Alphavirus-derived small RNAs modulate pathogenesis in disease vector mosquitoes

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Mosquito-borne viruses cause significant levels of morbidity and mortality in humans and domesticated animals. Maintenance of mosquito-borne viruses in nature requires a biological transmission cycle that involves alternating virus replication in a susceptible vertebrate and mosquito host. Although the vertebrate infection is acute and often associated with disease, continual transmission of these viruses in nature depends on the establishment of a persistent, nonpathogenic infection in the mosquito vector. An antiviral RNAi response has been shown to limit the replication of RNA viruses in flies. However, the importance of the RNAi pathway as an antiviral defense in mammals is unclear. Differences in the immune responses of mammals and mosquitoes may explain why these viruses are not generally associated with pathology in the invertebrate host. We identified virus-derived small interfering RNAs (viRNAs), 21 nt in length, in Aedes aegypti infected with the mosquito-borne virus, Sindbis (SINV). viRNAs had an asymmetric distribution that spanned the length of the SINV genome. To determine the role of viRNAs in controlling pathogenic potential, mosquitoes were infected with recombinant alphaviruses expressing suppressors of RNA silencing. Decreased survival was observed in mosquitoes in which the accumulation of viRNAs was suppressed. These results suggest that an exogenous siRNA pathway is essential to the survival of mosquitoes infected with alphaviruses and, thus, the maintenance of these viruses in nature.

RNAi | Sindbis | siRNA | Arbovirus | Aedes aegypti

Although the transmission cycles of many mosquito-borne viruses have been described, this group of pathogens continues to negatively impact public health and national economies on a global scale. One recent example of this is the reemergence of chikungunya virus (family Togaviridae, genus Alphavirus) in Southeast Asia and India with subsequent spread to the European continent (1, 2). The genome of the type alphavirus, Sindbis (SINV), is a positive-sense (+), nonsegmented, single-stranded (ss) RNA 11,703 nt in length (3). The 5’ 2/3 of the genome encode the nonstructural or replicase proteins, whereas the structural genes are encoded in the 3’ 1/3 (3). The structural genes are translated from a subgenomic mRNA (26S RNA) that is transcribed from an internal subgenomic promoter present in a full-length negative sense RNA copy (3). The negative sense copy also functions as the template for production of new genomes (49S RNA). Alphaviruses, like many other viruses, produce double-stranded RNA (dsRNA) during replication (4, 5).

RNAi refers to the process by which intracellular dsRNA is cleaved into siRNAs, and homologous transcripts silenced. Multiple pathways exist within somatic cells for sorting small RNAs. In Drosophila melanogaster, RNAs transcribed from the genome with short inverted repeats are processed by RNase III enzymes in the nucleus (Drosa) and cytoplasm (Dicer-1) to generate microRNAs (miRNAs). These ~22-nt small RNAs are bound by Argonaute proteins and primarily function to regulate endogenous targets. dsRNA produced during virus replication, as well as in vitro-generated dsRNAs are processed by an exogenous siRNA (exo-siRNA) pathway. Several studies have also recently revealed the existence of an endogenous siRNA (endo-siRNA) pathway, suggesting a broad function for endo-siRNAs in the regulation of endogenous gene expression (6). Viral dsRNA is cleaved in the cytoplasm (Dicer-2) to generate viRNAs. These 21-nt small RNAs are mostly bound by “slicer” (Argonaute-2) and facilitate an antiviral response. D. melanogaster loss-of-function mutants that inactivate RNAi exhibit elevated levels of viral RNA accumulation and increased mortality when infected with several (+) ssRNA viruses (including SINV), and knockdown of RNAi protein components in mosquito cells and adults has been shown to increase viral titers (7–12).

Although it is clear that dsRNA functions as a molecular trigger of an antiviral pathway capable of restricting virus replication, less clear is the origin of viRNAs. Some (+) ssRNA plant viruses have been shown to generate viRNAs from both (+) and negative (−) strands in approximately equal proportions, consistent with a replicative intermediate origin, whereas others have been found to generate a majority of viRNAs from (+) strands, suggesting hairpin structures present in genomic RNA can also be processed by the plant antiviral pathway (13–15). However, the origins of viRNAs generated by invertebrate exo-siRNA pathways have not been characterized (16).

In response to viral restriction, suppressors of RNA silencing (SRSs) have been identified in several viruses that infect invertebrate hosts (17). However, it remains unclear whether arboviruses encode similar proteins. The most well-characterized SRS identified in an animal virus thus far is the B2 protein of flock house virus (FHV) (5). A protein of similar function is also present in the distantly related Nodamura virus (NoV) (17). B2 protein is required for FHV replication in cells possessing a fully functional siRNA pathway, and similar phenomena have been observed in cells infected with NoV (7, 8, 10, 18). The structure of B2 suggests that it indiscriminately binds both siRNA duplexes and long dsRNAs, and experimental results are consistent with this (19–22). Thus, B2 acts by preventing the protein components of the siRNA pathway access to dsRNAs associated with FHV infection, but the protection is not absolute (5, 19–22). Accumulation of FHV-derived siRNAs has been shown to correlate with decreased accumulation of viral RNA in infected cells, whereas increases in virus replication are observed after the depletion or mutations of protein components involved in the siRNA pathway (7, 8, 10, 18).

A distinguishing characteristic of alphavirus infections, and more generally arbovirus infections, of the vector host is the establishment of a persistent, nonpathogenic state (23–26). Typically, viral titers increase to high levels during the initial “acute” phase of infection in susceptible cells and tissues but...
Characterization of Alphavirus-Derived siRNAs in *Ae. aegypti*. We characterized the small RNA content of *Ae. aegypti* infected with recombinant SINV viruses. Small RNA libraries were prepared from mosquitoes injected with double subgenomic SIN virus (dsSINV) derived from the infectious cDNA clone pTE/3’2J (28), from mosquitoes injected with dsSINV expressing the FHV B2 protein [dsSINV-B2 (FHV)], or from uninfected mosquitoes. After sequencing by synthesis, we analyzed the total 18- to 24-nt content in each of the 3 small RNA libraries. We expected miRNAs to make up a significant fraction of the total reads, and that this would be evident by a peak at 22 nt (29, 30). Indeed, 22 nt was the most abundant size class in both the uninfected and dsSINV-B2 (FHV) libraries, and was a significant percentage of the dsSINV library (Fig. 1A). Similarly, approximately half of the sequences in each library mapped to mature *D. melanogaster* miRNAs (Table 1). Interestingly, 40% of the sequences in the dsSINV library were 21 nt, compared with 18% and 14% in the dsSINV-B2 (FHV) and uninfected libraries, respectively (Fig. 1A). To determine whether viRNAs were present, small RNAs were mapped (see Materials and Methods) to the dsSINV or dsSINV-B2 (FHV) genome. As expected, the predominant size of sequences mapping to either dsSINV or dsSINV-B2 (FHV) was 21 nt (Fig. 1B). The total number of 21-nt matching small RNA sequences as well as the total number of unique 21-nt matching sequences and the total number of viRNA-generating loci for each genome are shown in Table 1. Importantly, sequences from the uninfected mosquito library could not be mapped to either viral genome with these criteria (Table 1), demonstrating that we had indeed identified exogenously derived small RNAs. Statistically significant reductions in viRNA abundance were observed in mosquitoes infected with dsSINV-B2 (FHV) relative to mosquitoes infected with dsSINV (Table 1). After normalizing for variable sequencing depth between samples, the total abundance of 21-nt viRNAs in the dsSINV-B2 (FHV) sample decreased by a factor of 9.8 (*P* = 0.0000; Fisher’s exact test). Detection of specific small RNAs confirmed that the absolute abundance of viRNAs in mosquitoes infected with dsSINV-B2 (FHV) decreased relative to those in mosquitoes infected with dsSINV (Fig. 2A). Normalized sequencing results indicated that loci corresponding to the probe sequence in dsSINV-B2 (FHV) generated fewer 21-nt viRNAs than identical loci in dsSINV (by a factor of 2.7; Figs. 1C and 2A). These data suggest that the accumulation of viRNAs in mosquitoes infected with dsSINV-B2 (FHV) is suppressed. Sequencing results indicated an asymmetric distribution of overlapping 21-nt viRNAs that mapped the length of dsSINV (Fig. 1C Upper) and dsSINV-B2 (FHV) (Fig. 1C Lower) genomes. The nonrandom distribution of sequences mapping to the SINV genome suggests that some loci are hot spots for

**Table 1. Analysis of small RNA libraries**

<table>
<thead>
<tr>
<th>Category</th>
<th>Uninfected</th>
<th>dsSINV*</th>
<th>dsSINV-B2(FHV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total small RNAs 18–24 nt</td>
<td>1,807,467</td>
<td>3,270,494</td>
<td>867,813</td>
</tr>
<tr>
<td>Total 21-nt viRNAs (unique, 100% match)</td>
<td>0</td>
<td>453,360</td>
<td>(120,297) 12,276</td>
</tr>
<tr>
<td>(+) strand 21-nt viRNAs</td>
<td>0</td>
<td>243,624</td>
<td>64,645</td>
</tr>
<tr>
<td>(-) strand 21-nt viRNAs</td>
<td>0</td>
<td>209,736</td>
<td>55,653</td>
</tr>
<tr>
<td>Total unique viRNA loci</td>
<td>0</td>
<td>15,246</td>
<td>3,911</td>
</tr>
<tr>
<td>(+) strand viRNA loci</td>
<td>0</td>
<td>7,749</td>
<td>2,236</td>
</tr>
<tr>
<td>(-) strand viRNA loci</td>
<td>0</td>
<td>7,497</td>
<td>1,675</td>
</tr>
<tr>
<td>viRNAs/unique loci</td>
<td>0</td>
<td>29.7</td>
<td>3.1</td>
</tr>
<tr>
<td>Total miRNAs (18–24 nt, unique, 100% match)</td>
<td>1,198,435</td>
<td>1,270,991</td>
<td>480,879</td>
</tr>
<tr>
<td>Percentage of total small RNAs = miRNAs</td>
<td>66.3</td>
<td>45.1†</td>
<td>56.2†</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are normalized to the smaller sequence set.
†After excluding viRNAs.

Fig. 1. Small RNAs in *Ae. aegypti* infected with SIN viruses (96 h). (A) Distribution of small RNA sequences (18–24 nt) in libraries prepared from mosquitoes infected with dsSINV or dsSINV-B2 (FHV), or from uninfected mosquitoes. (B) Length of small RNA sequences (18–24 nt) matching the dsSINV or dsSINV-B2 (FHV) genomes (100% match). (C) Distribution of 21-nt viRNA-generating loci in dsSINV and dsSINV-B2 (FHV) genomes. Vertical axis indicates the number of times a nucleotide position in the viral genome was represented in a unique viRNA sequence (normalized by smaller sequence set). Red peaks indicate viRNAs derived from virus (+) strands (siRNAs). Blue peaks indicate viRNAs derived from virus (-) strands (as viRNAs).

then are modulated to lower levels during the persistent phase of the infection (23, 24, 27). The exact mechanism by which viral titers are modulated in mosquito cells is unknown.

The work presented here characterizes in detail viRNAs produced in *Aedes aegypti* infected with an alphavirus. We also demonstrate here that suppressing the accumulation of these viRNAs results in elevated levels of viral RNA and increased mortality. These results suggest that an exogenous siRNA (exo-siRNA) pathway is essential to the survival of mosquitoes infected with alphaviruses and, thus, the maintenance of these viruses in nature.
viRNA generation. Comparison of siRNAs derived from viral positive-sense strands with those derived from negative-sense strands indicated a significant bias for accumulation from positive strands in both the dsSINV (P < 0.0001; χ² test) and dsSINV-B2 (FHV) (P < 0.0001; χ² test) samples (Table 1). These results are consistent with a model in which both long dsRNA replicative intermediates and highly structured regions present in viral RNA transcripts are processed by 1 or more mosquito dicer enzymes; although other explanations are also possible.

Suppressing the Accumulation of viRNAs Results in Elevated Levels of Viral RNA and Increased Mortality. The C44 residue of FHV B2 has been demonstrated to participate in binding the minor groove of dsRNA through Van der Waals interactions (20). C44A and C44S B2 mutants exhibit a >100-fold reduction in binding affinity to dsRNA (20). For this study, a C44Y mutant was generated. The C44Y mutation affects only the primary amino acid sequence of B2 and not that of FHV protein A. In vitro-transcribed FHV RNA1 possessing the C44Y mutation in B2 (RNA1-B2C44Y) self-directed RNA 1 replication and transcription of RNA 3 after electroporation into BHK-21 cells [supporting information (SI) Fig. S1]. However, RNA1-B2C44Y failed to accumulate to detectable levels after electroporation into S2 cells (Fig. S1). Previous studies have shown that B2 is not required for RNA 1 self-replication in BHK-21 cells but is essential for FHV RNA 1 accumulation in S2 cells because the protein suppresses an RNA silencing response that targets FHV RNAs for degradation (18, 31). viRNAs accumulated to similar levels in mosquitoes infected with dsSINV-B2C44Y (FHV) relative to mosquitoes infected with dsSINV at the same time point (Fig. 2A). These results suggest that the C44Y mutation knocks out or significantly reduces the ability of B2 to bind dsRNA.

To investigate the role of viRNAs in modulating alphavirus replication in the vector, mosquitoes were injected with equivalent titers of dsSINV-B2 (FHV) or dsSINV-B2C44Y (FHV). SINV RNA accumulated more rapidly and to higher levels in the presence of a functional FHV B2 protein (Fig. 2B). Although the survival of mosquitoes infected with dsSINV-B2C44Y (FHV) was indistinguishable from uninfected mosquitoes, 100% mortality was observed in mosquitoes injected with dsSINV-B2 (FHV) 6 days after inoculation (Fig. 2C).

Likewise, infection of C6/36 mosquito cells with dsSINV-B2 (FHV) resulted in cytopathological changes that continued to increase in severity over a period of 16 days (Fig. S2A). In contrast, cells infected with dsSINV or dsSINV-B2C44Y (FHV) were morphologically similar to uninfected cells at all time points (Fig. S2A). SINV RNA also accumulated more rapidly and to higher levels in cells infected with dsSINV-B2 (FHV) (Fig. S2B). Taken together, these results suggest that an exo-siRNA pathway is essential to modulating alphavirus replication and pathogenesis in the mosquito.

RNA Silencing Protects Adult Mosquitoes from Alphavirus Infection. To investigate whether other vectors would also be sensitive to alphaviruses expressing SRS proteins, we injected Anopheles gambiae with double subgenomic o’nyong-nyong virus (dsONNV) derived from the infectious cDNA clone p5’dONN/icyo (32), or dsONNV expressing the NoV B2 protein [dsONNV-B2 (NoV)]. NoV B2 shares <30% identity with FHV B2 at the amino acid level. ONNV is unique among arboviruses in that Anopheline mosquitoes have been implicated as the primary vectors during epidemic transmission (33). Although complete mortality was not observed in mosquitoes injected with dsONNV-B2 (NoV) until 11 days after infection, the survival of mosquitoes infected with the control virus (dsONNV) was again indistinguishable from uninfected mosquitoes (Fig. 3). Decreases in the survival of Ae. aegypti injected with TE/3’2J virus expressing the NoV B2 were similar to those observed in mosquitoes infected with TE/3’2J-B2 (FHV) virus (Fig. 3).

The specific infectivity of dsONNV-B2 (NoV) was also examined by infecting An. gambiae mosquitoes with virus-containing artificial blood meals. We have shown previously that ONNV is able to infect 33.1% and 39.8% of An. gambiae 8 and 10 days after virus ingestion, respectively (34). Similarly,
dsONNV expressing GFP has previously been shown to infect 48% of *An. gambiae* 7 days after virus ingestion (32). In contrast, 93.3% (n = 30) and 85.7% (n = 28) of mosquitoes ingesting a similar titer (∼5.9 log_{10} PFU/ml) of dsONNV-B2 (NoV) had detectable infections at 8 and 10 days, respectively. These increases in infectivity are significant when compared with the previously reported data for 7 (P = 0.0058; Fisher’s exact test) days (34), suggesting that an exo-siRNA pathway protects *An. gambiae* from ONNV infection.

**Discussion**

This report describes an example of sequencing and in depth characterization of small RNAs from mosquitoes infected with an arbovirus. Although it is not surprising that viRNAs were recovered from infected mosquitoes, the proportion of small RNAs that were viRNAs was somewhat surprising; viRNAs were found to comprise >10% of the total cellular small RNAs in the 18- to 24-nt size range. This is likely an underestimate, for several reasons. First, we constructed our library using total mosquito RNA. However, SINV does not infect all mosquito tissues, in particular the ovaries, and yet these tissues contributed to the total small RNAs sequenced. Second, we defined viRNAs as sequences exhibiting a 100% match to the SINV genome. These criteria exclude reads possessing mismatches from low sequencing fidelity as well as mismatches derived from high fidelity sequencing of natural variants within the viral quasispecies population. Last, similar to other recent studies we used a heuristic, rather than exhaustive, approach to map small RNAs (29, 30, 35–37).

Analysis of our small RNA libraries appears to suggest that SINV infection is associated with a decrease in total miRNA abundance, an effect that appears to be at least partially restored in the presence of FHV B2 (Table 1). However, these numbers must be interpreted with care, because complete miRNA sequences have not yet been annotated in the *Ae. aegypti* genome. Thus, it is possible that there may be additional unique, unannotated *Ae. aegypti* miRNAs or miRNA* variants in SINV-infected mosquitoes that we are at present unable to map. Comparing the relative abundance (normalized for sequencing depth) of specific *D. melanogaster* miRNAs among all 3 libraries provided individual examples of decreased, increased, and unchanged representation between samples (data not shown). Northern blot analysis also demonstrated that the abundance of mature miR-1 was unchanged between dsSINV and dsSINV-B2 (FHV)-infected mosquitoes (data not shown). Ultimately, however, it will be necessary to obtain more information, such as pre-miRNA sequences of mosquito miRNAs, before we can draw conclusions about representational differences in the abundance of miRNAs in these samples.

The transmission of an arbovirus is influenced by several complex parameters, such as the vector population density in relation to the vertebrate host, host preference, feeding frequency, length of extrinsic incubation period, vector competence, and the probability of daily survival for the vector. These parameters have been described by Macdonald’s equation, as reviewed by Black and Moore (38). A primary tenet of vectorial capacity theory is that for all values of vector competence, as the probability of daily survival decreases, the vector capacity of that mosquito population decreases exponentially. In particular, we note that a population with extremely low vector competence (for example, where only 1% of mosquitoes can become infected), but where viral infection has no effect on survival, is predicted to have a higher overall vectorial capacity than a similar population with high vector competence (for example, where 100% of vectors are capable of transmitting) but where viral infection lowers the probability of daily survival (<70%). Thus, from an evolutionary standpoint it is more beneficial to the virus to minimize effects on mosquito survival rather than maximize infection rates when there is a corresponding cost to survival.

The use of genetically modified mosquitoes has been proposed as a method of interrupting the transmission cycles of arboviruses, as well as other vector-borne pathogens such as malaria parasites (39). In many of these strategies, effector genes that lower the vector competence of mosquitoes would be incorporated into the mosquito genome and driven into wild populations. MacDonald’s equation suggests that unless vector competence was reduced to 0, reductions in vector competence will have limited success in eliminating vectorial capacity. Thus, the more logical target for genetic intervention is the probability of daily survival because of its exponential relationship with vectorial capacity. However, any gene with a strong negative effect on mosquito fitness will likely be lost from a population rather than fixed. One solution to this problem would be the development of effector genes that are neutral in uninfected mosquitoes but negatively affect survival upon infection with the viral pathogen.

Work presented here demonstrates that infection of mosquitoes with an alphavirus triggers a strong antiviral RNAi response resulting in the accumulation of viRNAs. We also demonstrate that suppressing the accumulation of these viRNAs results in elevated levels of viral RNA replication and increased mortality in the insect host. Collectively, our results suggest that an antiviral RNAi response protects the mosquito from alphavirus infection. Because it is true that arboviruses are not generally associated with detrimental effects in their natural vectors, and nearly all arboviruses produce dsRNA (the ultimate trigger of an antiviral RNAi response) during replication, these results have potentially broad implications for the control of mosquito-borne diseases. By understanding how both virus infectivity and pathogenicity are controlled in the disease vector, it may be possible to produce mosquitoes that, when infected with arboviruses, have significantly shortened life spans, thus, abrogating transmission to humans.

Increasingly, evidence appears to suggest that the siRNA pathway does not play a prominent role in mammalian antiviral responses (40). In particular, one recent study demonstrated that targeting components of the mammalian RNAi pathway with siRNAs actually inhibits the replication of hepatitis C virus (41). This is in marked contrast to results we have presented here. It is possible that the IFN system has replaced the RNAi pathway as the primary antiviral response in mammals (40, 42). Nonetheless, introduction of artificial exogenous nucleic acids has been used to successfully control virus replication in mammalian cells via host RNAi machinery (43). We have presented evidence here that viRNAs play an important role in modulating the pathogenic potential of alphaviruses in the mosquito. We have also demonstrated that these viRNAs appear to be generated by a nonrandom mechanism. Therapeutic approaches employing RNAi may prove to be particularly effective methods for controlling the pathogenesis of alphaviruses in mammalian hosts. Sequencing small RNAs from alphavirus infected mosquitoes may suggest effective targets for such approaches.

**Materials and Methods**

**Virology.** Cells used in these experiments were obtained from ATCC. Cells were maintained in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 10% FBS, plus nonessential amino acids (NEAA), L-glutamine, and antibiotics. A multiple cloning site (MCS) was inserted into the XbaI site immediately downstream of the duplicated subgenomic promoter sequence in pT7/3/2). Sequences encoding SRS proteins were inserted into full-length virus clones at Ascl and Paci sites present in each MCS. Infectious RNA transcripts were generated from linearized plasmid templates in a standard in vitro transcription reaction. BHK-21 cells were electrotansfected with RNA transcripts as described previously (34). Culture supernatant was harvested, clarified by centrifugation, and stored at −80°C. Virus titers were

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determined in triplicate by plaque assay on Vero cells. A full-length cDNA clone of FHV RNA 1 was constructed from the GenBank nucleotide sequence (accession no. X77156) using conventional methods (44). A T7 RNA polymerase promoter with +1 transcriptional start at the first nucleotide of FHV RNA 1 was included in the plasmid. The ribozyme sequence from the antigenomic strand was inserted in the thorax with 103 pfu of each SINV. One-day-old adult female Ae. aegypti mosquitos were injected into wild-type clones and sequenced. In vitro-transcribed FHV RNA1 was electroporated into BHK-21 and S2 cells by using a BTX ECM 630 at 460 V, 725 Ω, 75 μF and 715 V, 1575 Ω, 50 μF, respectively.

**Virus Infections.** Ae. aegypti (Liverpool strain) and An. gambiae (G3 strain) were reared at 28 °C and 80% relative humidity with a photoperiod of 15 h light/9 h dark. One-day-old adult female Ae. aegypti mosquitoes were reared at 28 °C and 80% relative humidity with a photoperiod of 15 h light/9 h dark. One-day-old adult female Ae. aegypti virus infections.

Small RNA Library Preparation, Sequencing, and Analysis. Small RNA libraries were prepared from whole mosquitoes 96 h after virus infection as described previously (46), with the following modifications. Total RNA was isolated by using the mirVana miRNA isolation kit (Ambion) according to the manufacturer’s instructions. Small RNAs (18–30 nt) were recovered by PAGE and eluted with 0.3 M NaCl for 3 h at RT. Eluate and gel debris was filtered by using a Costar Spin X celluose acetate filter (Corning Life Sciences) and ethanol precipitated (2×). Sequences (3′ and 5′) were ligated by using TA ligase 1 (NEB). Ligation products were recovered as described above. PCR amplification was done by using Phusion* DNA polymerase (Finnzymes Oy).

**Acknowledgments.** The full-length SINV cDNA clone, pTE3/2J, was provided by Charles Rice (The Rockefeller University, New York, NY). The full-length ONNV cDNA clone, p5s dONNV/c, was provided by Brian Foy (Colorado State University, Fort Collins, CO). We thank Mihaela Zavolan for reading the manuscript and providing helpful comments about the analysis of the small RNA libraries and Robert Aronson for help with small RNA database analysis. This work was supported by National Institute of Allergy and Infectious Diseases Grant AI077726, the Virginia Polytechnic and State University Fralin Life Science Institute, and Virginia Polytechnic and State University startup funds (to K.M.M. and Z.N.A.).


24. Amplicated cDNA constructs were recovered by gel purification and sequenced on an Illumina genome analyzer. Small RNA datasets were analyzed as described by others (29, 30, 35). Briefly, to determine the total number of 18–24 small RNA reads, linker sequences were first identified and removed. Small RNA reads without an identifiable linker sequence were excluded. Next, small RNAs that mapped to noncoding RNAs such as rRNA, tRNA, and snRNA were excluded. Remaining reads were filtered, and all reads <18 nt or >24 nt were discarded. Small RNA reads were mapped to dsSINV, dsSINV-B2 (FHV) or to mature miRNA sequences by using National Center for Biotechnology Information (NCBI)-BLAST, with a word size of 7 and expected cutoff of 100. Only small RNA reads with 100% match to the SINV genome or to mature miRNA sequences were accepted. For comparisons between libraries, numbers of small RNA reads were subject to linear normalization as described by Kascnau (47) and Ghildiyal (29).

**RNA Isolation and Detection.** Total RNA was extracted from whole mosquitoes or cells by using TRI Reagent (MRC) according to the manufacturer’s instructions. Accumulation of viral RNAs was analyzed by Northern blot using standard procedures. The NEBlot Phototope kit (NEB) was used to biotinylate a fragment derived from pTE3/2J corresponding to the SINV 3′ UTR (Xbal to XhoI). Biotinylated FHV probe was derived from a 578-bp fragment present in both RNA and RNAs (PspOMI and SacI). After hybridization under standard conditions, the Phototopa-Star Detection kit (NEB) was used to detect the bound probe by exposure to X-ray film. viRNAs were detected (96 h after virus infection) using the mirVana miRNA Detection kit (Ambion) according to the manufacturer’s instructions. A probe was made to correspond to a portion of the TE3/2J virus genome (nucleotides 8885–8920) by using the mirVana miRNA Probe Construction kit (Ambion) according to the manufacturer’s instructions. Ambion’s Decade Markers were used for size determination.

**References.**
Supporting Information

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Fig. S1. FHV RNA 1 (B2-C44Y) replication in BHK-21 and S2 cells. Northern blot analysis of FHV RNA1 and RNA3 accumulation after the electroporation of in vitro-transcribed wild-type (wt) or B2 C44Y mutant (C44Y) FHV RNA1 into vertebrate (BHK-21) or invertebrate (S2) cultured cells. Ribosomal RNA (rRNA) indicates equivalent loading of total RNA in each lane.
Fig. S2. C6/36 cells infected with dsSINV-B2 (FHV) exhibit cytopathological changes. (A) Bright-field images of C6/36 cells 0, 3, 9, and 16 days after virus infection. Uninfected cells (un) are shown for comparison. (B) Accumulation of SINV genomic (49S), subgenomic (26S), and 2nd subgenomic (S2) RNAs in C6/36 cells. Numbers indicate the time after infection (in days) that total RNA was extracted.
Fig. S3. Decreased survival of *Ae. aegypti* after infection with recombinant SIN virus expressing a suppressor of RNA silencing. Survival curves for mosquitoes intrathoracically injected with recombinant SIN virus (1 day old). Survival curves for uninjected, uninfected mosquitoes (1 day old) are shown for comparison. Error bars indicate the standard deviation among 3 replicate cohorts (50 mosquitoes per cohort).