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Analysis of La Crosse Virus S-Segment RNA and Its Positive-Sense Transcripts in Persistently Infected Mosquito Tissues

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La Crosse (LAC) virus is an important cause of pediatric arboviral encephalitis in the United States. LAC virus is biologically transmitted by the mosquito *Aedes triseriatus*, and, like other arthropod-borne viruses, it establishes a persistent, nonpathogenic infection in its vector following oral infection. To investigate LAC virus persistent infection of mosquitoes, a reverse transcription-PCR assay was developed for the amplification of LAC virus negative-sense small (S) genome RNA segment, its full-length complement, and its mRNA transcript for qualitative analysis of transcription and replication in persistently infected mosquito tissues. RNAs were assayed from midguts removed at predetermined times after infection with a LAC virus-containing blood meal. LAC virus genome was detected almost uniformly in midguts at days 3 to 28 postinfection (p.i.) and, as the time p.i. progressed, in more of the samples than either mRNA or viral cRNA (vcRNA). Thus, persistent LAC virus infection of *A. triseriatus* midguts was correlated with a reduction in detectable viral mRNA and vcRNA. The assay was also used for analysis of virus-specified RNA in both quiescent and biosynthetically active mosquito ovaries. Viral replication decreased, as indicated by the absence of viral mRNA and vcRNA, in the ovaries of mosquitoes that did not receive further blood meals after their original oral infection. Viral replication increased in ovaries of mosquitoes that took an additional blood meal 30 days p.i. and was continuous in mosquitoes that took multiple meals to stimulate oogenesis. Thus, virus replication in persistently infected mosquito ovaries was dependent on host cell biosynthetic status.

La Crosse (LAC) virus is a member of the *Bunyavirus* genus in the family *Bunyaviridae* (4). The genome of bunyaviruses consists of three segments of negative-sense RNA. During replication, each bunyavirus genome segment serves as a template for transcription of truncated mRNA, which uses host cell mRNA primers, and for transcription of full-length viral cRNA (vcRNA). The LAC virus small (S) segment encodes the nucleocapsid (N) protein and a small nonstructural protein (11, 18).

LAC virus is a mosquito-borne arbovirus and the causative agent of an important form of pediatric encephalitis in the United States (6). The virus is biologically transmitted among susceptible vertebrate hosts by the mosquito *Aedes triseriatus* (3, 4). Following mosquito ingestion of a virus-containing blood meal, the midgut is the first tissue infected. After replication in the midgut, the virus is disseminated to infect other tissues, including the salivary glands and ovaries (4). Ovarian infection can result in transovarial transmission of the virus (23), which permits overwintering of the virus in the mosquito eggs (5, 12, 24). In contrast to potentially pathogenic infections in vertebrates, mosquito tissues become persistently infected with LAC virus for life without apparent untoward effects. The molecular events involved in establishment and maintenance of persistent, noncytopathic infections in mosquitoes and mosquito cell culture (10, 17) are poorly understood. Previous studies revealed that the percentage of mosquito progeny infected by transovarial transmission correlated with the titer of virus in the ovaries. Female mosquitoes with the highest ovarian titers produced the highest proportion of infected progeny; virus titer in the ovaries was increased 6- to 12-fold by ingestion of uninfected blood meals (7). Total RNA synthesis in mosquito ovaries increases after ingestion of blood, reaching peak levels 36 to 48 h after the blood meal (2). In non-blood-stimulated ovaries, RNA levels are comparatively reduced (9). The requirement of bunyaviruses for capped 5′ ends of host mRNAs to prime viral mRNA synthesis (14) suggests a possible connection between increase of host RNA synthesis following a blood meal and stimulation of viral replication in mosquito ovaries.

Arbovirus-vector interactions have traditionally been studied by virus isolation from and/or antigen detection in the vector, but these techniques do not distinguish between virus replication and the presence of stable, nonreplicating virions. Hybridization assays and other techniques used to detect nucleic acids have been used as alternatives to virus isolation and immunoassay for characterizing virus infection (13, 19); however, hybridization assays are relatively insensitive and have limited practicality for studying virus transcription and replication in samples containing minute amounts of viral RNA, such as tissues from individual vectors (22).

We report here the use of reverse transcription (RT)-PCR for studying LAC viral persistence and replication in mosquito tissues. The LAC virus RT-PCR provided strand-specific amplification of LAC virus S-segment viral RNA (vRNA), mRNA, and vcRNA. This technique was used to test the hypotheses that persistent LAC virus infection of *A. triseriatus* midguts is regulated at the level of RNA synthesis and that viral replication in persistently infected ovaries is influenced by the biosynthetic and/or reproductive state of the organ.

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TABLE 1. Primers for RT-PCR of LAC virus S-segment RNA

RT primer name	Sequence (5′→3′)	Location relative to LAC S RNA genome segment[a]	Paired primer for PCR	Template	Product size (bp)
LNF (LAC virus N-gene forward primer)	TCAAGAGTGTGATGTCGGATTTGG	Complement of nt 71–94	LVC	vRNA	860
LNR (LAC virus N-gene reverse primer)	GGAAGCCTGATGCCAAATTTCTG	Nt 741–763	LNF	mRNA, vcRNA	715
LVC (LAC virus vcRNA reverse primer)	TTTTGCTGTCCCCTACCACC	Nt 892–911	LNF	vcRNA	860

[a] Nt, nucleotides.

MATERIALS AND METHODS

Virus. Prototype LAC virus was obtained from the World Health Organization Arbovirus Reference Center at the Yale Arbovirus Research Unit, New Haven, Conn. This virus was originally isolated in 1965 from the brain of a patient with LAC virus encephalitis (20) and had been passaged eight times in mice and vertebrate cell culture. Stock virus was prepared in BHK-21 cell cultures.

***A. triseriatus* mosquito colony.** *A. triseriatus* (Bluff strain) mosquitoes were originally collected as eggs from breeding sites near La Crosse, Wis., in August 1992. Mosquitoes were maintained in an insectary at 20 to 23°C and 80% relative humidity, with a daily cycle of 16 h of light–8 h of darkness. Larvae were fed Tetra-Min fish food. Adult mosquitoes were provided sugar cubes, raisins, and distilled H_2O. As a source of blood for egg development, an adult mouse was offered to the mosquitoes once a week. Each generation was maintained separately, and laboratory generations F6 and F7 were used in these studies.

Mosquito infection and processing. (i) Positive-control mosquitoes. Positive-control mosquitoes were infected by intrathoracic inoculation of 10 to 50 50% BHK cell infective doses of LAC virus and held for 14 days; they were then cold anesthetized, and their severed heads were squashed on slides and examined by fluorescent-antibody staining (5). Fluorescent-antibody-positive mosquitoes were frozen at −70°C prior to RNA extraction.

(ii) Oral infection of mosquitoes with LAC virus. Experimental mosquitoes were orally infected with LAC virus in a blood meal. Blood meals were prepared by mixing equal volumes of infected cell culture medium containing $10^{7.5}$ PFU of LAC virus per ml and a suspension of washed sheep erythrocytes in fetal bovine serum containing 10% sucrose; the mixture was offered to mosquitoes in droplets placed onto the netting of their cartons for 1 h. Fully engorged mosquitoes were selected for use in the studies. On selected days postinfection (p.i.), midguts or ovaries were removed from mosquitoes, rinsed in phosphate-buffered saline, and stored at −70°C prior to RNA extraction. All mosquitoes were monitored for disseminated virus infections by fluorescent-antibody staining of LAC virus antigen in their heads (5); only mosquitoes with disseminated infections were used in the ovary analyses.

RNA extraction from mosquitoes. RNA was extracted from individual mosquitoes or mosquito organs by using the acid guanidinium thiocyanate-phenol-chloroform method (8), modified for whole mosquitoes (22), and RNA was purified with an RNaid kit (Bio 101, Inc., La Jolla, Calif.). Samples were stored at −70°C prior to RT-PCR. Each sample was analyzed for the presence of all three RNA species.

RT and PCR primers. Primers for specific RT of LAC virus S-segment vRNA, mRNA, and vcRNA were selected from published sequences (1) with OLIGO 4.0 computer software (National Biosciences, Plymouth, Minn.) (21). The locations and sequences of primers and the sizes of products are given in Table 1. LAC virus S-segment vcRNA transcripts were approximately 100 nucleotides longer at the 3′ end than S-segment mRNA transcripts. The LAC virus vcRNA reverse primer (LVC) bound to the S-segment complement downstream from the 3′ end (nucleotide 886) of the S-segment mRNA; thus, it primed RT of vcRNA but not mRNA. The LAC virus N-gene reverse primer (LNR) primed RT of both mRNA and vcRNA.

LAC virus strand-specific RT-PCR. RNA was incubated at 70°C for 10 min with 15 pmol of the appropriate RT primer in a total volume of 10 μl. After cooling to room temperature, the RNA was reverse transcribed with 67 U of Superscript II (Gibco-BRL, Gaithersburg, Md.) in 1× first-strand buffer (10 mM dithiothreitol, 10 μmol of deoxynucleoside triphosphates, and 40 U of RNasin) in a 20-μl reaction mixture. Two microliters of cDNA was added to 48 μl of 1× PCR buffer (1.5 U of *Taq* DNA polymerase [Promega, Madison, Wis.], 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl [pH 9.0], 0.1% Triton X-100, 200 μM each nucleotide, and 50 pmol of each primer), and the mixture was incubated at 94°C for 1 min, 58°C for 1 min, and 70°C for 2 min through 25 cycles; a final extension was done at 72°C for 7 min. PCR products were analyzed by electrophoresis in 1.5% agarose gels. Plasmids containing vRNA and mRNA cDNA inserts were designated pLVC and pLNR, respectively (21). Runoff transcription from linearized plasmid templates was used to produce vRNA, vcRNA, or mRNA, all of which were used as positive controls for the RT-PCR.

Internal control for RT-PCR of mosquito RNA. An RT-PCR technique for detection of mosquito β-actin mRNA was developed as an internal control for RT reactions. The sequence of *A. triseriatus* actin is not known; however, primers based on the *Bombyx mori* β-actin sequence were used to reverse transcribe and amplify by PCR a 396-bp product from *A. triseriatus* actin mRNA. The forward primer sequence was 5′TCAGGTAGTCGGTCAGAT; the reverse primer sequence was 5′TCCAGAGCAAGAGAGGTA. The positive-control template for actin PCRs was part of the *A. triseriatus* β-actin gene cloned into the plasmid vector pGEM and designated pA396 (21).

RESULTS

LAC virus strand-specific RT-PCR. The specificity of the RT-PCR assay was verified with vRNA, mRNA, and vcRNA transcribed from pLVC and pLNR. Products of the expected sizes (Table 1) were obtained in each assay (data not shown). The LAC virus S-segment vRNA and vcRNA RT-PCRs were specific for vRNA and vcRNA, respectively. The mRNA RT-PCR detected both mRNA and vcRNA. When mosquito RNA was subjected to RT-PCR with primers specific for β-actin, a 396-bp product resulted. A second, minor band, approximately 550 bp in size, was occasionally obtained (data not shown).

Analysis of LAC virus RNA synthesis in *A. triseriatus* midguts by strand-specific RT-PCR. The S-segment RT-PCR was used for qualitative analysis of LAC virus RNA species in individual midguts. Mosquitoes were orally infected with LAC virus, and on days 1, 3, 6, 9, 14, and 21 p.i., midguts were removed and each was assayed for LAC virus-specified RNAs.

LAC virus genomic RNA (vRNA) was detected in more of the samples from persistently infected mosquitoes than either mRNA or vcRNA; it was detected in a total of 84% (27 of 32) of the midguts from days 3 to 28 (Table 2). LAC virus S-segment mRNA was detectable at day 1 p.i. and in 33 to 70% of the individual midguts on days 3 through 28 (Table 2). At day 3 p.i. only 50% of the midguts contained detectable

TABLE 2. LAC virus S-segment-specific RNA in infected mosquito midguts

Day p.i.	Detection rate [no. positive for RNA/no. infected with LAC (% positive)] for:		
	vRNA[a]	mRNA[b]	vcRNA[c]
1	0/1 (0)	1/1 (100)	0/1 (0)
3	6/10 (60)	7/10 (70)	5/10 (50)
6	5/5 (100)	3/5 (60)	2/5 (40)
9	4/5 (80)	3/5 (60)	3/5 (60)
14	6/6 (100)	4/6 (67)	2/6 (33)
21	3/3 (100)	1/3 (33)	1/3 (33)
28	3/3 (100)	2/3 (67)	0/3 (0)

[a] $\chi^2 = 2.8$; $P = 0.83$.
[b] $\chi^2 = 4.8$; $P = 1.0$.
[c] $\chi^2 = 13.1$; $P = 0.04$.

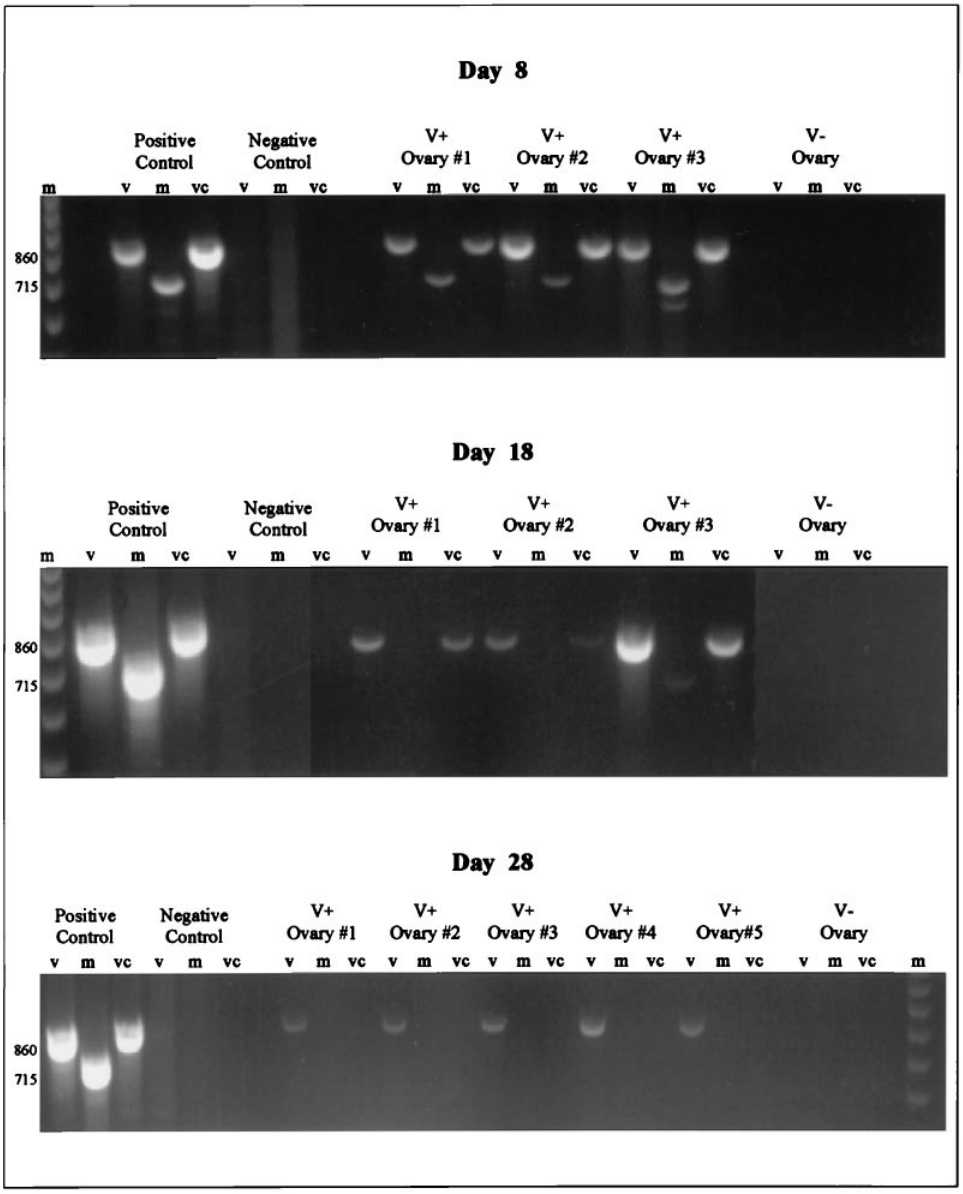

FIG. 1. RT-PCR analysis of LAC virus replication in mosquito ovaries at days 8, 18, and 28 p.i. Mosquitoes were orally infected with LAC virus on day 0 and sampled on day 8, 18, or 28 p.i. One-fifth of the total RNA obtained from a pair of individual mosquito ovaries was subjected to RT-PCR for detection of vRNA (v), viral mRNA (m), or vcRNA (vc). V+, virus-infected; V−, uninfected. Numbers on the left are molecular sizes, in base pairs.

vcRNA, and the proportion containing detectable replicative template declined during days 14 to 28. Chi-square analysis revealed that there was not a significant decline in mRNA ($P > 0.05$). In contrast, the decline in vcRNA was statistically significant ($P < 0.05$). These results suggest that a persistent infection of the midgut of mosquitoes orally infected with LAC virus was characterized by stable maintenance of vRNA, a possible reduction in mRNA, and a reduction in detectable vcRNA.

LAC virus RNA synthesis in inactive ovaries. To examine replication and persistence of LAC virus in the ovaries, two experiments were performed. In the first experiment, mosquitoes were blood fed LAC virus on day 0 and then follicular resting, or quiescence, was induced by deprivation of additional blood meals. LAC virus RNA in the ovaries was monitored by RT-PCR every 3 to 5 days from 14 to 30 days p.i. As in persistently infected midguts, viral replication decreased over time (Fig. 1). Genomic RNA was detectable in 100% of the mosquito ovaries through day 28 and in 7 of 10 mosquitoes on day 30 (Table 3). Fewer mosquitoes had detectable mRNA and vcRNA, and the proportion declined over time. At days 14 and 18, two of three mosquitoes did not have detectable viral mRNA in the ovaries but did have vcRNA. At day 21 p.i., no viral mRNA or vcRNA was detectable in the three mosquitoes examined. By day 30, only 10% of mosquitoes had mRNA and vcRNA detectable in the ovaries (Table 3). These results confirmed the hypothesis that when ovaries were inactive, virus replication was concomitantly down-regulated. Chi-square analysis revealed that the reduction of mRNA and vcRNA,

TABLE 3. LAC virus S-segment-specific RNA in the ovaries of mosquitoes that were not refed after the initial infection (experiment 1)

Day p.i.	Virus antigen in heads (no. positive/total no.)[a]	RT-PCR detection rate [no. positive/total no. (% positive)]			
		vRNA[b]	mRNA[c]	vcRNA[d]	β-Actin mRNA[e]
14	3/3	3/3 (100)	1/3 (33)	3/3 (100)	3/3
18	3/3	3/3 (100)	1/3 (33)	3/3 (100)	3/3
21	3/3	3/3 (100)	0/3 (0)	0/3 (0)	3/3
25	5/5	5/5 (100)	1/5 (20)	2/5 (40)	5/5
28	10/10	10/10 (100)	1/10 (10)	3/10 (30)	9/9
30	10/10	7/10 (70)	1/10 (10)	1/10 (10)	10/10

[a] $P = 0.9625$.
[b] $P = 0.9702$.
[c] $P = 0.0014$.
[d] $P = 0.0032$.
[e] $P = 0.9625$.

indicative of reduced virus replication, was statistically significant ($P < 0.05$) (Table 3).

LAC virus RNA synthesis in active ovaries. A second experiment was performed to compare viral replication in active (egg-producing) ovaries with that in quiescent ovaries. Mosquitoes were blood fed with LAC virus on day 0 and then divided into two groups: (i) mosquitoes that blood fed on an uninfected mouse three or four times over the course of 30 days and (ii) mosquitoes that received one uninfected blood meal 30 days later. Mosquitoes that were exposed to but did not blood feed on the adult mouse at day 30 were used as non-blood-fed controls.

(i) LAC viral replication in the ovaries of mosquitoes blood fed at weekly intervals. Among a total of 46 mosquitoes with disseminated infections that had blood fed three or four times, 100% had vRNA, mRNA, and vcRNA detectable in their ovaries 35 days p.i. (data not shown). In the mosquitoes that were exposed once to the blood source but did not engorge, viral replication in the ovaries was decreased: 80% (8 of 10) of these mosquitoes had detectable vRNA, whereas only 30% had detectable mRNA and 50% had detectable vcRNA (data not shown). This experiment confirmed the hypothesis that when ovary activity is maintained by blood feeding, virus replication is ongoing.

(ii) LAC viral replication in the ovaries of mosquitoes blood fed again on day 30. One group of mosquitoes blood fed on day 0 was held for 29 days with no further blood meals, to allow follicular quiescence to occur. They were blood fed a second time on day 30 and assayed 24, 48, 72, or 96 h after the second feeding to determine if stimulation of ovary activity would reactivate viral replication. Viral replication in the ovaries was increased after the mosquitoes received the second blood meal (Table 4). Viral mRNA and vcRNA were detectable in 100% of the ovaries up to 96 h postfeeding. However, the differences in detectable mRNA and vcRNA between the refed mosquitoes and the non-refed controls were not statistically significant (for mRNA, $\chi^2 = 4.45$; for vcRNA, $\chi^2 = 2.27$; in both cases, $P > 0.05$). Of the 11 control mosquitoes, assayed on day 30 without refeeding, 4 had mRNA detectable in the ovaries and 6 had vcRNA detectable in the ovaries (Table 4). It is noteworthy that these control mosquitoes had been exposed to the vertebrate host at day 30 but did not ingest detectable blood. The prevalence of vcRNA and mRNA was greater than in mosquitoes that had not been exposed to the mouse again on day 30 (Table 3). This suggests that vertebrate host exposure itself may stimulate ovarian activity. Nonetheless, the results of this experiment suggest that virus replication can be activated when ovaries are stimulated by a blood meal.

DISCUSSION

Although the ability of LAC virus to persist in mosquitoes is well known, the molecular events underlying establishment and maintenance of persistent infections are not understood. Traditional studies of the vector-virus interaction, using virus isolation or detection of viral antigen, have not distinguished between active viral replication and the presence of stable, nonreplicating virions in mosquito tissues. We have developed and used an RT-PCR assay to specifically identify LAC virus vRNA, mRNA, and vcRNA in tissues of individual mosquitoes. We have assumed that S-segment vcRNA and mRNA are markers for genome replication and gene expression, respectively, and that their detection in biologically relevant mosquito tissues indicates active viral replication. The assay has

TABLE 4. LAC virus S-segment-specific RNA in the ovaries of mosquitoes blood fed on day 30 after the initial infection (experiment 2)

Time of assay (h)[a]	Virus antigen in heads (no. positive/total no.)[b]	RT-PCR detection rate [no. positive/total (% positive)]			
		vRNA[c]	mRNA[d]	vcRNA[e]	β-Actin mRNA[f]
24	10/10	10/10 (100)	10/10 (100)	10/10 (100)	10/10
48	10/10	10/10 (100)	10/10 (100)	10/10 (100)	10/10
72	10/10	10/10 (100)	10/10 (100)	10/10 (100)	10/10
96	12/12	12/12 (100)	12/12 (100)	12/12 (100)	12/12
Controls (not refed)	11/11	10/11 (91)	4/11 (36)	6/11 (55)	11/11

[a] Hours after second blood feeding.
[b] $P = 0.9987$.
[c] $P = 0.9990$.
[d] $P = 0.3485$.
[e] $P = 0.6862$.
[f] $P = 0.9987$.

been used to examine midguts, the initial site of viral amplification after oral infection, and both quiescent and biosynthetically active ovaries of individual mosquitoes.

Our results suggest that persistent LAC virus infection of *A. triseriatus* midguts is regulated at the level of RNA synthesis (Table 2). A decrease in the proportion of mosquitoes with detectable LAC virus vcRNA in the midgut began shortly after infection and was followed by a decrease in detectable LAC virus mRNA, while vRNA was detectable in 100% of the mosquitoes even 28 days p.i. In addition, although the RT-PCR assays used to investigate viral persistence were not quantitative, the results (Fig. 1) also suggest that virus transcription was reduced in many samples with time p.i. These observations are consistent with the report that the rate of synthesis of LAC virus RNAs declines in persistently infected mosquito cell cultures (17) and suggest that a reduction in active LAC virus replication occurs in the midguts of persistently infected mosquitoes, although stable virions remain in these tissues for long periods, perhaps for the life of the mosquito.

Our studies confirmed the hypothesis that reduced viral replication was correlated with reduced host ovarian biosynthetic activity. The persistent virus infection in quiescent ovaries was characterized by reduced numbers of mosquitoes with detectable S-segment vcRNA and mRNA, with uniform maintenance of vRNA (Table 3). In contrast, mosquitoes fed repeated blood meals maintained vcRNA and mRNA in the ovaries throughout the course of the study, and among mosquitoes that were held to establish a persistent viral infection in the ovaries and were then refed, the proportion with mRNA and vcRNA, as well as vRNA, in ovaries increased immediately following the blood meal (Table 4). Previous studies have shown a correlation between repeated blood feeding and high virus titers in ovaries (7); this suggests a possible connection between increased ovarian RNA synthesis due to stimulation by a blood meal (2) and enhanced viral replication. Conversely, the reduction in biosynthetic activity that occurs in quiescent ovaries (9) may affect viral replication by limiting availability of needed host cell mRNAs. Our results support the hypothesis that the ovaries of mosquitoes actively developing eggs are more permissive to virus replication. Virus replication can thus be reactivated when oogenesis is stimulated, which may also result in more efficient transovarial transmission.

These studies did not address the mechanisms for reduction or increase of viral replicative RNAs. During ovarian quiescence, RNA and protein synthesis are reduced (2); thus, host mRNA 5′ oligonucleotide primers, which are required for viral mRNA transcription (14), may be greatly reduced. Bunyavirus transcription has been reported to require ongoing protein synthesis in vertebrate cells (15); however, in invertebrate cells, this requirement apparently does not exist (16). Alternatively, the decreased proportion of mosquitoes with viral mRNA and vcRNA may have been due to degradation, whereas vRNA in virions would be more stable. Virus replication in active ovaries may be increased because large amounts of mRNA and rRNA are being synthesized by the nurse cells (2). In addition, other host cell macromolecules necessary for viral replication may be more available in active ovaries. Because these experiments were designed to detect steady-state levels of RNA, it was not possible to measure the rate of transcription of new mRNA and vcRNA in the ovaries. Nonetheless, the results suggest that host and virus transcription are coregulated during persistent infections.

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REFERENCES

1. **Akashi, H., and D. H. L. Bishop.** 1983. Comparison of the sequences and coding of La Crosse and snowshoe hare bunyavirus S RNA species. J. Virol. **45:**1155–1158.
2. **Banks, G. K., C. A. Malcolm, and A. N. Clements.** 1994. Temporal pattern of RNA and protein synthesis in the ovary of *Aedes aegypti*. J. Insect Physiol. **40:**463–468.
3. **Beaty, B. J., and D. H. L. Bishop.** 1988. Bunyavirus-vector interactions. Virus Res. **10:**289–302.
4. **Beaty, B. J., and C. H. Calisher.** 1991. Bunyaviridae: natural history. Curr. Top. Microbiol. Immunol. **169:**27–78.
5. **Beaty, B. J., and W. H. Thompson.** 1975. Emergence of La Crosse virus from endemic foci. Fluorescent antibody studies of overwintered *Aedes triseriatus*. Am. J. Trop. Med. Hyg. **24:**685–691.
6. **Calisher, C. H.** 1994. Medically important arboviruses of the United States and Canada. Clin. Microbiol. Rev. **7:**89–116.
7. **Chandler, L. J., B. J. Beaty, G. D. Baldridge, D. H. L. Bishop, and M. J. Hewlett.** 1990. Heterologous reassortment of bunyaviruses in *Aedes triseriatus* mosquitoes and transovarial and oral transmission of newly evolved genotypes. J. Gen. Virol. **71:**1045–1050.
8. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
9. **Clements, A. N., and M. R. Boocock.** 1984. Ovarian development in mosquitoes: stages of growth and arrest, and follicular resorption. Physiol. Entomol. **9:**1–8.
10. **Elliott, R. M., and M. L. Wilkie.** 1986. Persistent infection of *Aedes albopictus* C6/36 cells by Bunyamwera virus. Virology **150:**21–32.
11. **Kolakofsky, D., and D. Hacker.** 1991. Bunyaviridae: bunyavirus RNA synthesis: genome transcription and replication. Curr. Top. Microbiol. Immunol. **169:**143–159.
12. **LeDuc, J. W.** 1987. Epidemiology and ecology of the California serogroup viruses. Am. J. Trop. Med. Hyg. **37:**60S–68S.
13. **Olson, K. E., C. D. Blair, and B. J. Beaty.** 1990. Detection of dengue viral RNA in mosquito vectors by mixed phase and solution hybridization. Mol. Cell. Probes **4:**307–320.
14. **Patterson, J. L., B. Holloway, and D. Kolakofsky.** 1984. LaCrosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. J. Virol. **52:**215–222.
15. **Raju, R., and D. Kolakofsky.** 1987. Translational requirement of La Crosse virus S-mRNA synthesis: in vivo studies. J. Virol. **61:**96–103.
16. **Raju, R., L. Raju, and D. Kolakofsky.** 1989. The translational requirement for complete La Crosse virus mRNA synthesis is cell-type dependent. J. Virol. **63:**5159–5165.
17. **Rossier, C., R. Raju, and D. Kolakofsky.** 1988. La Crosse virus gene expression in mammalian and mosquito cells. Virology **165:**539–548.
18. **Schmaljohn, C. S., and J. L. Patterson.** 1990. Bunyaviridae and their replication. Part II. Replication of the Bunyaviridae, p. 1175–1194. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
19. **Schoepp, R. J., J. F. Bray, K. E. Olson, A. El-Hussein, F. R. Holbrook, C. D. Blair, P. Roy, and B. J. Beaty.** 1989. Detection of bluetongue virus serotype 17 in *Culicoides variipennis* by nucleic acid blot and sandwich hybridization techniques. J. Clin. Microbiol. **28:**1952–1956.
20. **Thompson, W. H., B. Kalfayan, and R. O. Anslow.** 1965. Isolation of California encephalitis group virus from a fatal human illness. Am. J. Epidemiol. **81:**245–253.
21. **Wasieloski, L. P.** 1995. La Crosse virus gene expression in *Aedes triseriatus*. Ph.D. dissertation. Colorado State University, Fort Collins.
22. **Wasieloski, L. P., A. Rayms-Keller, L. A. Curtis, C. D. Blair, and B. J. Beaty.** 1994. Reverse transcription-PCR detection of La Crosse virus in mosquitoes and comparison with enzyme immunoassay and virus isolation. J. Clin. Microbiol. **32:**2076–2080.
23. **Watts, D. M., S. Pantuwatana, G. R. DeFoliart, T. M. Yuill, and W. H. Thompson.** 1973. Transovarial transmission of La Crosse virus (California encephalitis group) in the mosquito, *Aedes triseriatus*. Science **182:**1140–1141.
24. **Watts, D. M., W. H. Thompson, T. M. Yuill, G. R. DeFoliart, and R. P. Hanson.** 1974. Overwintering of La Crosse virus in *Aedes triseriatus*. Am. J. Trop. Med. Hyg. **23:**694–700.