Noncoding Subgenomic Flavivirus RNA Is Processed by the Mosquito RNA Interference Machinery and Determines West Nile Virus Transmission by Culex pipiens Mosquitoes

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ABSTRACT
Flaviviruses, such as Zika virus, yellow fever virus, dengue virus, and West Nile virus (WNV), are a serious concern for human health. Flaviviruses produce an abundant noncoding subgenomic flavivirus RNA (sfRNA) in infected cells. sfRNA results from stalling of the host 5’-3’ exoribonuclease XRN1/Pacman on conserved RNA structures in the 3’ untranslated region (UTR) of the viral genomic RNA. sfRNA production is conserved in insect-specific, mosquito-borne, and tick-borne flaviviruses and flaviviruses with no known vector, suggesting a pivotal role for sfRNA in the flavivirus life cycle. Here, we investigated the function of sfRNA during WNV infection of Culex pipiens mosquitoes and evaluated its role in determining vector competence. An sfRNA1-deficient WNV was generated that displayed growth kinetics similar to those of wild-type WNV in both RNA interference (RNAi)-competent and -compromised mosquito cell lines. Small-RNA deep sequencing of WNV-infected mosquitoes indicated an active small interfering RNA (siRNA)-based antiviral response for both the wild-type and sfRNA1-deficient viruses. Additionally, we provide the first evidence that sfRNA is an RNAi substrate in vivo. Two reproducible small-RNA hot spots within the 3’ UTR/sfRNA of the wild-type virus mapped to RNA stem-loops SL-III and SL, which stick out of the three-dimensional (3D) sfRNA structure model. Importantly, we demonstrate that sfRNA-deficient WNV displays significantly decreased infection and transmission rates in vivo when administered via the blood meal. Finally, we show that transmission and infection rates are not affected by sfRNA after intrathoracic injection, thereby identifying sfRNA as a key driver to overcome the mosquito midgut infection barrier. This is the first report to describe a key biological function of sfRNA for flavivirus infection of the arthropod vector, providing an explanation for the strict conservation of sfRNA production.

IMPORTANCE
Understanding the flavivirus transmission cycle is important to identify novel targets to interfere with disease and to aid development of virus control strategies. Flaviviruses produce an abundant noncoding viral RNA called sfRNA in both arthropod and mammalian cells. To evaluate the role of sfRNA in flavivirus transmission, we infected mosquitoes with the flavivirus West Nile virus and an sfRNA-deficient mutant West Nile virus. We demonstrate that sfRNA determines the infection and transmission rates of West Nile virus in Culex pipiens mosquitoes. Comparison of infection via the blood meal versus intrathoracic injection, which bypasses the midgut, revealed that sfRNA is important to overcome the mosquito midgut barrier. We also show that sfRNA is processed by the antiviral RNA interference machinery in mosquitoes. This is the first report to describe a pivotal biological function of sfRNA in arthropods. The results explain why sfRNA production is evolutionarily conserved.

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of transmission of these flaviviruses is important to evaluate their
epidemic potential and is the first step in designing novel ways to
break the flavivirus transmission cycle.

The overall capacity of a mosquito to become orally infected
after an infectious blood meal and to transmit the virus to the next
vertebrate host is expressed as the vector competence (11). For a
successful infection of the mosquito vector, arboviruses have to
overcome the midgut infection and escape barriers, as well as the
salivary gland barriers (12). After ingestion of a viremic blood
meal, the midgut epithelial cells have to become infected. These
cells have a strong RNA interference (RNAi) response that re-
stricts virus infection and acts as a bottleneck on virus population
diversity (13, 14). Next, the virus has to replicate in the midgut
cells, produce progeny virus, and escape from the midgut cells
through the basal lamina into the hemolymph. Subsequently, the
virus disseminates to other organs and ultimately infects the sali-
vary glands. After sufficiently high virus titers in the saliva have
been reached, viruses can be transmitted to a new vertebrate host
via a bite by the infected mosquito.

Vector competence is influenced by viral, environmental (e.g.,
temperature and the microbiome), and vector-related factors, in-
cluding vector genetics and antiviral responses (11). In arthro-
pods, antiviral responses are predominantly mediated by RNAi
(13, 15), and also by Toll, IMD, and JAK/STAT signaling pathways
and apoptosis (16, 17). Three distinct insect small-RNA pathways
can be discriminated: the 21-nucleotide (nt) small interfering
RNA (siRNA) response, the ~21- to 22-nt microRNA (miRNA)
pathway, and the 25- to 30-nt PIWI-interacting RNA (piRNA)
response (18–20). In Culex mosquitoes—the predominant group
of WNV vectors—virus-specific siRNAs are highly abundant,
whereas viral piRNAs (vpiRNAs) appear to be absent (14, 21). For
flaviviruses, three putative viral suppressors of RNAi (VSRs) have
been identified in vitro: the NS4B and NS3 proteins of DENV type
2 (DENV-2) and the noncoding subgenomic flavivirus RNA
(sRNA) for WNV (22–24).

During viral replication in both vertebrate and invertebrate
host cells, flaviviruses produce a highly abundant noncoding
sRNA approximately 0.5 kb in size. sRNA is produced via a
unique mechanism involving degradation of the viral genomic
RNA (vRNA) by the host S-3’ exoribonuclease XRN1/Pacman
(25). XRN1 stalls at stem-loop (SL) and dumbbell (DB) RNA
structures within the flaviviral 3’ untranslated region (UTR), re-
sulting in the accumulation of sRNA (26–29). The stalling of
XRN1 occurs due to steric hindrance caused by interactions of
pseudoknots (PK) and other tertiary RNA structures (30). During
WNV infection, three species of sRNA, named sRNA1, -2, and
-3, are produced by stalling of XRN1 on SL-II, SL-IV, and DB-1
(26, 29). The formation of sRNA is important for replication in
insect cells and several types of vertebrate cells, since sRNA mu-
tants that produce only sRNA3 have attenuated replication rates,
even in Dicer-2-deficient C6/36 cells (25, 28, 29, 31). In mamma-
lian cells, sRNA is essential for flavivirus-induced cytotoxicity and
pathogenicity (25). Importantly, sRNA acts as an antagonist of
both interferon- and retinoic acid-inducible gene-I-like recep-
tor-dependent innate immune responses (31, 32). Accordingly,
WNV mutants deficient in sRNA production are attenuated in
mice (25, 29).

Despite the clear functions of sRNA in flavivirus pathogenesis
in vertebrates, the role of sRNA in the arthropod vector is poorly
understood. sRNA production is conserved for all MBFs and
TBFs, and although recent RNA structure analysis of ISF 3’ UTRs
failed to identify XRN1-resistant structures (33, 34), it was previ-
ously reported that ISFs and NKVFs have conserved SL structures
with putative pseudoknot structures in their 3’ UTRs (35). In-
deed, sRNA production has been experimentally shown for sev-
eral NKVFs and the ISF cell-fusing agent virus (36). The conser-
vation of sRNA production by MBFs, TBFs, and ISFs emphasizes
the putative importance of sRNA production in arthropods (35).

So far, it has been demonstrated that sRNA acts as a suppressor of
miRNA- and sRNA-mediated RNAi in vitro in both mammalian
and arthropod cells (23, 37). The highly abundant sRNA most
likely acts as a decoy substrate for Dicer to prevent it from cleaving
other double-stranded RNA (dsRNA) molecules. Recently, sRNA
has been demonstrated to mildly suppress the RNAi pathway in
vivo, but the biological significance of this suppression requires
more detailed studies (38). Based on the high abundance of sRNA
in infected arthropod cells and the conservation of sRNA in most,
if not all, flavivirus genomes known to date (35), including those
restricted to replication in arthropods, we hypothesize that sRNA
is a crucial factor for flavivirus transmission by the arthropod
vector.

In this study, the importance of sRNA for dissemination and
transmission of WNV by Culex pipiens mosquitoes was investi-
gated. By using a lineage II WNV infectious clone deficient in
sRNA formation, we investigated replication kinetics in mamma-
lian cells and both RNAi-competent and -deficient mosquito cell
lines. The putative role of sRNA in modulating the RNAi re-
response in vivo was determined by a deep-sequencing approach
using viral small RNAs (vsRNAs). Notably, the importance of
sRNA in WNV infection and transmission by Culex mosquitoes
was studied in vivo, and the role of the midgut epithelium was
assessed by comparison of infections via the blood meal and via
intrathoracic injections. The outcomes of this research highlight
the importance of sRNA for flavivirus transmission by mosqui-
toes and provide a biological explanation as to why sRNA pro-
duction is strictly conserved in the genus Flavivirus.

**MATERIALS AND METHODS**

**Cell culture.** *Aedes albopictus* U4.4 and C6/36 (ATCC CRL-1660) mos-
quito cells were cultured in Leibovitz L-15 medium (Gibco) supple-
mented with 10% fetal bovine serum (FBS) (Gibco), 2% tryptose phos-
phate broth (Gibco), and 1% nonessential amino acids (Gibco). *Culex
tarsalis* Cx.t cells (39) (CDC, Fort Collins, CO) were cultured in Schne-
ieder’s medium (Gibco) supplemented with 10% FBS. All the mosquito
cells were maintained as a monolayer in T25 cell culture flasks at 28°C.

**Generation of infectious clones and virus stocks.** The wild-type
WNV lineage 2 isolate used in this study was isolated in southeastern
Europe (GenBank KC496015.1) and is referred to below as WNVGR. The
infectious clone of WNVGR, based on the same virus isolate, was supplied
by Tamás Bakonyi from the Department of Microbiology and Infectious
Diseases, Szent István University, Budapest, Hungary (40). The infectious
cloned is referred to as below as WNVIC. A subclone of the virus was made
by PCR amplification of the NS4-3’ UTR region with Phusion polymerase
(New England Biolabs), using primers FW (5’-TGGCTGAAAGTCGCCAG
GAACGA-3’) and RV (5’-TGGAAGTCCGAGCTCATGCGT-3’). The re-
sulting amplicon was cloned into pJET1.2 (Fermentas), and site-directed
mutagenesis was used to produce pJE1.2/subGR10ΔSF1, with mutations in the pseudoknot site of SL-II, with Phusion PCR using primers FW (5′-GAAGCTCTACACTAGGGTGCTTGCTGCG-3′) and RV (5′-CTAGTGCCTCGGACAGCAATTTAC-3′). Site-directed mutagenesis of pJE1.2/subGR10ΔSF1 with primers FW (5′-CTCATGGTTG CACTCTCGGAGAGTGCAC-3′) and RV (5′-CGCAAGAGTGGCA CACTAGTGGTGGTGCAC-3′) was used to produce pJE1.2/subGR10ΔSF1 +2 with mutations in SL-II and SL-IV. The NS4B-3 CACTAGAGTGTGGTCTGAC-3′ probes were made in culture media, and a Vero cell suspension (1.0 % FBS, penicillin, and streptomycin) was inoculated with 1 ml of samples of cell culture medium were added to each well of 6-well plates. Three days posttransfection (dpt), the supernatant was harvested and used to inoculate a 75 flask of C6/36 cells seeded 1 day in advance. Six days postinfection (dpi), the supernatant of C6/36 cells was harvested and stored at −80°C. All the viruses used in this study are from a second passage (P2).

Cell viability assay. Cell monolayers were washed with 1× phosphate-buffered saline (PBS) and lysed using passive lysis buffer (PLB) (Promega) for 10 min at room temperature. Subsequently, CellTitre-Glo 2.0 reagent (Promega) was mixed with the PLB in a 1:1 ratio and incubated in the dark for 10 min at room temperature before measuring the luminescence using a Fluostar Optima microplate reader (BMG Labtech).

Virus titrations. The 50% tissue culture infectious dose (TCID50) was determined using an endpoint dilution assay (EPDA) on Vero cells, as described previously (41). Briefly, Vero cell monolayers were detached using trypsin (Gibco) and diluted in DMEM supplemented with 10% FBS, penicillin, and streptomycin. Tenfold dilutions of the virus samples were made in culture media, and a Vero cell suspension (1.0 × 10⁵ cells) was added in a 1:1 ratio. For each dilution, 1.0 μl was plated at 6-fold in a 60-well Micro-Well plate (Nunc) and scored 3 dpi for the presence of WNV by either cytopathic effect (CPE) or immunostaining using anti-E monoclonal antibodies.

Virus growth curves. Monolayers of the indicated cell types were infected in a 6-well plate at a multiplicity of infection (MOI) of 1 by adding 1 ml of diluted virus to the cells. The cells were incubated in the presence of virus for 2 h and washed three times with 1× PBS. Two milliliters of fresh cell culture medium was added, and the cells were incubated at either 37°C with 5% CO₂ (Vero cells) or at 28°C (mosquito cells). At the indicated times postinfection, 30 μl of samples of cell culture medium were frozen at −80°C until further processing. Reverse transcription, and PCR amplification, the cDNA libraries were gel purified from 6% polyacrylamide-1× TBE gels. For the sequencing of small RNAs, WNVΔC, WNVΔSF infected northern European mosquitoes, the 19- to 24-nucleotide reads were cut from the gel using radioactively labeled RNA oligonucleotides and loaded in adjacent lanes of the gel as rulers. Subsequently, the gel slices were crushed using gel breaker tubes, and small RNAs were eluted in 800 μl 0.3 M sodium acetate (NaOAc), precipitated with 80% ethanol (EtOH), and dissolved in 11 μl H₂O. Small-RNA deep-sequencing libraries were prepared using the TrueSeq small-RNA kit (15016914; Illumina) according to the manufacturer’s protocol, as described previously (21). Briefly, after adapter ligation, reverse transcription, and PCR amplification, the cDNA libraries were gel purified from 6% polyacrylamide-1× TBE gels, eluted in 500 μl 0.3 M NaOAc, precipitated with 80% EtOH, and dissolved in 11 μl 10 mM Tris-HCl, pH 8.5. The small-RNA library was sequenced on an Illumina HiSeq 2500 (Basecable, Leiden, The Netherlands), and single-end FASTQ reads were generated using the Illumina Casava pipeline version 1.8.3. Initial quality assessments were performed with Basecable using in-house filtering protocols and the FASTQC quality control tool version 0.10.0. Further sequence analysis was performed on the Galaxy server (41). Adapter sequences were removed from each read using the Clip tool version 1.0.1 and mapped to either the WNVΔC or WNVΔSF genome with Bowtie version 1.1.2 (41). Size profiles of the viral small RNAs were retrieved from all the mapped reads, allowing 1 mismatch. For genome profiles, the 5′ ends of the 21-nucleotide reads were mapped along the viral genome.

Mosquito rearing. A C. pipiens colony originating from Brummen, The Netherlands, was established in 2010 and maintained at 23°C and 60% relative humidity (RH) on a 16-h/8-h light-dark (L-D) cycle. The mosquitoes were maintained in Bugdorm cages and provided with 6% glucose solution ad libitum. Hemotek PS5 feeders (Discovery Workshops) filled with bovine or chicken whole blood (Kemperkip, Uden, The Netherlands) were used to administer a blood meal for egg production. Egg rafts were allowed to hatch in tap water supplemented with Liquify no. 1 (Interpet Ltd., Dorking, United Kingdom). The larvae were fed with a 1:1 mixture of ground koi food, ground rabbit food, and bovine liver powder (Sagam-Aldrich, Zwijndrecht, The Netherlands).

In vivo infections. (i) Infectious blood meal. Two days before blood feeding, the mosquitoes were starved by providing them with a tissue soaked in water. One milliliter of whole chicken blood (Kemperkip, Uden, The Netherlands) was mixed with 1 ml virus solution in Leibovitz-L15 supplemented with 10% FBS to a final concentration of 4.0 × 10⁵ TCID₅₀/ml. Two- to 7-day-old mosquitoes were allowed to feed ad libitum through a Parafilm membrane using a Hemotek feeder in a controlled dark room at 24°C and 70% RH for 1 h. After blood feeding, the mosqui-
toes were anesthetized with 100% CO₂ and engorged females were selected on a CO₂ pad.

(ii) Intrathoracic injections. Two- to 5-day-old female mosquitoes were anesthetized with 100% CO₂ and placed on a CO₂ pad, and /H11011/H11003 103 or /H11011/H11003 102 TCID₅₀ of virus was injected in a total volume of 69 nl using a Drummond Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company). Blood-fed or injected mosquitoes were incubated at 28°C on a 16-h/8-h L-D cycle and fed with 6% sugar water during the course of the experiment. At 14 dpi (or as indicated), mosquitoes were anesthetized with 100% CO₂, and the legs and wings were removed from the mosquito bodies. Saliva from the mosquitoes was collected by putting the proboscis in 5/H9262 3% sugar water, 50% FBS for 45 min. After salivating, the mosquito bodies were added to a 1.5-ml Eppendorf tube containing 0.5-mm zirconium beads (Next Advance, New York, NY) and frozen at 80°C. Fifty-five microliters of HEPES-buffered DMEM–10% FBS–penicillin/streptomycin–gentamicin was added to the saliva samples. The mosquito homogenates and saliva samples were stored at 80°C until further processing.

WNV infectivity assay. WNV infectivity assays were performed as described previously (42). Briefly, mosquito bodies were homogenized in a Bullet blender (Next Advance, New York, NY; two rounds of 2 min each at maximum speed); 100 µl of HEPES-buffered DMEM–10% FBS–gentamicin was added to the mosquito homogenates, and 30 µl mosquito homogenate was used to inoculate Vero cell monolayers in 96-well plates. For saliva samples, 30 µl saliva in DMEM-10% FBS-PS-gentamicin was used to inoculate a Vero cell monolayer in a 96-well plate. At 3 dpi, the wells were scored for the presence of virus by CPE and immunostaining using anti-E monoclonal antibodies (43, 44).

RNA structure modeling in silico. Secondary structures were folded using the Mfold Web server with standard settings and flat exterior loop type (45). The secondary RNA structure was visualized using the VARNA RNA editing package (46). Tertiary-structure folding was performed using the RNAComposer Web server with standard settings (47). The tertiary RNA structure was visualized using the PyMOL viewer (48).

Statistics. All statistical analyses were performed in GraphPad Prism 5. Significant differences between virus infection and transmission rates were determined by Fisher’s exact test (α = 0.05). t tests were performed using a 95% confidence interval. The D’Agostino and Pearson omnibus normality test was used to check for Gaussian distributions. In cases where the data did not follow a Gaussian distribution, a Mann-Whitney U test was used (α = 0.05) to replace the t test. Statistics on the virus growth curves were done using a two-way analysis of variance (ANOVA).

RESULTS

An sfRNA1-deficient clone of WNV is not attenuated in vitro. WNV mutants that are deficient in the formation of full-length sfRNA (sfRNA1) cause progressively less CPE in cell culture when only shorter and/or less abundant sfRNA species can be produced (25, 29). To study the effects of sfRNA on transmission independent of effects on replication and cytopathicity, we used a mutant that is deficient only in sfRNA1. This mutant has previously been shown to have little effect on the replication rate and cytopathicity of WNV, as opposed to sfRNA1-sfRNA2-deficient mutants, which are attenuated in both insect and invertebrate cell lines (25, 29, 31). An infectious clone of WNV (WNVIC) was compared to an sfRNA1-deficient infectious clone of WNV (WNVΔSF1) that was generated by mutating the top loop of SL-II (Fig. 1A). The
wild-type virus (WNVGR) was used for comparison in the growth curve and cell viability experiments. As expected, Northern blot analysis of RNA isolated from Vero cells using a 3′ UTR-specific probe indicated that WNVIC predominantly produced sfRNA1. Conversely, WNV/H9004 SF1 produced only sfRNA2 as a result of mutated SL-II, resulting in XRN1 stalling on SL-IV (Fig. 1B). An unexpected additional band was observed for WNVIC between sfRNA1 and sfRNA2, which was not produced in cells infected with WNV/H9004 SF1 (Fig. 1B). Comparison with the flaviviruses Usutu virus (USUV) and WNV New York 1999 (WNV NY99) demonstrated that the size of full-length sfRNA from WNVIC was similar, confirming that the top band is sfRNA1 (data not shown).

One-step growth curves in Vero cells demonstrated that WNV/H9004 SF1 had growth kinetics similar to those of WNVIC and the wild-type WNVGR virus isolate (Fig. 1C). In addition, WNV/H9004 SF1 caused a level of CPE comparable to that of WNVGR and WNVIC, as observed by a steady decrease in cell viability between 1 and 3 dpi (Fig. 1D). These results validate WNV/H9004 SF1 as a model to study the effects of sfRNA without interference from altered cytopathicity and/or replication.

An sfRNA1-deficient WNV replicates efficiently in RNAi-competent mosquito cells. To investigate whether sfRNA1 is required for replication of WNV in mosquito cells, one-step virus growth curves were performed on C. tarsalis Cx.t, A. albopictus U4.4, and A. albopictus U4.4 cells infected with WNVGR, WNVIC, or WNVΔSF1 at an MOI of 1. Supernatants were taken at 0, 1, 2, 3, and 4 dpi and titrated by EPDA. Shown are the mean titers ± SEM from two independent experiments. The statistics were done using a two-way ANOVA. *, P < 0.05. (B) Northern blot of RNA isolated 4 dpi from U4.4, C6/36, or Cx.t cells using a 3′ UTR-specific probe. Numbers at right are kilobases. (C) Mean sfRNA1/sfRNA2 signal ratio. Shown are the mean results and SEM from two independent experiments. The statistics were performed using a two-tailed unpaired t test.

Viral siRNAs derived from the 3′ UTR are produced in NA and NWE C. pipiens mosquitoes infected with WNVGR. While sfRNA suppresses reporter-based RNAi in mosquito cells (23, 37) and in vivo in mosquitoes and interacts with the RNAi machinery in mammalian cell lines (38), it is still unclear whether during an in vivo infection in mosquitoes the 3′ UTR sfRNA is processed by the RNAi machinery. To investigate if WNVGR 3′ UTR-derived small RNAs are produced in C. pipiens mosquitoes, we analyzed the vsRNA profiles generated in a set of independent experiments with a wild-type WNV lineage II isolate (WNVGR) in northwestern European (NWE) and North American (NA) C. pipiens strains (Fig. 3) (21). Size distributions of vsRNAs showed that 21-nt viral
siRNAs (vsiRNAs) were highly abundant and that no obvious >25-nt vsRNAs were produced (Fig. 3A), indicating that no WNV-specific piRNAs are produced in *C. pipiens* mosquitoes. A small shoulder of 25- to 32-nt sRNAs was present; however, these sRNAs did not have the 10A-biased piRNA signature (21). Hot- and cold-spot analysis of the 21-nt vsRNAs along the WNV genome indicated that vsiRNAs were produced across the whole length of the genome (Fig. 3B). NA mosquitoes produced smaller amounts of vsiRNAs and mapped differentially across the genome, indicating that different mosquito populations can influence the sRNA profile. Detailed hot- and cold-spot analysis of the viral 3′ UTR showed that in both NWE and NA mosquitoes two hot spots were present in SL-III and the 3′ SL, respectively, while NA mosquitoes also displayed a hot spot in SL-IV (Fig. 3C). These results show that the 3′ UTR of the WNV*G* rRNA is targeted by the RNAi machinery and processed into 21-nt vsRNA, indicating that sRNA might be processed by the RNAi machinery *in vivo*.

*C. pipiens* mosquitoes mount an active RNAi response against wild-type and sRNA-deficient WNV. Our experiments clearly show that an sRNA-deficient mutant replicates efficiently in RNAi-competent mosquito cells, although the 3′ UTR appears to be processed by the RNAi machinery *in vivo*. To investigate if sRNA is a substrate for Dicer and/or influences the mosquito RNAi response *in vivo*, 19- to 24-nt small-RNA populations were sequenced from pools (n > 12) of WNV*IC* or WNV*ΔSF1* blood meal-infected mosquitoes at 14 dpi (Fig. 4). RNAs larger than 25 nt were excluded from deep sequencing, since it was previously shown that *Culex* mosquitoes do not produce 25- to 30-nt vpiRNAs (Fig. 3A) (14, 21). To rule out potential effects of differentiation between the two mosquito pools, we first verified the virus titers and vgRNA levels at 14 dpi (Fig. 4A). The virus titers in the two pools of mosquitoes were marginally different, with a slightly but not significantly higher titer for WNV*IC* (1.1 × 10⁶ versus 6.3 × 10⁵ TCID₅₀/ml; P = 0.272) (Fig. 4A), while qPCR on the vgRNA demonstrated that the numbers of viral genome copies were similar (Fig. 4B). Northern blot analysis of total RNA isolated from pools of WNV-positive mosquitoes confirmed that in mosquitoes, as well, WNV*IC* predominantly produced sRNA1 while WNV*ΔSF1*
Small-RNA sequences of WNVIC or WNVΔSF1 blood-fed mosquitoes. (A) WNV titers of mosquito bodies used for small-RNA sequencing. The statistics were performed using an unpaired t test. The error bars indicate SEM. (B) WNV genome copies in RNA samples used for small-RNA sequencing determined by qRT-PCR. (C) Northern blot from the same RNA sample that was used for deep sequencing using a 3' UTR-specific probe. Numbers at right are kilobases. (D and E) Size distributions of small-RNA reads mapping to the viral genome of WNVIC or WNVΔSF1 blood-fed mosquitoes normalized to the total number of reads (D) or the total number of viral reads (E) in the library. (F and G) The 5' ends of the reads were aligned to the viral genome and normalized against the number of viral reads. The positive-strand (F) and negative-strand (G) reads were mapped for WNVIC and WNVΔSF1. (H and I) Detailed view of the 21-nt RNA distribution on the 3' UTR for the positive strand (H) and the negative strand (I). The green arrows indicate siRNA hot spots that are siRNA specific. The blue arrow indicates the presence of KUN-miR-1.
produced only sfRNA2 (Fig. 4C). The vsRNA size distributions were very similar in WNVIC- and WNV/H9/H9004SF1-infected mosquitoes (Fig. 4D and E). A distinctive peak of 21-nt vsRNAs was observed for both WNVIC and WNV/H9/H9004SF1 (Fig. 4D and E). This 21-nt vsRNA population was present on both the positive and negative strands, indicating that they can be regarded as vsiRNAs. WNVIC produced slightly higher total levels of vsiRNAs than WNV/H9/H9004SF1 (Fig. 4D), although when normalized to the number of viral reads (Fig. 4E), there was no difference in the percentages of 21-nt reads. This indicates that the difference in percentages of total reads can be explained by the slightly elevated titer of WNVIC, although the numbers of viral genome copies were similar (Fig. 4A and B).

Next, we investigated whether sfRNA affected the genome distribution of vsRNAs by mapping the 21-nt reads along the vgRNA for the positive (Fig. 4F) and negative (Fig. 4G) strands. The vsiRNA genome distributions were highly similar between WNVIC and WNV/H9/H9004SF1 on both the positive and negative strands, as illustrated by the mirrored hot- and cold-spot plots (Fig. 4F and G), providing no direct evidence that sfRNA1 interferes with the antiviral RNAi response in vivo.

sfRNA-derived vsiRNAs should be positive-strand biased due to the nature of sfRNA biogenesis (25). Indeed, two distinct hot spots that occur on the positive strand in WNVIC-infected mosquitoes, but not on the positive strand of WNV/H9/H9004SF1-infected mosquitoes (Fig. 4F) or on the negative strands of both viruses, were identified (Fig. 4I). Hot spots 10642 and 10976 were present only in WNVIC-infected mosquitoes and not in mosquitoes infected with WNV/H9/H9004SF1, indicating that the vsiRNAs from these hot spots are derived from sfRNA1. Interestingly, these sfRNA-specific hot spots cooccurred with predicted secondary structures of SL-III (hot spot 10642) and the 3′ SL (hot spot 10976) (Fig. 4H and 5A). Finally, Kunjin virus miR-1 (KUN-miR-1), previously discovered in vitro (50), was also found at position 11030, indicating that KUN-miR-1 could have a potential function in vivo in mosquitoes. These results show for the first time that sfRNA is processed by the mosquito RNAi machinery and that an sfRNA1-defective WNV mutant has a differential vsiRNA profile in the viral 3′ UTR.

**FIG 5** Three-dimensional RNA structure model of sfRNA revealing that SL-III and the 3′ SL are more accessible to the mosquito RNAi machinery. (A) Schematic presentation of the WNVGR sfRNA secondary RNA structure with projections of the vsiRNA hot spots. Pseudoknot interacting bases are colored red. sfRNA-specific vsiRNAs are indicated in green, and each SL and DB was given a separate color. (B and C) 3D RNA structure model of the first 240 nt (B) and last 288 nt (C) of WNV sfRNA. The colors match the color scheme of the secondary structures in panel A.
however, sticks out of the 3' UTR coil and thus could also present an accessible dsRNA substrate. These results provide insight into the tertiary fold of the 3' UTR/sfRNA and explain why vsiRNAs are mostly derived from SL-III and the 3' SL.

**sfRNA1 determines WNV transmission by mosquitoes.** The conservation of sfRNA production in all flaviviruses suggests an important biological function in both mammalian and insect hosts. Importantly, the sequence of the flaviviral 3' UTR can be phylogenetically divided based on the virus vector species (33), indicating a role for sfRNA in infection and/or transmission of the arthropod vector. To investigate if sfRNA is important for dissemination and transmission of WNV, *C. pipiens* mosquitoes were allowed to feed for 1 h on a blood meal containing either WNVIC or WNV/H9004 SF1. After 14 days, the titer of WNV in the mosquito bodies was determined by EPDA, and the presence of WNV in the saliva was determined by inoculating Vero cells (Fig. 6A). Importantly, the infection rate, as determined by the percentage of engorged mosquitoes with a measurable virus titer, was significantly lower for WNV/H9004 SF1 than for WNVIC (13% versus 32%; P < 0.001) (Fig. 6B and Table 1). In addition, the transmission rate, as determined by the percentage of saliva-positive mosquitoes out of the total number of engorged mosquitoes, was significantly lower for mosquitoes infected with WNV/H9004 SF1 than for mosquitoes infected with WNVIC (10% versus 26%; P < 0.001) (Fig. 6B and Table 1), indicating that sfRNA1 production was important for transmission when mosquitoes were orally infected. Dissemination rates, as determined by the percentage of saliva-positive mosquitoes out of the number of virus-positive mosquitoes, was not affected by sfRNA1 (82% versus 79%; P = 1.000) (Fig. 6B and Table 1). This indicates that sfRNA1 mainly affects infection of the midgut and consequently increases the transmission rate. The viral titers of mosquito bodies that were positive for the presence of

**Table 1** Four replicate blood-feeding experiments demonstrate that sfRNA determines the infection and transmission rates of WNV in *C. pipiens* mosquitoes

<table>
<thead>
<tr>
<th>Virus</th>
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* Shown are infection, transmission, and dissemination rates of *C. pipiens* mosquitoes orally infected with either WNVIC or WNVΔSF1. In addition, the sample size (n) as the number of engorged females after a blood meal and absolute numbers of positive bodies and saliva samples are shown for each replicate.
virus by EPDA were not significantly different between WNVIC and WNV/H9004SF1 (P = 0.360) (Fig. 6C). However, the viral titers in the saliva were significantly higher for WNVIC-infected than for WNV/H9004SF-infected mosquitoes (2.2 × 10^5 versus 8.0 × 10^3; P = 0.01) (Fig. 6C). These results suggest that sfRNA1 determines WNV transmission by increasing the overall infection rates and by boosting viral titers in the mosquito saliva.

**sfRNA1 is a key driver to overcome the mosquito midgut infection barrier.** The infection rate of mosquitoes and the subsequent dissemination to the salivary glands are important parameters for arbovirus transmission (11). Clearly, WNVΔSF1 displayed significantly reduced transmission rates, which could be (i) the result of reduced virus dissemination through an effect of sfRNA1 on a postmidgut barrier or (ii) the consequence of reduced initial infection rates. Although our data suggest that the main effect of sfRNA occurs in the midgut, we discriminated between these two possibilities by intrathoracic injections of *C. pipiens* mosquitoes with 7.7 × 10^3 TCID50 of either WNVIC or WNV/H9004SF1. In this way, the midgut barriers are bypassed to allow the study of postmidgut virus dissemination and transmission. At 14 dpi, an infection rate of 100% was achieved for mosquitoes injected with either WNVIC or WNVΔSF1 (Fig. 7A and Table 2), suggesting that the two viruses are equally capable of establishing a systemic, fully disseminated infection when the

![Graph showing infection and transmission rates](image)

**FIG 7** Intrathoracic injections of *C. pipiens* mosquitoes with WNVIC and WNVΔSF1. (A) Infection (Bodies), transmission (Saliva), and dissemination (Saliva/Bodies) rates of WNVIC (n = 34) and WNVΔSF1 (n = 50) in *C. pipiens* mosquitoes after intrathoracic injections with 7.7 × 10^3 TCID50 per mosquito. The data represent cumulative numbers from three independent experiments. The statistics were performed using Fisher exact tests. (B) Titers of WNV-positive mosquito bodies and saliva samples from mosquitoes injected with WNVIC or WNVΔSF1. Shown are the mean titers ± SEM. The statistics were performed using a two-tailed unpaired Mann-Whitney U test. (C) Transmission rates in *C. pipiens* mosquitoes of WNVIC or WNVΔSF1 after intrathoracic injection at 1, 6, and 14 dpi. The statistics were performed using Fisher exact tests. (D) Infection and transmission rates at 7 dpi in *C. pipiens* mosquitoes after intrathoracic injection of a low dose (7.7 × 10^2 TCID50 versus 7.7 × 10^3 TCID50) of WNVIC or WNVΔSF1. The statistics were performed using Fisher exact tests.

**TABLE 2** Three replicate intrathoracic injection experiments indicate no effect of sfRNA on postmidgut dissemination of WNV in *C. pipiens* mosquitoes

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<th>Positive saliva (no.)</th>
<th>Infection (%)</th>
<th>Transmission (%)</th>
<th>Dissemination (%)</th>
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*Shown are infection, transmission, and dissemination rates of *C. pipiens* mosquitoes that were intrathoracically injected with either WNVIC or WNVΔSF1. In addition, the sample size (n) and absolute numbers of positive bodies and saliva samples are shown for each replicate.
midgut infection barrier is bypassed. Instead, the saliva of all mosquitoes was tested for the presence of WNV. There was no significant difference in transmission rates (94% versus 90%; P = 0.696) (Fig. 7A and Table 2) or dissemination rates (94% versus 90%; P = 0.696) (Fig. 7A) between WNVIC and WNVΔsf1, despite a significant difference in average body titers (WNVIC, 2.4 × 10^6; WNVΔsf1, 4.4 × 10^6; P < 0.001) (Fig. 7B). Titration of the mosquito saliva showed no significant differences (WNVIC, 4.4 × 10^6; WNVΔsf1, 1.0 × 10^6; P = 0.873). Additionally, at 1, 6, and 14 dpi, no significant difference between the transmission rates of WNVIC and WNVΔsf1 was observed (1 dpi, 14% versus 11% [P = 1.000]; 6 dpi, 100% versus 100% [P = 1.000]; 14 dpi, 100% versus 86% [P = 0.492]) (Fig. 7C). To rule out the possibility that the lack of difference between WNVIC and WNVΔsf1 was due to a viral dose that was too high, we repeated the experiment with a 10-fold-lower injection dose of 7 × 10^2 TCID50 per mosquito and scored the infection and transmission rates at an earlier time point of 7 dpi (Fig. 7D). Again, no difference between WNVIC and WNVΔsf1 was observed in either the infection or transmission rate, indicating that there is no clear postmidgut effect of sfRNA1 on transmission. These results demonstrate that sfRNA1 does not play a determining role in postmidgut dissemination to the salivary glands. Instead, sfRNA1 is vital to efficiently overcome the midgut infection and/or escape barrier, resulting in enhanced virus dissemination and subsequent transmission.

An sfRNA1-sfRNA2-deficient WNV has attenuated replication in Culex cells and decreased transmission by Culex mosquitoes. We showed that sfRNA1 is required for successful passage of the mosquito midgut barrier, but WNVΔsf1 still produces the shorter sfRNA2. To investigate whether combined abrogation of both sfRNA1 and sfRNA2 production would further attenuate WNV, we generated an infectious clone with mutations in both SL-II and SL-IV (WNVΔ2) (Fig. 8A). The mutation introduced in SL-IV resides in the conserved three-way-junction that was previously shown to be important for XRN1 resistance (30). Northern blot analysis of RNA isolated from C6/36 mosquito cells infected with WNVIC, or WNVΔsf1 + 2 demonstrated that the double mutant indeed fails to produce sfRNA1 and sfRNA2 (Fig. 8B). The effect of sfRNA1-sfRNA2 deletion on replication was tested by performing growth curves on Cx.t and Vero cells with WNVIC, and WNVΔsf1 + 2. No difference in replication was observed in Vero cells, although WNVΔsf1 + 2 presented slightly lower titers (Fig. 8C). However, in Cx.t cells, WNVΔsf1 + 2 was severely attenuated in virus growth, indicating that sfRNA is important for successful replication in Culex (Fig. 8D). Since we showed that deletion of sfRNA1 production results in decreased transmission rates in C. pipiens after an infectious blood meal, we hypothesized that combined abrogation of sfRNA1 and sfRNA2 production further attenuates WNV in mosquitoes. To test this hypothesis, C. pipiens mosquitoes were given an infectious blood meal with 5.0 × 10^6 TCID50/ml of either WNVIC or WNVΔsf1 + 2 (Fig. 8E) (Table 3). The titer of this blood meal was slightly lower than in the previous experiments due to a lower plateau titer of WNVΔsf1 + 2 in C6/36 cells. WNVIC-infected mosquitoes reached an infection rate of 16% and an ~14% transmission rate, consistent with the data in Fig. 6. However, the WNVΔsf1 + 2 blood-fed mosquitoes reached significantly lower infection (2%; P = 0.004) and transmission (2%; P = 0.04) rates, indicating that sfRNA1 and sfRNA2 are vital for infection of mosquitoes. Sequencing of the 3' UTR of the WNVΔsf1 + 2-infected mosquitoes showed that the mutations in SL-II and SL-IV were still present, indicating that no reversions occurred (data not shown). In conclusion, these results provide the first evidence that sfRNA plays an important role in mosquito transmission of WNV, which could underpin the strict conservation of sfRNA production by flaviviruses.

DISCUSSION

Flavivirus epidemics occur as a result of a complex three-way interplay between virus, host, and vector and are influenced by a range of environmental factors. Where and when flavivirus outbreaks will occur cannot be readily predicted, but it is clear that the ability of a mosquito population to transmit a certain flavivirus is essential for efficient viral spread from one vertebrate host to another. This vector competence is primarily dictated by the specific virus-vector combination, yet the viral determinants of vector competence are not well defined. For WNV, it has been reported that a single amino acid change in the envelope glycoprotein E resulted in increased mosquito transmission rates compared to the original NY99 strain (51). This example illustrates that flaviviral products (i.e., viral proteins and viral RNA) may determine vector competence by overcoming infection barriers to establish a transmissible infection in the mosquito (52).

Here, we have identified a novel determinant of WNV vector competence, a noncoding viral RNA whose abundant production in infected cells is widely conserved in the large group of flaviviruses. This sfRNA, as well as the flavivirus 3' UTR it is derived from, contains various conserved RNA structures that may influence virus transmission by the arthropod vector (35, 53). sfRNA production is highly conserved among all members of the genus Flavivirus, including the NKVFs, ISFs, TBFs, and MBFs (25, 35, 36). Despite the many functions of sfRNA described in mammalian cells, the biological significance for sfRNA production in the arthropod vector has remained unknown (35). We now demonstrate that sfRNA determines WNV transmission by C. pipiens mosquitoes. An sfRNA1-deficient WNV mutant, WNVΔsf1, was attenuated in the mosquito and had significantly decreased infection and transmission rates. A mutant deficient in sfRNA1 and sfRNA2, WNVΔsf1 + 2, was also attenuated for replication in Culex cells and demonstrated decreased transmission and infection rates in C. pipiens mosquitoes after an infectious blood meal. From these studies, we conclude that sfRNA is produced abundantly in the mosquito to facilitate efficient transmission to the next host.

It has been firmly established that infection of and escape from midgut epithelial cells present crucial barriers for arbovirus transmission (recently reviewed in reference 12). Our results show that sfRNA affects the transmission rate at the level of the mosquito midgut, since WNV transmission was negatively affected by sfRNA deficiency when the virus was administered through an infectious blood meal but not after intrathoracic injection. After administration through intrathoracic injections, the infection and transmission rates between WNVIC and WNVΔsf1 were not significantly different at all tested virus doses and time points, although we did observe a slight difference in the overall virus titer in the mosquito bodies. This may be an indication of a potential additional postmidgut effect of sfRNA on virus dissemination, which is supported by the lower titers in the saliva for WNVΔsf1 after infection via an infectious blood meal. However, the transmission rates of wild-type and sfRNA-defective WNV in the in-
Injection experiment were always close to 100%, and no significant difference in the virus titer in the saliva was observed.

Several intracellular mechanisms could be involved in establishing the mosquito midgut barrier against arbovirus infection, such as RNAi, Toll/IMD/JAK-STAT signaling pathways, or apoptosis. The importance of antiviral RNAi in midgut cells for virus transmission and population diversity has been shown convincingly in Culex mosquitoes (13, 14). We aimed to determine the strength of the mosquito RNAi response by analyzing the vsiRNA reads relative to the viral genome in a direct comparison between WNV<sub>IC</sub> and WNV<sub>ΔSF1+2</sub>. The abundance of vsiRNA is dependent on (i) the efficiency of the putative viral RNAi suppressor function of sfRNA and (ii) the amount of available viral dsRNA substrate in the mosquito body, which is a function of viral RNA replication and thus dependent on the success of RNAi suppression. In our study, we found no direct correlation between the expression of sfRNA1 and the abundance of (v)siRNAs produced <i>in vivo</i>. We cannot formally exclude the possibility that sfRNA1 facilitates WNV replication by RNAi suppression at an early stage so that later more viral dsRNA can be processed into vsiRNA, but the

**FIG 8** Transmission and infection rates of <i>C. pipiens</i> infected with WNV<sub>IC</sub> and WNV<sub>ΔSF1+2</sub>. (A) 2D RNA structure model of SL-II and SL-IV of WNV<sub>GR</sub>. Pseudoknot interaction is indicated by the lines; the nucleotides mutated in WNV<sub>ΔSF1+2</sub> are shaded. The arrows indicate the starting points of sfRNA1 and sfRNA2. (B) Northern blot of RNA harvested from C6/36 cells infected with WNV<sub>IC</sub> or WNV<sub>ΔSF1+2</sub>. (C and D) Vero (C) or Cx.t (D) cells were infected with WNV<sub>IC</sub> or WNV<sub>ΔSF1+2</sub> at an MOI of 1. Supernatants were taken at 0, 1, 2, 3, and 4 dpi and titrated by EPDA. Shown are the mean titers of two replicate samples ± SEM. (E) Infection (Bodies), transmission (Saliva), and dissemination (Saliva/Bodies) rates of <i>C. pipiens</i> mosquitoes infected through an infectious blood meal containing WNV<sub>IC</sub> (<i>n</i> = 73) or WNV<sub>ΔSF1+2</sub> (<i>n</i> = 83). The infection, transmission, and dissemination rates are presented as percentages of the total number of engorged female mosquitoes. The data represent cumulative numbers from three independent experiments. The statistics were performed using Fisher exact tests.
similar levels of WNVIC and WNVΔSF1 viral genomic RNAs in infected mosquitoes do not support this.

The effect of sRNA on midgut infection most likely occurs during initial infection, which takes place in a small number of cells (54). This “single-cell” interaction requires very high-resolution sequence data to detect potential RNAi suppression by sRNA, which can explain why we did not find this correlation in our experiments. Sequencing of individual midguts of WNVIC and WNVΔSF1 blood-fed mosquitoes could provide more detail on the mechanism of interplay between the RNAi response and sRNA production in the mosquito midgut. A recent study showed that sRNA mildly suppressed the dsRNA-induced silencing in Culex quinquefasciatus mosquitoes infected with Kunjin virus (38). The silencing suppression was demonstrated to be significant, although the difference between the wild-type and the sRNA-deficient virus was not large. Since complete knockdown of RNAi is associated with increased mosquito mortality upon arbovirus infection, it is quite possible that sRNA only mildly modulates the RNAi response to facilitate virus transmission without negatively affecting mosquito fitness (38).

Thus, because vsiRNA abundance cannot provide conclusive evidence for RNAi suppression by sRNA, we reasoned that analyzing the distribution of the vsiRNA on the viral genome would be more informative to reveal potential differences between WNVIC and WNVΔSF1. However, sRNA did not affect the genome distribution of vsiRNAs on either the positive or negative strand of the WNV genome. Despite the clear importance of the SL-III regions from the 3’ UTRs of several flaviviruses from the Japanese encephalitis virus (JEV) serogroup (including WNV, USUV, JEV, and Kunjin virus) showed that the sequence of this hot spot is poorly conserved (data not shown). This indicates that the SL-III-derived viral small RNA is unlikely to have an important biological function as an miRNA/siRNA but leaves open the possibility that processing of SL-III by Dicer may ultimately have an effect on virus dissemination in the mosquito midgut, e.g., via an RNA decoy mechanism. The hot spot in the 3’ SL occurred only in WNVIC-infected mosquitoes and not in WNVΔSF1-infected mosquitoes, indicating that this small RNA is derived from sRNA1 even though sRNA2 also contains the 3’ SL and is produced by WNVΔSF1. Perhaps the absence of sRNA1 decreases the amount of substrate to be processed into small RNAs. Alternatively, the different folding of the shorter sRNA2 compared to that of sRNA1 results in different processing by the RNAi machinery.

When the 3D RNA structure of sRNA is modeled, both SL-III and the 3’ SL are clearly sticking out of the compact fold of the predicted sRNA tertiary structure. These exposed stem-loops could potentially make them easily accessible substrates for Dicer and/or other host nucleases. Further studies are required to fully understand the molecular interplay of sfRNA, and the RNA structures within the molecule, with RNAi and the relative importance of the interaction for flavivirus transmission in vivo.

Our finding that sRNA is very important in mosquito infection is in line with recent research that studied the mutation rate of flavivirus 3’ UTR RNA sequences in the insect host. It was demonstrated for DENV-2 that the XRN1/Pacman-resistant RNA structures SL-I and SL-II (equivalent to SL-II and SL-IV, respectively, for WNV) evolve differently depending on the origin of the host cell. In mosquito cells, SL-II incorporated mutations very rapidly compared to the upstream SL-I, which was conserved in mosquito cells but mutated more frequently during infection of mammalian cells (34). This suggests that the XRN1/Pacman-resistant SL-I is under strong selective pressure to be maintained in the insect host, most likely to safeguard production of sRNA1. Consistent with this notion, it was recently reported that sRNA plays a role in flavivirus transmission during a DENV epidemic. A DENV clade from Puerto Rico was outcompeted by a new clade that produced higher sRNA/vgRNA ratios, indicating that sRNA...
production correlates with increased epidemiological fitness (32). Our results support and extend this hypothesis by demonstrating for the first time that sRNA is required for successful flavivirus transmission by mosquitoes.

In conclusion, we have shown that sRNA is a key driver to overcome the mosquito midgut infection barrier to establish a transmissible infection. Moreover, we provide novel evidence that sRNA is processed by the RNAi machinery in vivo in mosquitoes. Our results provide the first biological explanation for the highly conserved nature of sRNA among all flaviviruses, foremost those that replicate in mosquitoes, which may offer novel targets to interfere with the complex flavivirus transmission cycle in nature.

ACKNOWLEDGMENTS

We thank Aaron Braul for providing the Cx.t cells and Tamás Bakonyi for providing us the WNV lineage II infectious clone.

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REFERENCES

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REFERENCES

sfRNA Determines Flavivirus Transmission by Mosquitoes