uninfected blood meal to stimulate a second gonadotropic cycle and FIRs were determined for these progeny. The mean FIRs ranged from 68% for SM1-78 to only 1% for LAC ORI (Table 2).

Table 2. *Aedes triseriatus* oral and filial infection rates (FIRs) with three strains of LAC virus

Virus	Blood meal titre*	Oral infection rate†	FIR‡	FIR range (%)§
WT LAC	7·5	51·2 (21/41)	38·8 (19/49)	14–75
LAC ORI	7·2	28·3 (19/67)	1·2 (1/77)	0–3
SM1-78	5·8	51·6 (47/91)	68 (34/50)	0–92

* Log_{10} $TCID_{50}$ determined by end-point assay in Vero cells.
† Per cent adult female mosquitoes orally infected as determined by immunofluorescent assay of leg tissue (no. infected/no. fed).
‡ Per cent of total progeny mosquitoes transovarially infected as determined by immunofluorescent assay of head tissue (no. infected/total progeny)
§ Range of FIRs among the progeny of three, two and four female mosquitoes assayed after infection with WT LAC, LAC ORI and SM1-78, respectively.

Sequence comparison of LAC RNA from three virus strains after mosquito passage

For each of the three LAC virus strains, the genomic RNA sequences of virus used to prepare the blood meal (designated parental); viral RNA amplified from the midgut, ovaries and salivary glands of two to four orally infected mosquitoes; and viral RNA amplified from one to six transovarially infected progeny mosquitoes (designated virus strain adult number-progeny number, e.g. WT 9-5) were determined. A 266 nt region of the S segment nucleocapsid ORF, a 410 nt region of the M segment G1 ORF, and a 599 nt region of the M segment G2 ORF of each sample were sequenced. All sequences were compared to those of the parental WT LAC RNA. Overall, the N and G2 ORFs were found to be less variable than the G1 ORF.

WT LAC virus RNA sequences

No nucleotide changes from the parental WT LAC nucleocapsid ORF sequence were detected in any organs of the two orally infected and one transovarially infected mosquitoes examined.

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Table 3. Amino acid and nucleotide substitutions in the G1 ORF of WT LAC, LAC ORI and SM1-78

Six positions in a 410 nt region of the G1 ORF of WT LAC (WT) and LAC ORI (ORI) consistently acquired either synonymous (shown as nucleotide bases) or nonsynonymous (shown as amino acids) mutations as compared to parental (Par) sequence when virus infected the midgut (MG) and disseminated to the ovaries (OV) and salivary glands (SG). Six WT LAC-infected progeny (WT 9-5, 9-6, 9-9; WT 14-1, 14-2, 14-3) and one LAC ORI-infected progeny (ORI 82-1) were analysed also for mutations in transovarially transmitted virus. SM1-78 RNA acquired only a single mutation in one midgut sample after replication in mosquitoes (not shown).

	Nucleotide at position					
	1749	1912	1922	1928	1971	1974
	Amino acid at position (where changed)					
Sample		618	621	623		
WT Par	**T**	**Arg**	**Ala**	**Glu**	**G**	**T**
WT 5 MG	T	Trp	Ala+Val*	Glu	G	T
WT 5 OV	T	Trp	Val	Glu	G	T
WT 5 SG	T	Trp	Val	Glu	G	T
WT 9 MG	T	Trp	Ala	Glu	G	T
WT 9 OV	T	Trp	Val	Glu	G	T
WT 9 SG	T	Trp	Val	Glu	G	T
WT 9-5	C	Trp	Ala	Glu	G	C
WT 9-6	C	Trp	Ala	Glu	G	C
WT 9-9	T	Arg	Val	Glu	G	T
WT 14 MG	T	Trp	Ala	Gly	G	T
WT 14 OV	T	Trp	Ala	Gly	G	T
WT 14 SG	T	Trp	Ala	Gly	G	T
WT 14-1	T	Trp	Ala	Gly	G	T
WT 14-2	T	Trp	Ala	Gly	G	T
WT 14-3	T	Trp	Ala	Gly	G	T
ORI Par	**C**	**Trp**	**Ala**	**Glu**	**A**	**C**
ORI 1 MG	T	Arg	Ala	Glu	G	T
ORI 1 OV	T	Arg	Val	Glu	G	T
ORI 1 SG	T	Arg	Val	Glu	G	T
ORI 4 MG	T	Arg	Ala	Glu	G	T
ORI 4 OV	T	Arg	Ala	Glu	G	T
ORI 4 SG	T	Arg	Ala+Val*	Glu	G	T
ORI 82 MG	T	Arg	Ala	Glu	G	T
ORI 82 OV	T	Arg	Ala	Glu	G	T
ORI 82 SG	T	Arg	Ala	Glu	G	T
ORI 82-1	T	Arg	Ala	Glu	G	T
ORI 84 MG	C	Trp	Ala	Glu	G	C
ORI 84 OV	T	Arg	Ala	Glu	G	T
ORI 84 SG	T	Arg	Ala	Glu	G	T
SM1-78 Par	**C**	**Trp**	**Ala**	**Glu**	**G**	**C**

* Positions where overlapping electropherogram peaks indicated that there was a mixture of two virus sequences present in the same organ, one encoding Ala and the other Val at amino acid 621.

G2 ORF RNA from one or more organs of all three WT LAC orally infected mosquitoes had a synonymous mutation at nucleotide 743. This substitution was not present in the virus transmitted to the single progeny mosquito tested. A synonymous change also was detected at position 559 in the RNA from all three organs of one of the orally infected mosquitoes (WT 14).

WT LAC G1 ORF RNA was analysed from the organs of three orally infected mosquitoes and six transovarially infected progeny. Mutations had occurred in RNA from one or more

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organs of two of the WT LAC-infected mosquitoes at positions 1912 and 1922, resulting in amino acid changes of Arg_{618} to Trp and Ala_{621} to Val. The third WT LAC-infected mosquito had the same mutation at position 1912 and an A to G transition at position 1928, resulting in an amino acid change of Glu_{623} to Gly. One or two of the same mutations were detected in viral RNA extracted from all of the WT LAC-infected progeny tested, with two progeny showing additional synonymous mutations at nucleotides 1749 and 1974 (Table 3).

LAC ORI virus RNA sequences

The N ORF sequence analysed was identical in WT LAC and LAC ORI parental virus RNA. No nucleotide changes from the parental N ORF RNA sequence were detected in any sequences examined from two mosquitoes infected orally and one mosquito infected transovarially with LAC ORI virus.

The sequence of the G2 ORF region analysed also was identical for WT LAC and LAC ORI parental virus RNA. No nucleotide changes were detected in the G2 ORF of LAC ORI RNA from two of the three orally infected mosquitoes. A mutation occurred at nucleotide 609 in M RNA from the ovaries and salivary glands of the third mosquito. This substitution resulted in an amino acid change from Phe_{183} to Ser.

In contrast to the other two genome regions, in the G1 ORF region analysed, the LAC ORI parental virus RNA had mutations at nucleotide positions 1749, 1912, 1971 and 1974 as compared to WT LAC (Table 3). The mutation at nucleotide 1912 resulted in an amino acid change from Arg_{618} to Trp. LAC ORI G1 ORF RNA was analysed from the organs of four orally infected mosquitoes and one transovarially infected progeny. Upon replication and dissemination in the mosquito vector, LAC ORI acquired G1 mutations in RNA from most or all organs at positions 1749, 1912, 1922, 1971 and 1974 (Table 3). The mutation at position 1912 resulted in a Trp_{618} to Arg substitution, and that at 1922 in an Ala_{621} to Val substitution. Interestingly, the mutations at positions 1749, 1912, 1971 and 1974 resulted in restoration of the sequence of the parental WT LAC virus. Although 77 progeny mosquitoes from LAC ORI-infected females were tested by fluorescent antibody and RT–PCR, only one was found to be infected. The LAC ORI virus in this mosquito retained the same mutations that were detected in the parental female (Table 3).

SM1-78 virus RNA sequences

The parental SM1-78 N ORF sequence had a C to T transition at position 179 by comparison to WT LAC RNA, resulting in a predicted amino change of Ser_{33} to Leu. No nucleotide changes from the parental RNA sequence were detected in the two orally infected and one transovarially infected mosquitoes examined.

By comparison to WT LAC parental RNA, SM1-78 G2 ORF RNA had a single nucleotide transition of C to T at position 786, resulting in an amino acid change of Thr_{242} to Ile. No nucleotide changes from the parental RNA sequence were detected in this region of the G2 ORF from any of the SM1-78 orally infected mosquitoes or in the RNA of the single SM1-78-infected progeny mosquito tested.

In the G1 ORF region analysed, SM1-78 parental RNA differed from WT LAC at nucleotide positions 1749, 1912 and 1974, resulting in the amino acid change Arg_{618} to Trp (Table 3). LAC ORI and SM1-78 parental RNA differed from each other only at nucleotide 1971 and had identical amino acid sequences in this region. In the organs of the three SM1-78-infected mosquitoes analysed, only a single mutation in one midgut was detected in the LAC G1 ORF. The substitution occurred at nucleotide 1915, resulting in an amino acid change from Asp_{619} to Asn. None of the six SM1-78-infected progeny tested contained viruses with mutations in this region of the G1 ORF (data not shown).

In one organ of two mosquitoes tested (WT 5 and ORI 4), there appeared to be equal amounts of viral RNA with two different nucleotides present at the same position (Table 3). This was detected by the presence of two overlapping electropherogram peaks, which were present in both strands of DNA, at the same position. SSCP analysis confirmed the presence of more than one genotype in a single organ.

SSCP analysis of quasispecies

To characterize genetic diversity in a population of LAC viruses during replication and dissemination in a single vector, a sequence of 126 nt within the G1 ORF corresponding to the coding sequence for an antigenic determinant in the G1 glycoprotein (Grady *et al.*, 1983, 1987) was amplified from the organs of a WT LAC orally infected mosquito by RT–PCR and cloned. Thirty clones each from the midgut, ovaries and salivary glands were then amplified by PCR and analysed for SSCP (Fig. 1). To compare diversity in the more homogeneous environment of cultured mosquito cells, another 30 clones were prepared from WT LAC persistently infected C6/36 cells and analysed. Each group of DNA samples with an identical migration pattern was designated a genotype. The 30 midgut samples gave rise to 13 different genotypes, the ovary samples gave rise to 11 genotypes, salivary gland samples to nine genotypes, and the C6/36 cell culture samples to four genotypes. The predominant genotype for each organ was designated the consensus genotype. When samples with the consensus genotypes from each organ were analysed by SSCP together, they were shown to have the same migration pattern (Fig. 1).

Of the 120 samples that were analysed by SSCP, 68 (56·7 %) had the consensus genotype. The consensus genotype in the midgut comprised 10 samples (33·3 %) with the two next most predominant genotypes containing six (20 %) and two (6·7 %) samples respectively. The remaining 12 samples (40 %) had unique genotypes. The consensus genotype in the ovary comprised 20 samples (66·7 %), with the remaining 10 samples

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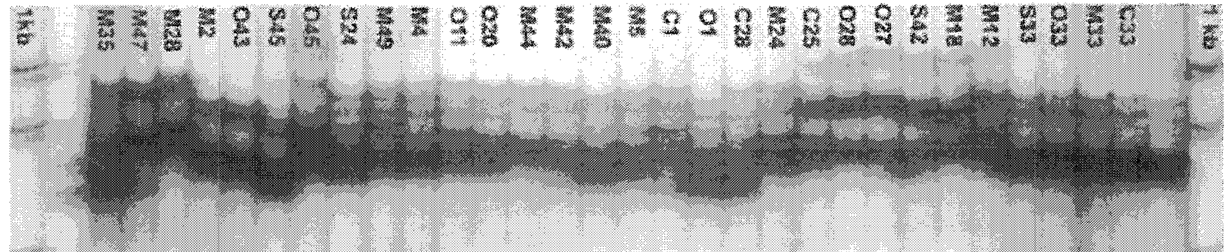

Fig. 1. SSCP gel of representative genotypes. Thirty clones each from the midgut, ovaries and salivary glands of an infected mosquito and from an infected C6/36 cell culture were analysed by SSCP. In this gel, several representative genotypes were analysed together with the consensus genotype from each sample group (C33, M33, O33, S33) and cDNA from ovary samples used for sequence analysis. C, C6/36 cell culture clones; M, midgut clones; O, ovary clones; S, salivary gland clones.

Table 4. Nucleotide differences between sequenced samples

Data give number of nucleotide differences between the 126 bp G1 ORF sequences with the midgut, salivary gland and ovary consensus SSCP genotype and the four ovary samples that had an SSCP pattern different from the consensus when they were compared by CLUSTAL W alignment.

	Midgut consensus	Salivary gland consensus	Ovary consensus	Ovary 1	Ovary 28	Ovary 43	Ovary 45
Midgut consensus	–	0	0	1	4	3	4
Salivary gland consensus		–	0	1	4	3	4
Ovary consensus			–	1	4	3	4
Ovary 1				–	5	4	5
Ovary 28					–	7	8
Ovary 43						–	6
Ovary 45							–

having unique genotypes (33·3%). The consensus genotype in the salivary gland comprised 19 samples (63·3%) with the two next most predominant genotypes each containing three samples (10% each). The remaining five samples (16·7%) had unique genotypes. In the C6/36 cells, 19 samples (63·3%) had the consensus genotype. The two next most predominant genotypes contained seven (23·3%) and three (10%) samples respectively. The remaining sample (3·3%) had a unique genotype.

Nucleotide sequences were determined for eight ovary samples, four of which had consensus and four had different genotypes, along with a consensus midgut sample, a consensus salivary gland sample and a consensus C6/36 sample. Sequences were aligned using the CLUSTAL W program and analysed for identity. All the consensus sequences had the same 126 bp sequence, which was identical to that determined for the G1 ORF from WT LAC parental RNA used for oral infection of mosquitoes (Table 3). The four ovary samples that had genotypes different from the consensus and from each other had from one to four nucleotide differences when compared to the consensus sequence and from four to eight nucleotide differences when compared to each other (Table 4).

Of the total of 12 mutations in non-consensus ovary sequences examined, only four resulted in amino acid changes. In ovary 28, a T to C transition at position 1919 resulted in a Phe_{620} to Leu change and an A to G transition at nucleotide 1958 resulted in a Thr_{633} to Ala change. In ovary 43, an A to G transition at position 1935 resulted in a Lys_{625} to Arg change and an A to T transversion at nucleotide 1968 resulted in an Asp_{636} to Gly change.

Discussion

These studies tested the hypothesis that passage in vectors results in purifying selection for certain LAC virus genotypes in the quasispecies that are more fit for replication in the respective organ systems. Viruses with dramatically different laboratory passage histories (and thus different genotypes) were used to orally infect *Ae. triseriatus* and the resulting progeny viruses were compared to parental virus for genetic changes during vector passage. Specifically, genetic changes in the ORFs for three structural proteins of LAC virus were monitored during replication in mosquitoes and after TOT of the virus to mosquito progeny. Genetic and phenotypic

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changes are epidemiologically significant only if mutant viruses are transmitted to vertebrate hosts orally and/or to progeny transovarially. Therefore, virus populations were sampled from the midgut, ovaries, salivary glands and progeny of infected mosquitoes.

Surprisingly, the analysed regions of the glycoprotein G2 ORF as well as the nucleocapsid ORF were relatively stable; however, changes regularly occurred at six positions within a 225 nt sequence of the 410 nt amplicon from the G1 ORF of WT LAC and LAC ORI (Table 3). Seventy-five percent of these substitutions occurred initially in the midgut (18/24 substitutions) and 21% (5/24) occurred initially in the ovaries and salivary glands. One synonymous substitution occurred only in two transovarially infected progeny. All of the substitutions detected in virus genomes from the midgut also were present in virus RNA from the salivary glands and ovaries of the same mosquito. Therefore, it appears that a majority of the genetic changes resulted from selection in the midgut, and these mutated viruses disseminated to the ovaries and the salivary glands. Of the mutations encoding amino acid substitutions that were detected in adult female mosquitoes, 77% were present in the genomes of viruses transmitted to progeny.

All of the G1 ORF mutations resulting in amino acid substitutions occurred in a 17 nt region of the 410 nt sequence analysed. The most frequent amino acid substitution occurred at position 618 of the G1 glycoprotein and involved either Arg replacing Trp (WT LAC) or Trp replacing Arg (LAC ORI). SM1-78 RNA had a Trp codon at this position that did not change upon mosquito passage. These data suggest that the presence of Arg at position 618 may be a result of passage in cell culture (Table 3). It is possible that mutations consistently occur at this position in response to changes in a region of the G1 ORF outside the sequence analysed in order to preserve structural properties of the protein, since amino acids 607 to 638 are proposed to occur on the surface of the G1 protein and form an antigenic determinant (Grady *et al.*, 1983, 1987). Other mutations resulting in amino acid substitutions occurred less frequently: Asp_{619} to Asn in the midgut of a single SM1-78-infected mosquito; Ala_{621} to Val in 29% (6/21) of midguts, ovaries and salivary glands of mosquitoes infected with either WT LAC or LAC ORI; and Glu_{623} to Gly in a single WT LAC-infected mosquito, also transmitted to progeny.

It has been proposed that in the mature virion, the G1 glycoprotein masks G2, making it unavailable for binding to mosquito midgut cells (Ludwig *et al.*, 1989), and that in the midgut G1 is cleaved by host proteases. The exposed G2 glycoprotein then mediates binding of the virus to midgut cells. However, following release of progeny virus into the haemocoel, G1 is no longer exposed to proteolytic conditions present in the midgut and may mediate virus attachment to cells in the ovaries and salivary glands (Ludwig *et al.*, 1991). Therefore, at least two modes of selection may act on the G1 glycoprotein after virus infection of a mosquito. Viruses with G1 glycoproteins that are most readily cleaved by midgut proteases may be most fit for infection of midgut cells upon ingestion in a blood meal. When the virus progeny disseminate, selection for viruses with G1 glycoproteins that bind efficiently to the receptors on secondary target organs may occur. The cellular receptor(s) for G1 has not been identified; it is possible that G1 binds to a receptor present on both vertebrate and invertebrate cells. In that case, viruses that have adapted to passage in cell culture may not be at a disadvantage when infecting the secondary organs of the mosquito vector. It is worth noting the correlation between the lower oral infection rate for mosquitoes by LAC ORI (Table 2) and the observation that 66% of the G1 ORF mutations detected were in LAC ORI RNA vs 30·5% of mutations in WT LAC and 6·6% in SM1-78.

Mutations resulting in a conservative change from Ala_{621} to Val in the G1 glycoprotein of WT LAC and LAC ORI and synonymous substitutions at nucleotide positions 1749, 1971 and 1974 in the M RNA of WT LAC and LAC ORI (Table 3) may reflect differences in codon usage between cultured vertebrate cells and mosquito cells.

The SSCP analysis of genetic diversity within the virus population in a single mosquito suggested that a greater variety of quasispecies were fit to replicate in mosquito organs than in cell culture. In persistently infected C6/36 cells only four genotypes were detected among 30 samples. The greatest variety of genotypes was detected in the mosquito midgut, and as the infection disseminated from the midgut to other organs, the number of genotypes was reduced. The consensus genotype was identical in midguts, salivary glands and ovaries as revealed by both SSCP and sequence analyses.

The results of this study demonstrate that strains of LAC virus that have been serially passaged in cell culture may undergo balancing selection when reintroduced into the mosquito host due to the presence of different selective constraints present in the two disparate amplification systems. Repeated passage of WT LAC and LAC ORI in cell culture may have selected for viruses that were less fit for replication in mosquitoes. When reintroduced into mosquitoes, these viruses underwent genetic changes necessary to readapt to efficient replication in the mosquito host. SM1-78 virus showed the least amount of change during oral and transovarial passage in mosquitoes. This may be attributable to the fact that SM1-78 virus had not been repeatedly passaged in vertebrate cells. Most of the genetic changes were detected in the G1 ORF, whereas the sequences of the G2 and N ORFs were relatively stable. If genetic changes are indeed a function of selection based on tissue tropisms, it is surprising that few changes occurred in the G2 sequence analysed. However, since it is postulated that glycoprotein G2 is involved only in mosquito midgut infection and not in binding to vertebrate cells, cell culture passage would not be expected to select for different genotypes.

It is important to note that virus mutations acquired during replication in parental mosquitoes were detected in their

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progeny. Thus, TOT can serve to amplify and to maintain new virus genotypes resulting from vector passage in nature.

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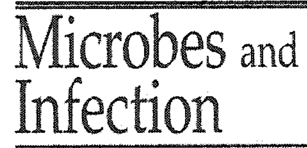
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Review

La Crosse virus: replication in vertebrate and invertebrate hosts

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Abstract

La Crosse virus is maintained in a cycle involving mosquitoes and small mammals. Vertebrate cell infection is generally cytolytic; vector cell infection results in persistent infection. Features of La Crosse virus replication that may permit the virus to traffic between vector and vertebrate hosts and condition different infection outcomes are described. © 2002 Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

Keywords: La Crosse arbovirus; Mosquitoes; Co-regulation of transcription; Apoptosis; Persistent infections; Quasispecies; Bunyaviridae

1. Introduction

La Crosse virus (LAC) is an arthropod-borne virus (arbovirus) in the family Bunyaviridae, the largest family of arboviruses with over 300 members [1–3]. As do all arboviruses, LAC virus in nature cycles between vertebrate and invertebrate hosts. Infections can cause serious disease in human hosts, but there are few untoward effects in the vector. Most remarkably, LAC virus can be maintained in nature by vertical transmission in mosquitoes and can replicate in potentially vulnerable tissues of mosquito ovaries and embryos without deleterious effects [3]. The mechanisms underlying differences in host–virus interactions will be emphasized in this review.

Many viruses in the Bunyaviridae family are significant human, veterinary, and plant pathogens. The family is divided into five genera: *Bunyavirus, Nairovirus, Phlebovirus, Tospovirus*, and *Hantavirus* [1,2]. All of the viruses, with the exception of the hantaviruses, are vector-borne. All infect vertebrates with the exception of Tospoviruses, which are plant viruses [2]. The genera are further divided into serogroups based on antigenic relationships [1]. LAC virus is a member of the genus *Bunyavirus*, California (CAL) serogroup, and is a major cause of encephalitis and aseptic meningitis in children in the U.S. [4,5]. The CAL serogroup includes other viruses that are known to cause human illness, such as Jamestown Canyon, California encephalitis, snowshoe hare (SSH), Tahyna, Inkoo, and Guaroa [6].

2. LAC virus structure and genome configuration

Bunyaviruses have tripartite, negative-sense RNA genomes (Fig. 1) [7,8]. Consensus nucleotide sequences are present on the 3' termini of all three RNA segments and cause the segment ends to bind to complementary sequences on 5' ends to form panhandle structures [7–9]. The consensus sequences may serve as transcriptase recognition structures.

The genomic coding strategies of LAC virus are known (Fig. 1): the large (L) RNA segment (6980 nucleotides (nt)) codes for the polymerase; the medium (M) RNA segment (4526 nt) codes for a polyprotein that is post-translationally processed, yielding the G1 and G2 glycoproteins and a nonstructural protein NS_M; and the small (S) RNA segment (984 nt) codes for the nucleocapsid (N) protein and a nonstructural protein NS_S in overlapping reading frames [7–9].

The pleomorphic virion is approximately 90–100 nm in diameter (Fig. 1). The virion consists of the three genome segments encapsidated with the N protein to form helical

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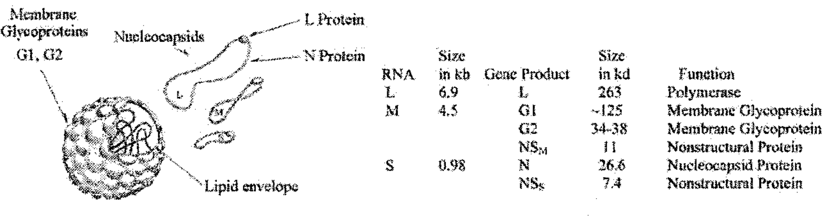
A: La Crosse Virus Structure



RNA	Size in kb	Gene Product	Size in kd	Function
L	6.9	L	263	Polymerase
M	4.5	G1	~125	Membrane Glycoprotein
		G2	34-38	Membrane Glycoprotein
		NS_M	11	Nonstructural Protein
S	0.98	N	26.6	Nucleocapsid Protein
		NS_S	7.4	Nonstructural Protein

B: Replication & Transcription of La Crosse Virus RNA

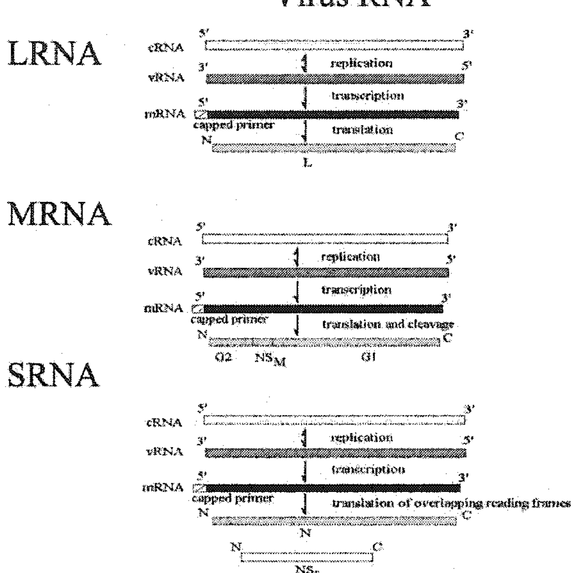


Fig. 1. **A**: LAC virus structure. **B**: replication and transcription of LAC RNA.

structures, a few nucleocapsid-associated polymerase molecules needed to transcribe the negative-sense viral genome, and a surrounding host-derived lipid envelope containing virus-encoded glycoprotein spikes [2,9].

3. LAC virus replication

Viral entry into the host cell and uncoating are thought to occur by receptor-mediated endocytosis and membrane fusion [2,7–9]. The host receptors bound by the viral surface glycoproteins have not been identified. The three nucleocapsids are released into the cytoplasm, where primary transcription is initiated. One subgenomic mRNA is transcribed from each of the three genome segments by the virion-associated polymerase. The three mRNA species possess short, nonviral-coded primer sequences at their 5' ends (Fig. 1), which are acquired from host mRNA in both vertebrate and invertebrate cells [9–12]. The LAC virion-associated L protein has methylated cap-dependent endonuclease as well as primer-stimulated RNA polymerase activities [10,11]. No other major arbovirus family uses host-derived transcription primers. Transcription of both S and M mRNA species terminates approximately 100 nucleotides before the end of the template RNA [7–9]. Little information is available concerning transcription of the L mRNA species. LAC mRNAs do not bind to oligo (dT).

Translation by host cell ribosomes yields the structural and nonstructural proteins. Replication of the viral genome and a second round of transcription follow primary transcription and translation. Virus maturation occurs at the Golgi apparatus [2,7–9]. Glycosylation of the G1 and G2 glycoproteins is initiated in the endoplasmic reticulum and completed in the Golgi [2,9]. The nucleocapsids bud through the modified Golgi membranes. Virions accumulate in the cisternae and are transported in vesicles to the cell surface and released by exocytosis.

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4. Emergence of LAC virus

LAC virus emerged as a significant human pathogen in the 1960s, and is the most common cause of pediatric arboviral encephalitis in the U.S. [4,5,13]. Isolation of the virus post-mortem from the brain of a child infected near La Crosse, Wisconsin [14] in 1960, led to the recognition of this virus as the causative agent of LAC encephalitis. Reports of LAC encephalitis range from 42 to 174 cases per year, but there is clearly great underreporting of the disease [13]. Recent epidemiologic analyses of case reports from 28 states estimate that the incidence in endemic areas is 20–30/100,000, which exceeds that of bacterial meningitis [5]. The estimated case fatality rate of LAC encephalitis is about 0.3% [4,5]. Children are more susceptible to illness resulting from LAC infection than are adults, with > 90% of individuals with symptomatic LAC virus infections being under 15 years of age.

Of patients hospitalized with LAC encephalitis in La Crosse, Wisconsin, 42% had seizures during or after the acute illness [15]. Thus, LAC infection in the Upper Midwestern U.S. is highly epileptogenic [4]. Learning disabilities and cognitive deficits as well as neurobehavioral sequelae, such as attention deficits and hyperactivity, are associated with LAC CNS infection [4,5,15]. In one study, 36% of patients had IQ scores of 79 or less [5]. Antibody to LAC occurs at twice the prevalence in people institutionalized in Wisconsin for permanent mental disorders as in the general population [16]. When patients with Down's syndrome and cranial abnormalities (other cerebral defects) were excluded, the rate was three times that in the general population. Clearly, LAC and its close relatives are significant human pathogens.

5. LAC virus distribution, maintenance, and amplification in nature

CAL serogroup viruses have been isolated in North and South America, Africa, Europe, and Asia [1,6]. Although antigenically closely related, CAL viruses are maintained in nature in distinct cycles involving preferred arthropod vectors and vertebrate hosts [1,6,17,18]. LAC virus is maintained and amplified in nature by several distinct transmission mechanisms (Fig. 2). The predominant horizontal transmission/amplification cycle involves *Aedes triseriatus* mosquitoes, the principal vector of LAC virus, and chipmunks (*Tamias striatus*) and squirrels (*Sciurus carolinensis*) as the principal vertebrate hosts [1,6]. Infection of chipmunks results in acute viremia of sufficient titer to infect blood-feeding *Ae. triseriatus* mosquitoes, but in contrast to children, rodent hosts exhibit no clinical manifestations. Vertical amplification and maintenance also occur (Fig. 2). Infected female mosquitoes transmit the virus to progeny by transovarial transmission (TOT) [1,19]. Virus can be amplified dramatically by this mechanism; during each gonadotropic cycle, multiple infected progeny result. LAC virus overwinters in the infected eggs, and infected progeny emerge in the spring to resume the transmission cycle [1,6]. LAC virus may also be horizontally transmitted venereally (Fig. 2) from male to female *Ae. triseriatus* [20].

The geographic range of *Ae. triseriatus* is approximately the eastern half of the U.S. *Ae. triseriatus* mosquitoes dwell primarily in hardwood forests and breed in tree holes and discarded automobile tires or other outdoor containers [21]. Most human infections occur between July and September. Risk factors include rural residence, outdoor activities in endemic areas, and presence of suitable breeding sites near residence [21].

6. LAC virus replication in the mouse

The laboratory mouse is the preferred animal model for LAC encephalitis. The susceptibility of mice (and humans) is age-dependent; young hosts are much more susceptible to serious infection [22]. Indeed, one plaque-forming unit (PFU) of LAC virus administered either peripherally or intracranially (IC) in young mice is lethal. In older mice, 1 PFU of virus IC is lethal, but these mice are resistant to large doses of virus administered peripherally. Pathogenesis studies have revealed the sequence of events following LAC virus infection [22–24]. Following subcutaneous injection of LAC virus into suckling mice, there is an extraneural phase of replication before virus invasion of the CNS. In the extraneural phase, virus infects and replicates in striated muscle and to a lesser extent in cardiac and smooth muscle. Virus somehow moves from skeletal muscle to plasma (perhaps by the lymphatics), resulting in a plasma viremia. High virus titer in blood is a determinant of neuroinvasion, which may lead to CNS infection, encephalitis and death. It is not known how LAC virus negotiates the blood–brain barrier. In the brain, virus replicates in neurons. In the CNS of infected mice, typical inflammation and encephalitis signs are seen, including perivascular cuffing. LAC virus can induce apoptosis in neuronal cells, which can be inhibited by expression of bcl-2 in certain cell lines [25].

Gene structure–biological function studies using reassortant viruses revealed viral determinants of pathogenesis in the mouse [23,24]. Peripheral virulence and neuroinvasiveness co-segregated with the M RNA segment. The G1 glycoprotein, which is the vertebrate cell receptor ligand, is likely the principal determinant of this phenotype. G1 undergoes a conformational change at the acidic pH of the endosome, leading to membrane fusion. Monoclonal antibody resistant variants that have limited fusion capability are attenuated in mice [23,24]. In contrast, neurovirulence co-segregates with the large RNA segment, which encodes the polymerase [23,24].

It is noteworthy that pathogenesis studies of LAC and related viruses in vertebrate hosts have always followed

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Natural cycle of La Crosse virus

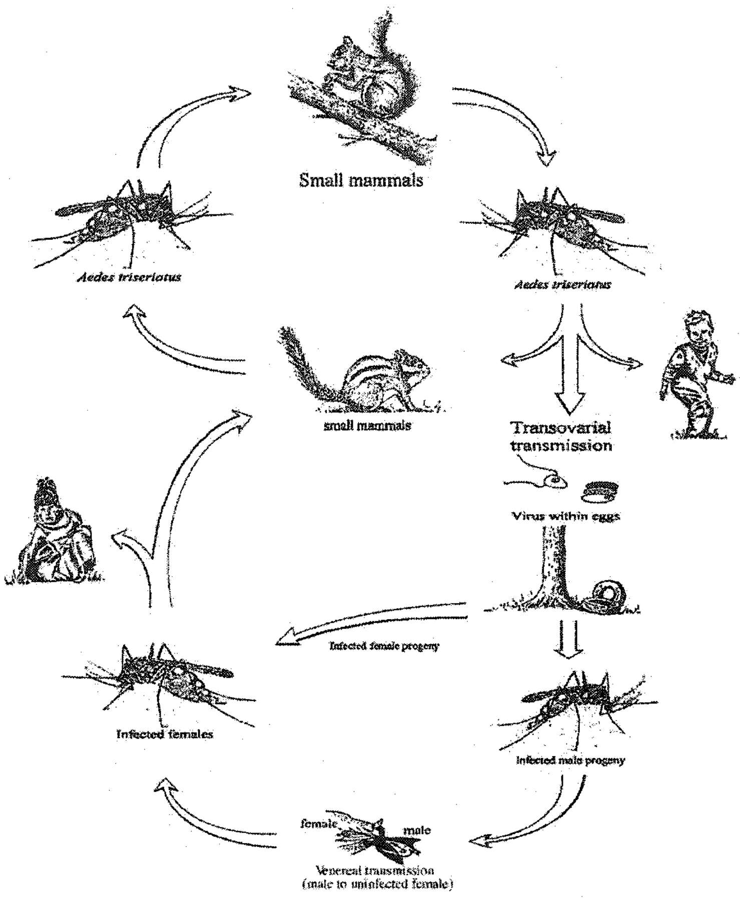

Fig. 2. LAC virus natural cycle.

inoculation of virus with a needle and syringe. This may have confounded the issue because arthropod saliva greatly potentiates transmission of bunyaviruses to vertebrate hosts. For example, high titered doses of LAC virus delivered peripherally by needle do not result in a productive infection of adult mice, whereas mosquito delivery of virus greatly enhances virus infection and virulence (unpublished data). Similarly, chipmunks exposed to LAC virus via infected mosquitoes had longer and higher titered viremias than those infected by needle inoculation [26]. Two publications are potentially important in this regard: 1) mosquito saliva can directly impact the host cytokine response [27]; and 2) alpha–beta interferon knockout mice are very susceptible to LAC virus even when delivered by needle and syringe [28]. Thus, it may be that mosquito saliva potentiates virus infection by perturbing the early interferon response to virus infection in the bite site.

7. LAC virus replication in the mosquito vector

Upon ingestion in an infected blood meal, LAC virus infects the epithelial cells lining the mosquito midgut [1,8]. The efficiency of viral dissemination to other tissues depends on both the virus and the vector [29–31]. If the virus is able to escape from the midgut, progeny particles then disseminate to the hemocoel and travel to and replicate in the heart, neural ganglia, fat body, ovaries, and salivary glands. The salivary glands are the last organs infected, at 7–16 d after ingestion of virus [1,8]. Once in the salivary gland, the virus replicates to high titers and is transmitted to the vertebrate host in the mosquito saliva. Infection of the ovaries results in transmission of LAC virus to the progeny. LAC virus accumulates in all tissues of the ovaries and maintains a persistent infection that allows efficient TOT of the virus [32]. The gonadal tissues of both male and female progeny are vertically infected, and infected males can venereally transmit the virus to uninfected female mosquitoes [20].

Gene structure–biological function studies using reassortant viruses revealed determinants of virus infection of mosquitoes. The M RNA segment was demonstrated to co-segregate with LAC virus midgut infection and transmission [1,8,24]. A mutant virus selected by resistance to neutralization by a G1 glycoprotein-specific monoclonal antibody was demonstrated to be incapable of infecting

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vector midguts [29]. Interestingly, this virus was the same one described above that had limited fusion capability and was attenuated in mice [24]. Although glycoprotein G1 is not the ligand that binds to midgut receptors, proteolytic cleavage of G1 is necessary to reveal either a ligand or hydrophobic region on G2 required to infect the vector midgut epithelium [33,34]. Proteolytic processing is apparently not necessary, however, for efficient infection of other vector tissues and organs after release to the hemocoel.

The efficient TOT of bunyaviruses by their vectors is truly a remarkable host–parasite relationship [3]. TOT is extremely efficient. Indeed, in laboratory studies the viruses seem to exert no significant deleterious effect on the developing oocyte and embryo, even during critical periods of follicular resting stages (ovarian diapause), embryogenesis, and embryo diapause [1,8]. In contrast, the same viruses can be extremely pathogenic in developing vertebrates, and certain bunyaviruses are major causes of abortion and teratogenesis [2].

8. Potential mechanisms that condition LAC virus attenuation in vectors

The reasons for the dramatic difference in LAC virus virulence in vectors and vertebrate hosts have been the subject of much speculation. One aspect of the molecular biology of bunyaviruses, cap scavenging, is especially intriguing in terms of persistence and efficient bunyavirus TOT [7–10]. As noted previously, bunyaviruses utilize an unusual mechanism for mRNA synthesis; they scavenge host cell mRNA 5' caps plus adjacent oligonucleotide sequences as primers for their own viral mRNA synthesis. During critical mosquito life stages, such as ovarian quiescence and embryo diapause, there could be a dramatic reduction in synthesis of host message, resulting in a reduced amount of capped mRNA available to provide transcriptional primers for viral mRNA. Thus, viral transcription could be reduced or co-regulated with host transcription, thereby modulating viral replication, minimizing detrimental effects to the host, and enhancing the overwintering efficiency of LAC virus. Here, we will describe our studies to investigate potential determinants of the remarkable host–parasite relationship of LAC virus in its vector.

9. Correlation of efficient TOT with diapause

During the winter months, temperate zone mosquito species undergo a state of dormancy known as diapause, which is characterized by reduced metabolic activity. In the northern part of their range, egg diapause is the mechanism for *Ae. triseriatus* overwintering [35]. In *Ae. triseriatus* the fully formed embryo is sensitive to diapause induction, which results from shortened photoperiod and reduced temperatures [35]. There is geographic variation (a north to south gradient) in the response of *Ae. triseriatus* eggs to shortened photoperiods.

Diapause could function to protect infected embryos when ambient temperatures would otherwise permit host metabolic activity and virus replication. Theoretically, mosquitoes capable of diapause would be the best vectors for transovarially transmitting virus. To determine if diapause and efficient TOT were genetically linked, strains of *Ae. triseriatus* from Wisconsin and Florida (diapausing and nondiapausing, respectively) were compared in their ability to vertically transmit LAC virus [36]. The geographic strains exhibited TOT rates that were almost identical, but filial infection rates (number of infected progeny) were significantly higher for the northern mosquitoes. Importantly, a nondiapausing strain of *Ae. triseriatus* selected from the Wisconsin diapausing strain exhibited reduced filial infection rates similar to the nondiapausing Florida mosquitoes [36].

These were laboratory studies and were conducted over relatively short periods of time. Field studies were then conducted to determine the effects of diapause on LAC virus overwintering [37]. Embryos from the TOT+ and TOT– *Ae. triseriatus* colonies were induced into diapause and shipped to Wisconsin in 1993 for overwintering in natural conditions. Samples were returned to the laboratory on a predetermined schedule, and mortality rates, diapause status, and infection rates were determined. The results revealed that LAC infection does affect survival—a greater proportion of noninfected eggs successfully overwintered than infected eggs. Nonetheless, most of the infected eggs did successfully overwinter. Importantly, mortality in LAC-infected eggs occurred after emergence of embryos from diapause in the spring of the year [37]. There were no differences between infected and noninfected mosquitoes in survivability in embryos in diapause (Fig. 3). This suggested that the diapause condition attenuated deleterious virus effects on embryos and that diapause intensity and duration could condition the efficiency of both vector and virus overwintering.

10. Co-regulation of vector and virus transcription

Studies were then conducted to determine the replicative state of LAC virus in *Ae. triseriatus* ovaries during oogenesis and in embryos during embryogenesis and overwintering. A reverse transcription-polymerase chain reaction (RT-PCR) technique was developed to detect LAC virus replicative forms [i.e. virion complementary (vcRNA) and messenger (mRNA) as well as genomic RNA (vRNA)] in mosquitoes and mosquito tissues [38]. The studies revealed that vector and virus transcription are co-regulated in mosquito midguts and ovaries. In orally infected mosquitoes, LAC virus mRNA, vcRNA, and in some instances, even vRNA became undetectable in mosquito ovaries that became gonadotropically inactive after long periods of

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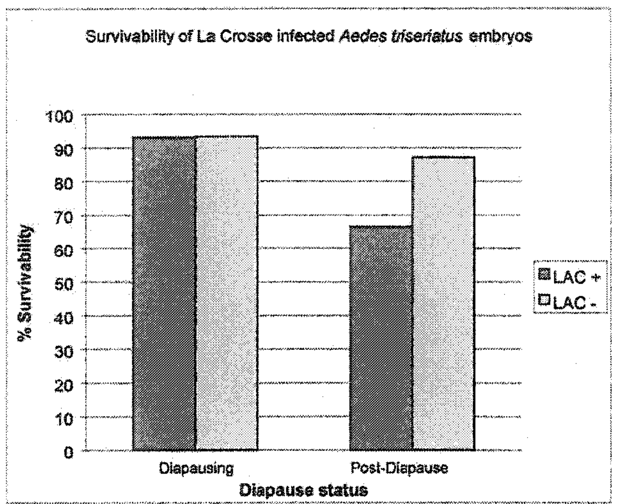

Fig. 3. Survivability of LAC virus infected diapausing and nondiapausing *Ae. triseriatus* embryos. LAC virus infected and noninfected *Ae. triseriatus* were induced into diapause and placed in natural conditions in Wisconsin to overwinter during the winter of 1993–1994. Eggs were returned to Colorado at predetermined times and assayed for embryo viability and virus infection. Diapause typically is broken in mid-April in Wisconsin when the photoperiod exceeds 13 h of daylight. Survivability rates are presented for diapausing and post-diapausing mosquitoes, respectively. Figure adapted from McGaw et al. [37].

extrinsic incubation. Upon ingestion of a noninfective blood meal 28 d after the infection, ovarian metabolic activity was induced and virus replication was reinitiated immediately [38] (Fig. 4). Virus replication may have been suppressed or the virus may have been cleared from the ovaries, which then again became permissive to virus infection and replication upon the reinitiation of gonadotropic activity.

11. Targeted cap scavenging from host mRNA species

Alternatively, cap scavenging could target abundant host mRNA species that condition embryo survival, which could also serve to modulate virus virulence in developmentally critical periods of the vector life cycle. In the studies to characterize the effect of LAC virus on overwintering of *Ae. triseriatus*, strand-specific RT-PCR analyses revealed that mRNA and vcRNA were detectable in both the diapausing and nondiapausing eggs throughout overwintering, suggesting that the virus was replicating during embryo diapause [37]. To determine if targeted cap scavenging was occurring, the 5' terminal host sequences on viral mRNAs were determined during and after emergence from diapause [11]. The results were striking: primer sequence heterogeneity in vectors was quite limited. One host mRNA donated most of the primers (5' CCACTCGCCACT) for virus transcription during embryogenesis and after emergence of embryos from diapause. Interestingly, the same primer sequence also predominated in persistent virus infections of cultured *A. albopictus* cells, suggesting that the donor gene is conserved in *Aedes* species. We have now identified a

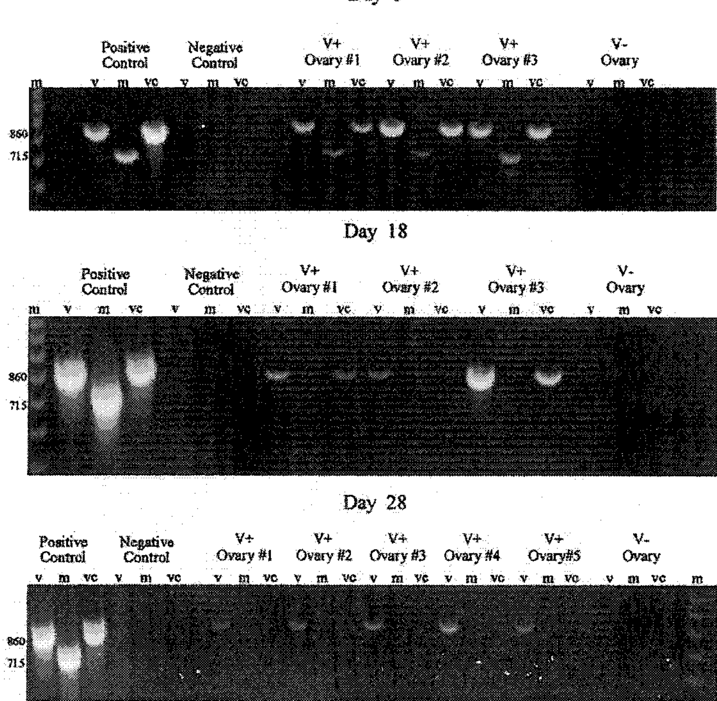

Fig. 4. RT-PCR analysis of LAC virus replication in mosquito ovaries. Ovaries were dissected from LAC virus infected mosquitoes on days 8, 18, and 28 post-infection, and assayed for LAC virus genomic RNA (v), mRNA (m), and virion complementary RNA (vc) by RT-PCR. By day 18, mRNA was no longer detectable in some ovaries, and by day 28, both mRNA and vcRNA were no longer detectable. The presence of mRNA and vcRNA indicates virus transcription and replication. Data originally published in Chandler et al. [38]. V+ = LAC virus infected ovaries; V− = uninfected ovaries.

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host mRNA with this sequence; it has significant similarity to the *Drosophila* inhibitor of apoptosis (IAP)1 gene (unpublished data) [39]. Virus perturbation of host cell apoptosis regulation would appear to compromise mosquito embryonic development, and thus jeopardize maintenance of persistent infection. However, the putative IAP mRNA has a 401-nt 5' untranslated region that is potentially highly structured and could serve as an internal ribosome entry site. This would allow cap-independent translation, as has been found for other cellular gene products implicated in apoptosis inhibition [40].

12. Apoptosis restriction in vector cells

Identification of an mRNA for an apoptosis regulatory gene as a potential target for LAC virus during long-term persistent infections prompted us to determine if there are fundamental differences in apoptosis regulation in LAC virus-infected vertebrate and invertebrate cells. Other studies have shown that LAC virus infection induces apoptosis in both mouse and human neural cell cultures and in the mouse CNS [25]. Since cleavage of genomic DNA into internucleosomal fragments at approximately 180–200 base pair intervals is a characteristic feature of apoptosis [39], we compared electrophoretic patterns of low molecular weight DNA isolated from cultured mammalian (BHK-21) and mosquito (C6/36) cells 36 h post LAC virus infection, at MOI = 1. We demonstrated a DNA laddering pattern typical of apoptosis in BHK-21 cells, but not in C6/36 cells (Fig. 5). Thus, LAC virus also induces apoptosis in non-neural mammalian cells. In contrast, LAC virus did not induce apoptosis in mosquito cells, which would be incompatible with establishing lifelong, noncytolytic infection in mosquito vectors.

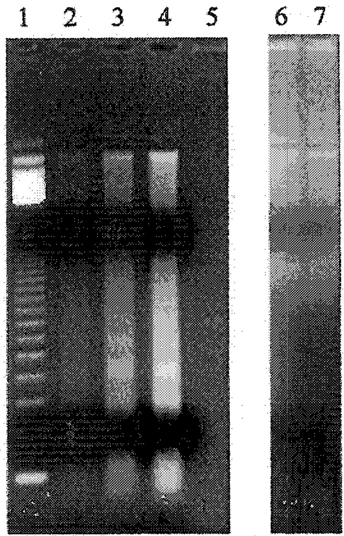

Fig. 5. Demonstration of LAC virus-induced apoptosis in BHK-21 cells, but not in *Aedes albopictus* C6/36 cells. Low molecular weight DNA from LAC virus-infected cultured cells was examined by agarose gel electrophoresis and ethidium bromide staining. Lane 1: 123 bp ladder, Lane 2: LAC-infected BHK-21 cells (100 ng of DNA), Lane 3: LAC-infected BHK-21 cells (500 ng), Lane 4: LAC-infected BHK-21 cells (1 μg), Lane 5: Mock-infected BHK-21 cells (1 μg), Lane 6: LAC-infected C6/36 cells (20 μg), Lane 7: Mock-infected C6/36 cells (20 μg). DNA laddering consistent with apoptosis is evident in Lanes 3 and 4 (vertebrate cells), but not in Lanes 6 and 7 (mosquito cells).

13. LAC virus passage between host systems

The viral mechanisms that permit efficient passage between vector and vertebrate hosts also have been the subject of much speculation [41]. The quasispecies model best accounts for the ability of LAC virus to adapt to the physiologically disparate vertebrate and invertebrate host systems so quickly [42]. RNA viruses, and especially negative-sense RNA viruses, exhibit extremely high base substitution error frequencies (e.g 10^{-3}), presumably due to poor fidelity of replicative enzymes and the lack of proofreading enzymes [43]. Due to this high error frequency, mutations occur in each replication of the genome. Thus, these viruses exist as quasispecies populations comprised of viruses that differ slightly from one another [43]. These subpopulations are present for rapid adaptation upon passage into new hosts.

We conducted studies to determine the effect of passage on the LAC virus genome and demonstrated that laboratory passage may rapidly introduce genetic changes. A 986-nt region of the G1 glycoprotein ORF was amplified directly from CNS specimens from two fatal cases of LAC encephalitis that occurred in the same geographic area in 1960 [14] and 1978 [44]. To investigate the effect of isolation and passage of LAC virus in suckling mice and cell culture, the nucleotide sequences were compared to those determined for the same isolates after limited passage in the laboratory [45]. When the sequence for the passaged 1960 M segment was compared to the nucleotide sequence obtained from the unpassaged viral RNA from the 1960 case [46], there were four nucleotide differences, three of which resulted in changes in the encoded amino acid (Table 1). The viral RNA from the 1978 passaged virus had four nucleotide differences when compared to the sequence of the M segment from the 1978 brain tissues, two of which resulted in changes in the deduced amino acid sequence (Table 1). However, when the nucleotide sequences of the 1960 and 1978 LAC RNA amplified directly from the brain tissues were compared, although four nucleotide differences were seen in this region of the M segment, only one of these resulted in an amino acid change (Table 1). Therefore, the few passages in the laboratory resulted in greater genetic change than that which occurred during 18 years of natural passage. Similarly, little genetic change was detected between the two unpassaged viruses when a 668-nt region of the S segment was analyzed [46].

There are several possible explanations for these regions of the LAC genome being conserved over a period of

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Table 1
Comparison of LAC virus M segment amino acid sequence in passaged and unpassaged viruses. LAC virus RNA sequences were amplified by RT–PCR from CNS tissues from human fatal cases and from viruses isolated from these tissues and passaged in suckling mice and/or cell culture [46]

LAC virus sample	Amino acid position			
	591	618	748	795
1960 Unpassaged	Asp	Trp	Thr	Gln
1960 Passaged	Asp	Arg	Ile	Pro
1978 Unpassaged	Asp	Trp	Thr	Arg
1978 Passaged	Asn	Trp	Ile	Arg

18 years. LAC virus may have achieved a fitness peak in its natural cycle in the La Crosse, WI area. There may be genotypes of LAC virus circulating in nature that are particularly neurovirulent in humans [45]. Alternatively, LAC CNS infection in human hosts may select for a member of the LAC quasispecies capable of causing a viremia titer high enough to allow the virus to become neuroinvasive.

14. Effect of vector passage on the LAC genotype

Studies were conducted to determine if the quasispecies model could explain efficient passage between vector and vertebrate hosts. Three different strains of LAC virus were used to orally infect *Ae. triseriatus* mosquitoes [47]. Two of the strains (WT LAC and LAC ORI) had been passed numerous times in the laboratory; the third strain (SM1-78) had been passed only once in mice. A portion of the N, G1 and G2 ORFs of each LAC strain was sequenced before the virus was used to infect the mosquitoes, after the virus replicated in the midgut of infected mosquitoes, after dissemination to the ovaries and salivary glands, and after the virus had been transmitted transovarially to progeny. The analyzed regions of N and G2 ORFs were found to be relatively stable in all three strains of LAC. After mosquito passage, however, both nucleotide and amino acid changes were detected in the G1 ORF of the two LAC strains that had been passed several times in the laboratory prior to use in this study. A number of these mutations were passed on to the progeny. The LAC strain that had been passed only once in mice did not show significant changes in the G1 ORF after replication in mosquitoes [47]. The genetic variability in LAC virus during passage in a single vector was determined using single strand conformation polymorphism analysis (SSCP) [48]. SSCP analysis (Fig. 6) revealed that LAC quasispecies selected by growth in cultured mosquito cells showed less haplotype variability than did viruses from an infected mosquito and that as infection disseminated from the midgut of the infected mosquito to other organ systems, the number of haplotypes also decreased [47].

15. Evolutionary potential of LAC virus

Long-term persistent infection of arthropods enhances viral evolutionary potential [41]. LAC virus evolves by antigenic drift (RNA intramolecular changes) and antigenic shift (RNA segment reassortment), both of which occur more frequently during long-term persistent infections [1,17,40]. Intramolecular change is probably the most important evolutionary mechanism for LAC virus. Indeed, no two isolates of LAC virus from nature are identical [8,17], which would be predicted by the quasispecies model. In addition, long-term persistent infections during TOT would allow the accumulation of genetic diversity in these populations in nature [41]. Then passage in alternate host systems as noted above could result in selection of the most fit virus genotypes in the new system.

Antigenic shift of bunyaviruses, including LAC virus, occurs at high frequency in laboratory systems, and has been documented to occur in nature [1,17]. The group C viruses of Brazil are especially noteworthy in this regard [1,17]. In nature, segment reassortment could occur in the vertebrate host or in the vector. Despite high-frequency reassortment of LAC and SSH virus in vertebrate cells in vitro, reassortment between these viruses has not been detected in mice in laboratory studies [17]. This is presumably due to the ephemeral nature of the infection in most vertebrate hosts due to production of antibody and the lack of dual infection of cells. In contrast, high-frequency reassortment occurs in vectors that are dually infected orally by ingesting two viruses simultaneously or asynchronously [49]. More than 25% of vectors thus exposed to two viruses become dually infected and, significantly, transmit newly reassorted viruses to vertebrate hosts [49]. Importantly, there is a limited window of time for dual infections to

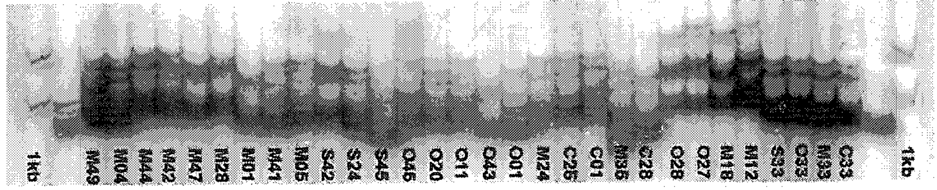

Fig. 6. SSCP analysis of LAC haplotypes in organs from a single infected mosquito. Multiple molecular clones of a region of G1 glycoprotein cDNA from a midgut, ovaries, and salivary glands from a LAC infected mosquito as well as multiple clones from LAC infected C6/36 cells were analyzed by SSCP for LAC haplotypes [47]. Representatives from several haplotypes were analyzed together with the consensus haplotype found in each tissue. The consensus haplotypes are samples C33, M33, O33, and S33. M = midgut clones, O = ovary clones, S = salivary gland clones, C = C6/36 cell culture clones.

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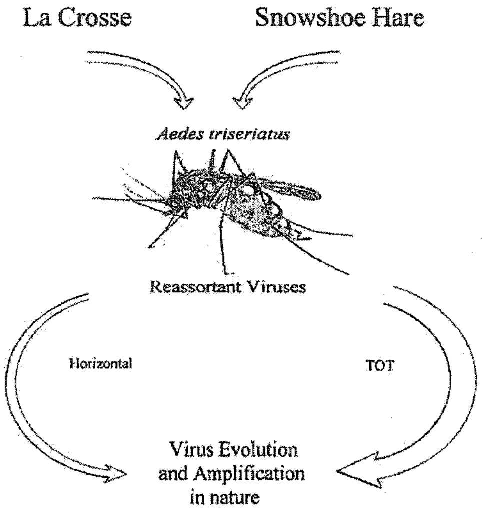

Fig. 7. LAC virus evolution in dually infected mosquitoes. Mosquitoes can become dually infected by ingestion of two viruses simultaneously or via interrupted feeding. Alternatively, mosquitoes infected transovarially with one virus can be superinfected with an alternate virus. Dually infected mosquitoes serve as the site for segment reassortment, and the newly evolved viruses can then be transmitted orally or transovarially to be expressed in nature.

occur; if a mosquito ingests a blood meal containing a potentially superinfecting virus two to three days after the initial virus, there is resistance to superinfection. The molecular basis for this is unclear. In nature, however, many mosquitoes exhibit an interrupted feeding behavior that can preclude interference. If a mosquito begins a blood meal and subsequently defensive behavior of the host causes the mosquito to interrupt its feeding, it will frequently feed to repletion on an alternate host in a short period of time. Thus, if both hosts were viremic, dual infection and segment reassortment could occur (Fig. 7).

There is an alternate, more biologically relevant mechanism for dual infection of mosquitoes to occur (Fig. 7). As noted, *Ae. triseriatus* mosquitoes are efficiently transovarially infected (TI+) with LAC virus. The nature of the infection differs in TI+ mosquitoes; titers are generally lower in all tissues than in orally infected mosquitoes. Thus, we assessed the potential for oral superinfection of TI+ mosquitoes [50]. Approximately 20% of LAC TI+ mosquitoes became superinfected by ingesting blood meals containing wild-type LAC or SSH viruses. LAC TI+ mosquitoes superinfected with SSH were detected by RT-PCR. Viruses from these mosquitoes were plaque-purified and genotyped using RT-PCR. Reassortant genomes were detected in 2.3% of the viruses genotyped, and 4.0% of the genomes tested were heterodiploid. This would seem to be an efficient means for generation of reassortant viruses in nature. TI+ females would emerge and perhaps feed upon a viremic vertebrate host. Since the females are infected for life, each meal would provide the opportunity for ingestion of a new virus. Superinfecting virus could then infect ovaries, where dual infection of the metabolically active tissues would result, and segment reassortment would occur. New virus genotypes would then be expressed in the progeny (Fig. 7).

16. Summary

LAC virus replication in its vector and vertebrate hosts differs dramatically. Replication in vertebrate hosts is typically cytolytic, ephemeral, and inconsequential in terms of virus evolutionary potential. In contrast, infection in vectors results in a lifelong persistent infection, which can enhance the evolutionary potential of the virus. Persistent infection of vectors may be conditioned by co-regulation of virus and vector transcription, by targeted cap scavenging , and by repression of apoptosis. The tripartite, negative-sense genome of LAC virus provides opportunities for virus evolution via both intramolecular changes (antigenic drift) and segment reassortment (antigenic shift), especially during long-term persistent infections. The genomic plasticity of LAC virus (i.e. the quasispecies) also conditions efficient passage of virus between host systems.

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