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Cell-to-Cell Spread of the RNA Interference Response Suppresses Semliki Forest Virus (SFV) Infection of Mosquito Cell Cultures and Cannot Be Antagonized by SFV[∇]

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In their vertebrate hosts, arboviruses such as Semliki Forest virus (SFV) (*Togaviridae*) generally counteract innate defenses and trigger cell death. In contrast, in mosquito cells, following an early phase of efficient virus production, a persistent infection with low levels of virus production is established. Whether arboviruses counteract RNA interference (RNAi), which provides an important antiviral defense system in mosquitoes, is an important question. Here we show that in *Aedes albopictus*-derived mosquito cells, SFV cannot prevent the establishment of an antiviral RNAi response or prevent the spread of protective antiviral double-stranded RNA/small interfering RNA (siRNA) from cell to cell, which can inhibit the replication of incoming virus. The expression of tombusvirus siRNA-binding protein p19 by SFV strongly enhanced virus spread between cultured cells rather than virus replication in initially infected cells. Our results indicate that the spread of the RNAi signal contributes to limiting virus dissemination.

In animals, RNA interference (RNAi) was first described for *Caenorhabditis elegans* (27). The production or introduction of double-stranded RNA (dsRNA) in cells leads to the degradation of mRNAs containing homologous sequences by sequence-specific cleavage of mRNAs. Central to RNAi is the production of 21- to 26-nucleotide small interfering RNAs (siRNAs) from dsRNA and the assembly of an RNA-induced silencing complex (RISC), followed by the degradation of the target mRNA (23, 84). RNAi is a known antiviral strategy of plants (3, 53) and insects (21, 39, 51). Study of *Drosophila melanogaster* in particular has given important insights into RNAi responses against pathogenic viruses and viral RNAi inhibitors (31, 54, 83, 86, 91). RNAi is well characterized for *Drosophila*, and orthologs of antiviral RNAi genes have been found in *Aedes* and *Culex* spp. (13, 63).

Arboviruses, or arthropod-borne viruses, are RNA viruses mainly of the families *Bunyaviridae*, *Flaviviridae*, and *Togaviridae*. The genus *Alphavirus* within the family *Togaviridae* contains several mosquito-borne pathogens: arboviruses such as Chikungunya virus (16) and equine encephalitis viruses (88). Replication of the prototype Sindbis virus and Semliki Forest virus (SFV) is well understood (44, 71, 74, 79). Their genome consists of a positive-stranded RNA with a 5' cap and a 3' poly(A) tail. The 5' two-thirds encodes the nonstructural polyprotein P1234, which is cleaved into four replicase pro-

teins, nsP1 to nsP4 (47, 58, 60). The structural polyprotein is encoded in the 3' one-third of the genome and cleaved into capsid and glycoproteins after translation from a subgenomic mRNA (79). Cytoplasmic replication complexes are associated with cellular membranes (71). Viruses mature by budding at the plasma membrane (35).

In nature, arboviruses are spread by arthropod vectors (predominantly mosquitoes, ticks, flies, and midges) to vertebrate hosts (87). Little is known about how arthropod cells react to arbovirus infection. In mosquito cell cultures, an acute phase with efficient virus production is generally followed by the establishment of a persistent infection with low levels of virus production (9). This is fundamentally different from the cytoplasmic events following arbovirus interactions with mammalian cells and pathogenic insect viruses with insect cells. Alphaviruses encode host response antagonists for mammalian cells (2, 7, 34, 38).

RNAi has been described for mosquitoes (56) and, when induced before infection, antagonizes arboviruses and their replicons (1, 4, 14, 15, 29, 30, 32, 42, 64, 65). RNAi is also functional in various mosquito cell lines (1, 8, 43, 49, 52). In the absence of RNAi, alphavirus and flavivirus replication and/or dissemination is enhanced in both mosquitoes and *Drosophila* (14, 17, 31, 45, 72). RNAi inhibitors weakly enhance SFV replicon replication in tick and mosquito cells (5, 33), posing the questions of how, when, and where RNAi interferes with alphavirus infection in mosquito cells.

Here we use an *A. albopictus*-derived mosquito cell line to study RNAi responses to SFV. Using reporter-based assays, we demonstrate that SFV cannot avoid or efficiently inhibit the establishment of an RNAi response. We also demonstrate that the RNAi signal can spread between mosquito cells. SFV can-

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not inhibit cell-to-cell spread of the RNAi signal, and spread of the virus-induced RNAi signal (dsRNA/siRNA) can inhibit the replication of incoming SFV in neighboring cells. Furthermore, we show that SFV expression of a siRNA-binding protein increases levels of virus replication mainly by enhancing virus spread between cells rather than replication in initially infected cells. Taken together, these findings suggest a novel mechanism, cell-to-cell spread of antiviral dsRNA/siRNA, by which RNAi limits SFV dissemination in mosquito cells.

MATERIALS AND METHODS

Cells and viruses. *A. albopictus*-derived U4.4 cells were grown at 28°C in L-15 medium–10% fetal calf serum–8% tryptose phosphate broth. BHK-21 cells were grown in Glasgow minimum essential medium–10% newborn calf serum–10% tryptose phosphate broth (37°C in a 5% CO₂ atmosphere), unless otherwise stated. For all RNAi experiments described below, BHK-21 cells were kept in the same medium and at the same temperature as U4.4 cells for transfection and downstream experiments. The SFV4 strain or derived recombinant SFV was grown in BHK-21 cells in Glasgow minimum essential medium–2% newborn calf serum (37°C in a 5% CO₂ atmosphere). Details of reporter viruses used can be obtained from the authors. The production of SFV-derived virus-like particles (VLPs) was previously described (57). Viruses or VLPs were purified from supernatant by centrifugation (three times for 30 min at 15,000 rpm), concentrated on a 20% (wt/vol) sucrose–TNE buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA [pH 7.4]) cushion by ultracentrifugation (25,000 rpm for 90 min), and resuspended in TNE buffer. Virus was titrated by plaque assay on BHK-21 cells, and VLPs were titrated by indirect immunofluorescence and quantification of infected cells using anti-nsP3 antibody.

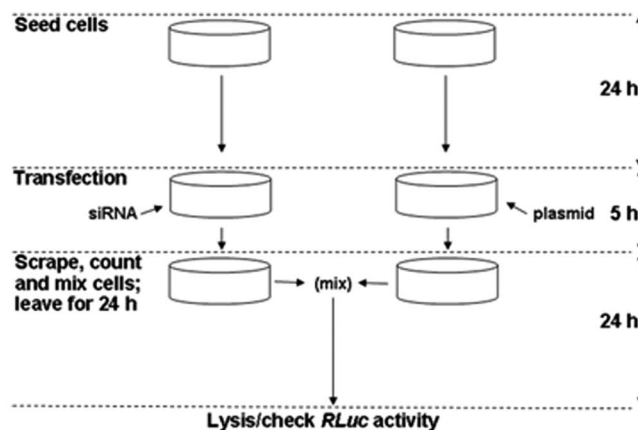
Plasmids and siRNA. Plasmid pRL-CMV (Promega) encodes *Renilla* luciferase (*RLuc*) under the control of the cytomegalovirus immediate-early promoter, which is active in mosquito cells (48). siRNAs for *RLuc* and negative control siRNA (siRNA 1) were obtained from Ambion (catalog numbers AM4630 and AM4635); other negative control siRNAs were found to be similar to the latter. Block-iT fluorescent siRNA (catalog number 2013; Invitrogen) is labeled with fluorescein.

dsRNA production. Long dsRNA of approximately 600 bp was produced using the MegascriptRNAi kit (Ambion). PCR products encoding a T7 promoter at each end and spanning the *RLuc* and enhanced green fluorescent protein (eGFP) genes from the start codon on were produced using primer pairs T7dsRenFD/RE (TAATACGACTCACTATAGGGATGACTTCGAAAAGTTT ATGATCCAG/TAATACGACTCACTATAGGGCTGCAAATTCCTCTGGT TCTAACTTTC) and dsT7eGFPFD/RE (TAATACGACTCACTATAGGGAT GGTGAGCAAGGGCGAGGAGCTGTTC/TAATACGACTCACTATAGGG CTGGGTGCTCAGGTAGTGGTGTCTGGGC) and pRL-CMV or pEGFP-N1 as templates, respectively. dsRNA was purified and aliquoted before use.

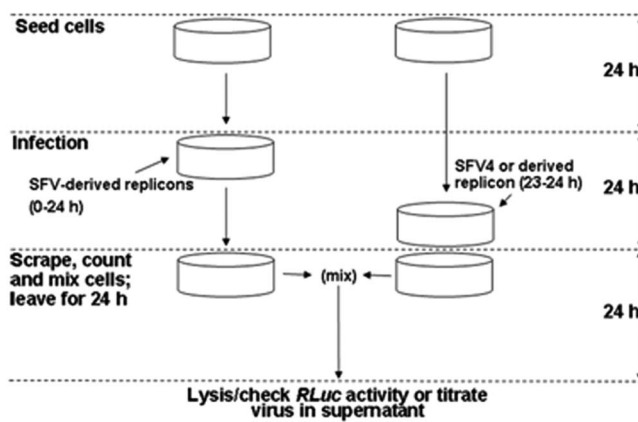
Transfection and cell contact experiments. A total of 1.8×10^5 to 2×10^5 U4.4 (or 1.5×10^5 BHK-21) cells/well were grown in 24-well plates. Before transfection, medium was replaced by fresh complete medium. DNA (20 ng) and/or siRNA (final concentration of 5 or 10 nM)/dsRNA (5 ng) was mixed with 1 μ l/well Lipofectamine 2000 (Invitrogen) in Opti-medium according to the manufacturer's instructions. One hundred microliters of the nucleic acid-Lipofectamine 2000 complexes was added to 400 μ l of medium in each well and incubated for 5 h at 28°C. After transfection, cells were washed twice to remove liposomes, and complete medium was added. For contact experiments to analyze cell-to-cell spread of RNAi using reporter genes, approximately 10^6 U4.4 (or BHK-21) cells/well (six-well plates) were transfected with DNA (80 ng) or siRNA (final concentration, 5 nM) using 1 μ l/well Lipofectamine 2000 for 5 h. Where indicated, SFV infection was carried out prior to siRNA transfection. At 5 h post-transfection, cells were scraped and mixed (two times; 1.5×10^5 or 1.5×10^4 cells/well for a high or low density of cells, respectively) in 24-well plates. Cells were lysed at 24 h postmixing. For contact experiments using SFV replicon- and virus-infected cells, the same cell numbers as described above were mixed for high and low densities. To mix BHK and U4.4 cells at a high density, 1.5×10^5 cells of each cell type were added per well. A flow chart of these experiments is shown in Fig. 1A and B.

Electroporation of mosquito cells. To electroporate DNA, 2×10^7 U4.4 cells were resuspended in 800 μ l ice-cold phosphate-buffered saline (PBS) and mixed with 500 ng pRL-CMV. Four hundred fifty microliters of the cell-DNA mixture was then pipetted into a 4-mm electroporation cuvette and pulsed twice (300 V with a pulse length of 4 ms and a pulse interval of 5 s) in a Bio-Rad electropo-

(A) Mixing of transfected cells



(B) Mixing of infected cells



(C) Scrape-loading of siRNA

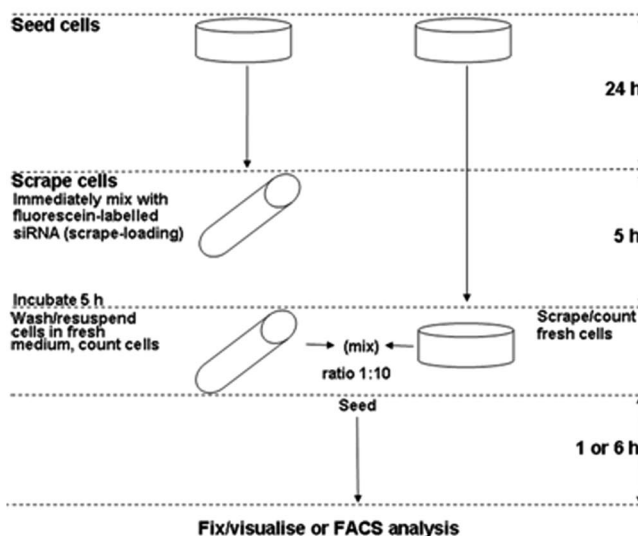


FIG. 1. Flow charts for cell mixing and scrape-loading experiments involving U4.4 mosquito cells. (A) Mixing of transfected cells. (B) Mixing of infected cells. (C) Scrape loading of fluorescein-labeled siRNA into cells. Experimental details such as cell numbers, etc., are described in Materials and Methods.

erator (Genepulser Xcell with CE module). Electroporated cells were then transferred into fresh complete medium and allowed to recover for 5 h; dead cells were removed.

Scrape loading of siRNA. The introduction of macromolecules via scrape loading was described previously (37, 59). For scrape loading of siRNA, six-well plates were seeded with 6.5×10^5 U4.4 cells/well and incubated at 28°C for 24 h. Cells were then scraped into 1 ml of fresh complete cell culture medium in a sterile round-bottom tube (catalog number 352054; BD Falcon), and fluorescein-labeled Block-iT siRNA (catalog number 2013; Invitrogen) was immediately added to a final concentration of 50 nM for 5 h at 28°C; membrane damage seals within less than 1 min (discussed in reference 26), leaving enough time for cells to recover. Cells were then centrifuged (5 min at 1,500 rpm), washed twice, resuspended in 1 ml of fresh complete medium, and counted. A total of 10^5 scrape-loaded cells were mixed with 10^6 nonfluorescent, fresh U4.4 cells in a total volume of 1 ml and seeded onto sterile coverslips in six-well plates. Cells were incubated at 28°C for 1 or 6 h and fixed in 10% neutral buffered formalin (catalog number 00600E; Surgipath Europe) for 1 h. Coverslips were then washed with PBS, mounted, and sealed, and fluorescence was visualized (Zeiss AxioSkop confocal microscope). Fluorescence-activated cell sorting (FACS) was performed on a Becton Dickinson FACSCalibur apparatus; cells were scrape loaded/mixed as described above, seeded into wells, removed from wells at 1 and 6 h postseeding, and immediately analyzed by FACS. For each experiment, 20,000 cells were gated, and numbers and percentages of fluorescent cells were measured. A flow chart of these experiments is shown in Fig. 1C.

Infection. Virus or VLPs were diluted in PBS with 0.75% bovine serum albumin; cells were infected at 28°C for 1 h and washed twice to remove any unbound particles. Multiplicity of infection (MOI) refers to PFU for viruses and infectious particles for VLPs. Complete medium was then added to cells. Infection efficiency was monitored by immunostaining using an anti-nsP3 antibody. For cell contact experiments with SFV4(3H)-*RLuc* and SFV VLPs, approximately 6.5×10^5 U4.4 cells/well (in six-well plates) were infected (MOI of 10) for the times indicated before cells were scraped and mixed at low or high densities (as described above). Virus-spreading experiments were carried out in six-well plates; confluent cell monolayers contained approximately 6.5×10^5 U4.4 cells/well at the time of infection with MOIs as indicated.

Luciferase assays. Cells were lysed in passive lysis buffer (Promega), and *RLuc* activities were measured using a dual-luciferase assay (Promega) with a GloMax 20/20 luminometer.

Real-time qPCR. Quantification of viral genome copy numbers was performed essentially as previously described (7). Briefly, RNA was isolated from U4.4 cells (three independent biological replicates per time point) using RNeasy (Qiagen). RNA quantity and quality were assessed with a NanoDrop spectrophotometer (Fisher Scientific). A total of 0.5 µg of total RNA from each sample was reverse transcribed, and each of those reactions was analyzed in triplicate by quantitative PCR (qPCR). The reaction mix contained 0.8 µM of each primer, 40 mM deoxynucleoside triphosphates, 3 mM MgCl₂, a 1:10,000 dilution of SYBR green (Biogene Ltd.), 0.75 U Fast Start *Taq* (Roche Applied Science), and 2 µl of template. Tubes were heated to 94°C for 5 min, and the PCR was then cycled through 94°C for 20 s, 62°C for 20 s, and 72°C for 20 s for 40 cycles on a RotorGene 3000 instrument (Corbett Research). Sequences of the primers were as follows: 5'-GCAAGAGGCAAACGAACAGA-3' (SFV-nsP3-for) and 5'-GGGAAAAGATGAGCAAACCA-3' (SFV-nsP3-rev).

Immunostaining. U4.4 cells were fixed with 4% paraformaldehyde. After two washes (always in PBS), cells were permeabilized with 0.3% Triton X-100 in PBS for 20 min and washed twice. After blocking with CAS block (Invitrogen) (20 min), primary anti-nsP3 antibody (in CAS block; anti-nsP3 at a 1:800 dilution) was added, followed by three washes. Incubation with secondary antibody (goat anti-rabbit biotinylated immunoglobulin G in CAS block at a 1:750 dilution) was followed by three washes, and streptavidin-conjugated Alexa Fluor 594 was added. After two washes, slides were mounted with mounting medium (Vector Laboratories), and images were acquired (Zeiss AxioSkop confocal microscope).

RESULTS

Like other arboviruses, SFV infection of cultured mosquito cells usually begins with an acute phase of efficient virus production between 12 and 24 h and then enters a persistent phase during which only a few cells (1 to 2%) produce virus (22). In this study, we used the *A. albopictus*-derived U4.4 cell line, which has been shown to closely resemble infectivity in the mosquito and has been previously used to study virus-host

interactions (19, 20, 61, 68). U4.4 cells have functional antimicrobial signaling pathways, and SFV4 infection starts with a burst of virus production between 12 and 24 h postinfection (p.i.), followed by persistent low-level virus production; infection at an MOI of 10 leads to an initial infection of all cells in the culture (28). Thus, U4.4 cells are a good model system in which to study SFV-mosquito cell interactions, and SFV4 displays the expected characteristics of an arbovirus in these cells.

SFV interference with siRNA-induced RNAi. Virus-induced dsRNA, the initiator of RNAi responses, is produced in Sindbis virus-infected mammalian and mosquito cells (78). Similarly, work in U4.4 cells infected with recombinant SFV encoding an nsP3-eGFP fusion protein, SFV(3F)4-eGFP (81), indicates that at 10 and 24 h p.i., large amounts of virus-induced dsRNA accumulates in replication complexes (not shown).

Previous work has shown that an RNAi response induced before infection can inhibit arbovirus replication in arthropod cells and that alphavirus infection of arthropod cells leads to the production of short, virus-derived siRNAs (32, 73); we also found that to be the case in SFV-infected U4.4 cells (our unpublished observations). To determine whether SFV infection of mosquito cells can interfere with the induction of RNAi or an ongoing RNAi process, the activity of *RLuc* produced from expression plasmid pRL-CMV in the presence of *RLuc*-specific siRNA or negative control siRNA was used to quantify RNAi activity in control and SFV4-infected U4.4 mosquito cells.

First, we determined whether SFV4 could suppress an established RNAi reaction. U4.4 cells were cotransfected with *RLuc* reporter plasmid pRL-CMV and *RLuc* or negative control siRNA and infected or mock infected with SFV4 24 h later. This timing allowed an accumulation of *RLuc* mRNA prior to infection and avoided any effects of the infection on plasmid expression. Luciferase activity was measured 24 h p.i. A strong reduction in levels of *RLuc* reporter gene expression was observed in cells receiving luciferase siRNA but not in cells receiving negative control siRNA. Neither luciferase expression nor its reduction by luciferase siRNA was affected by subsequent SFV4 infection (Fig. 2A). We conclude that SFV4 does not interfere with established RISC complexes.

Second, to determine if SFV4 can interfere with the induction of RNAi, U4.4 cells were transfected with *RLuc* reporter plasmid, infected with SFV4 24 h later, and then transfected with *RLuc* or negative control siRNAs. Luciferase activity was measured 24 h posttransfection (Fig. 2B). The established infection did not prevent silencing. We conclude that SFV4 does not prevent the induction of RNAi, at least to non-virus-related sequences. Infection up to 6 h (to allow increased protein expression) before siRNA transfection did not change the result (not shown).

It was previously shown that SFV-specific RNAi, established prior to infection, can suppress virus replication (15); this was also the case for U4.4 cells (not shown). To analyze whether virus can interfere specifically with antiviral RNAi induced after infection, the *RLuc* gene was cloned into the virus non-structural protein open reading frame (Fig. 3A) to create SFV4(3H)-*RLuc* (46). In this study, *RLuc* provides an easily quantifiable indicator of virus replication, and *RLuc* siRNAs should target the viral genome/mRNA for degradation. In

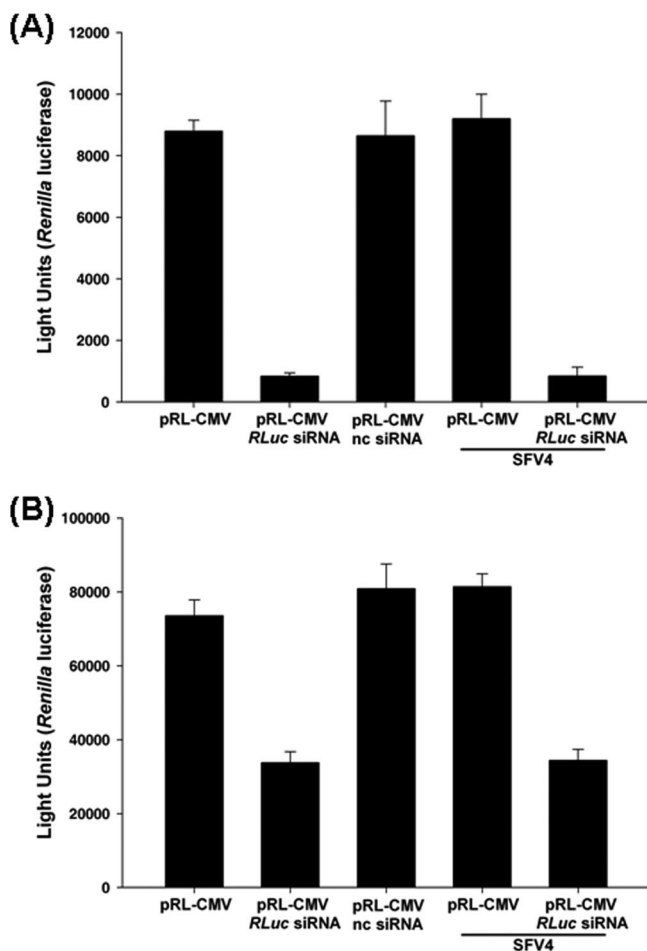


FIG. 2. SFV interactions with RNAi. (A) U4.4 mosquito cells were transfected with pRL-CMV (which expresses *RLuc*) (20 ng) and *RLuc* or negative control (nc) siRNAs (5 nM) for 5 h and infected with SFV4 (MOI of 10) at 24 h posttransfection, and luciferase activities were measured at 24 h p.i. (B) U4.4 mosquito cells were transfected with pRL-CMV (20 ng), infected with SFV4 (MOI of 10) at 24 h posttransfection, and then transfected with *RLuc* or negative control siRNAs (5 nM) for 5 h, and luciferase activities were measured 24 h posttransfection. Each bar represents the mean of three replicates; error bars indicate standard deviations. Every experiment was repeated at least twice.

vertebrate cells, reporter genes inserted into the nonstructural open reading frame directly reflect replication levels for up to 6 h p.i. (46); however, in insect cells, where host gene expression is much less affected by SFV infection, we found that *RLuc* reporter gene expression reflects virus replication and genome RNA levels over longer periods (28) and show here that this is the case (see Fig. 9) for a minimum of 48 h p.i.

When *RLuc* siRNA was transfected 1 or 8 h after a high-MOI infection (MOI of 10), the level of virus replication (as determined by luciferase activity) at 24 h posttransfection was decreased by approximately 75 or 50% compared to that of control cells or cells treated with negative control siRNA (Fig. 3B). We conclude that SFV4 cannot inhibit siRNA-induced antiviral RNAi.

SFV interference with long-dsRNA-induced RNAi. Despite not being able to interfere with RISC formation, the above-

described experiments with preformed siRNA do not exclude the possibility that SFV4 interferes with siRNA production, for example, cleavage of long dsRNA into siRNAs. To assess this possibility, experiments similar to those described above were carried out with *RLuc*-encoding SFV4(3H)-*RLuc* and 600-bp dsRNAs derived from *RLuc* or eGFP (see Materials and Methods). As shown in Fig. 3C, the transfection of *RLuc*-dsRNA but not eGFP-derived dsRNA 1 or 8 h following infection of U4.4 cells with SFV4(3H)-*RLuc* (MOI of 10) strongly reduced virus replication; this effect was slightly stronger when dsRNA was transfected immediately p.i. Similarly, silencing of plasmid-encoded *RLuc* activity by 600-bp dsRNA was not affected by infection (not shown).

The RNAi signal can spread between mosquito cells. In plants, the RNAi signal can spread cell to cell or through vasculature to affect the whole plant; this is generally referred to as non-cell-autonomous or systemic RNAi (85, 90). Some plant viruses can antagonize systemic RNAi responses (67). Gap junction-mediated cell-to-cell spread of an RNAi signal has been observed in mammalian cell culture (82, 89). In insects, it has been suggested that systemic RNAi can take place, but to date, direct cell-to-cell spread has not been demonstrated (6, 11, 24, 25). However, gap junctions and cytoplasmic bridges are present between *A. albopictus*-derived cells in culture (12).

Insects do not encode RNA-dependent RNA polymerase to amplify siRNAs, as occurs in plants during systemic RNAi, but short-distance cell-to-cell spread (10 to 15 cells) of the RNAi signal might not require prior amplification (85). To assess whether an RNAi signal can spread between mosquito cells, U4.4 cells were transfected with *RLuc* or negative control siRNA. These cells were then mixed with U4.4 cells transfected with pRL-CMV and plated at low (minimal contact) or high (many contacts) densities. At low density, there were few or no contacts between cells, whereas at high density, there were many contacts (Fig. 4A). Low-density seeding controls were necessary to avoid the formation of cytoplasmic bridges; reporter gene experiments showed that RNAi remains functional in cells at low density (not shown). As shown in Fig. 4B (bottom), when the two types of cells were in contact (high density), the *RLuc* siRNA-transfected cells were able to suppress reporter gene expression in pRL-CMV-transfected cells. In contrast, no suppression of the *RLuc* reporter was observed at low density (Fig. 4B, top). SFV4 infection for 1 h before siRNA transfection did not inhibit cell-to-cell spread of the RNAi signal. Extending the infection time (for up to 8 h) before siRNA transfection (to allow viral proteins more time to accumulate) or transfecting 600-bp dsRNA as source of siRNAs did not change the result (not shown). Similar results were also found with the *A. albopictus*-derived cell line C7-10 (not shown).

As a control for the presence of residual, nontransfected Lipofectamine-nucleic acid complexes, U4.4 siRNA donor cells were mixed with pRL-CMV-transfected BHK-21 cells, and we assumed that no communication could happen between cells of vertebrate and invertebrate origins. *RLuc* siRNA-mediated silencing of pRL-CMV does occur in BHK-21 cells at 27°C (Fig. 5A). When U4.4 cells (with siRNA) and BHK-21 cells (with pRL-CMV) were mixed at high density to allow cell contact, no reduction in *RLuc* activity was ob-

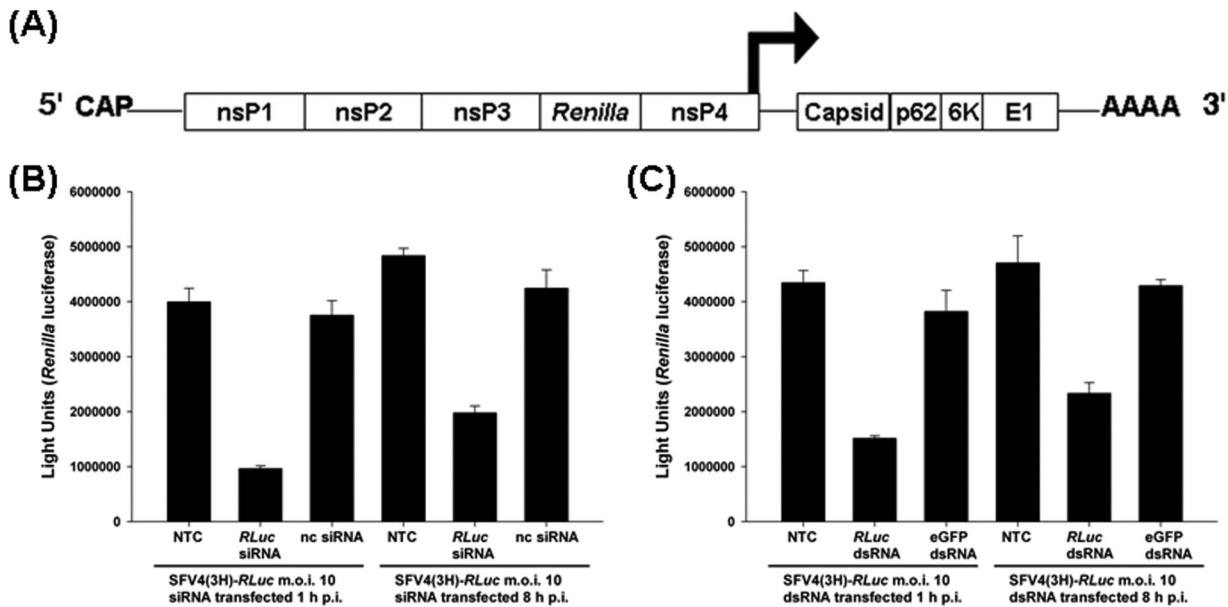


FIG. 3. SFV interactions with antiviral RNAi. (A) SFV4(3H)-*RLuc* encodes *RLuc* (flanked by duplicated nsP2-protease cleavage sites at the nsP3/4 junction) as part of the viral nonstructural polyprotein. (B) Transfection of *RLuc* but not negative control (nc) siRNA (10 nM) of U4.4 mosquito cells with SFV4(3H)-*RLuc* (MOI of 10) at 1 or 8 h p.i. strongly reduced virus replication. *RLuc* activities were determined at 24 h posttransfection. NTC, nontransfected control. (C) SFV fails to inhibit cleavage of dsRNA. Transfection of *RLuc* dsRNA but not eGFP-derived dsRNA (5 ng/well) of approximately 600 bp of U4.4 mosquito cells with SFV4(3H)-*RLuc* (MOI of 10) at 1 or 8 h p.i. strongly reduced virus replication. dsRNAs were derived from *RLuc* or control eGFP (see Materials and Methods); *RLuc* activities were determined at 24 h posttransfection. Each bar represents the mean of three replicates; error bars indicate standard deviations. Every experiment was repeated at least twice.

served (Fig. 5A). To show that siRNA transfer does not rely on damaged membranes in donor and/or recipient cells, an additional control experiment was carried out. U4.4 cells were transfected with siRNA or reporter plasmid pRL-CMV and then scraped and seeded at high density on attached U4.4 cells (and therefore with intact cell membranes) previously transfected with pRL-CMV or siRNA, respectively. As shown in Fig. 5B, *RLuc* siRNAs successfully silenced *RLuc* activity. This demonstrates that siRNAs transfer through intact cellular membranes, and membrane damage is not relevant for siRNA transfer.

To verify this finding using non-liposome-based methods, U4.4 reporter cells were transfected with pRL-CMV (expressing *RLuc*) by electroporation. U4.4 cells infected with SFV-derived VLPs (MOI of 10; infected for 24 h to allow siRNA accumulation) were used as siRNA donor cells. VLPs contain the virus genome coding sequences required for RNA replication, the replicon, but have the virus structural coding sequences deleted; they can efficiently infect cells, but VLP-infected cells cannot generate new virions. VLPs containing three different replicons were used: SFV(nseGFP/H)1 (as a control) had eGFP inserted between duplicated nsP2 cleavage sites in the nsP3/4 junction region, SFV(nseGFP/H)1-*RLuc*AS had *RLuc* cloned in the antisense orientation (*RLuc*AS) after the subgenomic promoter, and SFV(nseGFP/H)1-p19-*RLuc*AS expressed the siRNA-binding protein p19 and *RLuc*AS from a double-subgenomic promoter (Fig. 6A). Silencing by antisense gene expression in mosquito cells infected with SFV replicons was previously described to inhibit superinfection with Rift Valley fever virus (4), and the expression of antisense *RLuc* allows the production of *RLuc* siRNAs without generating

luciferase activity. The well-characterized plant tomosvirus RNAi inhibitor p19 (75) specifically binds to siRNAs and was previously shown to function in *Drosophila* embryo extracts (50). The siRNA-binding activity is a property of the p19 protein, and p19 RNAs display no biological activity (66, 80); we did not detect any effects of full-length or truncated p19-derived dsRNAs on reporter gene silencing (not shown). The reporter U4.4 cells were mixed shortly after electroporation (with pRL-CMV) with donor cells (infected 24 h earlier with VLPs) and incubated for 24 h. As shown in Fig. 6B, contact with siRNA donor cells infected with *RLuc*AS VLPs resulted in a significant decrease ($P < 0.0001$ by paired *t* test) of 20% in luciferase activity in reporter cells; this was rescued by expressing the siRNA-binding protein p19 in the donor cells. These results are consistent with those obtained using Lipofectamine-based studies (Fig. 4) and indicate that the cell-to-cell transfer of siRNA can inhibit gene expression in reporter cells.

Scrape loading of siRNA into mosquito leads to spread of fluorescein-labeled siRNA. As U4.4 cells do not take up siRNAs if simply added to the cells (our unpublished observations), we also verified that cell-to-cell spread of siRNA was occurring using a fluorescein-labeled siRNA (Block-iT fluorescent oligonucleotide; Invitrogen). Scraping of cells (see Materials and Methods for details) results in damaged membranes through which macromolecules such as RNA can be taken up. U4.4 mosquito cells were scraped from plastic dishes using a cell scraper and immediately incubated with fluorescein-labeled siRNA. These cells were then washed, counted, mixed with fresh U4.4 cells at a ratio of 1:10, seeded onto coverslips, and fixed after 1 h (to allow cell attachment and recovery of normal morphology) or 6 h. Total cell numbers and the num-

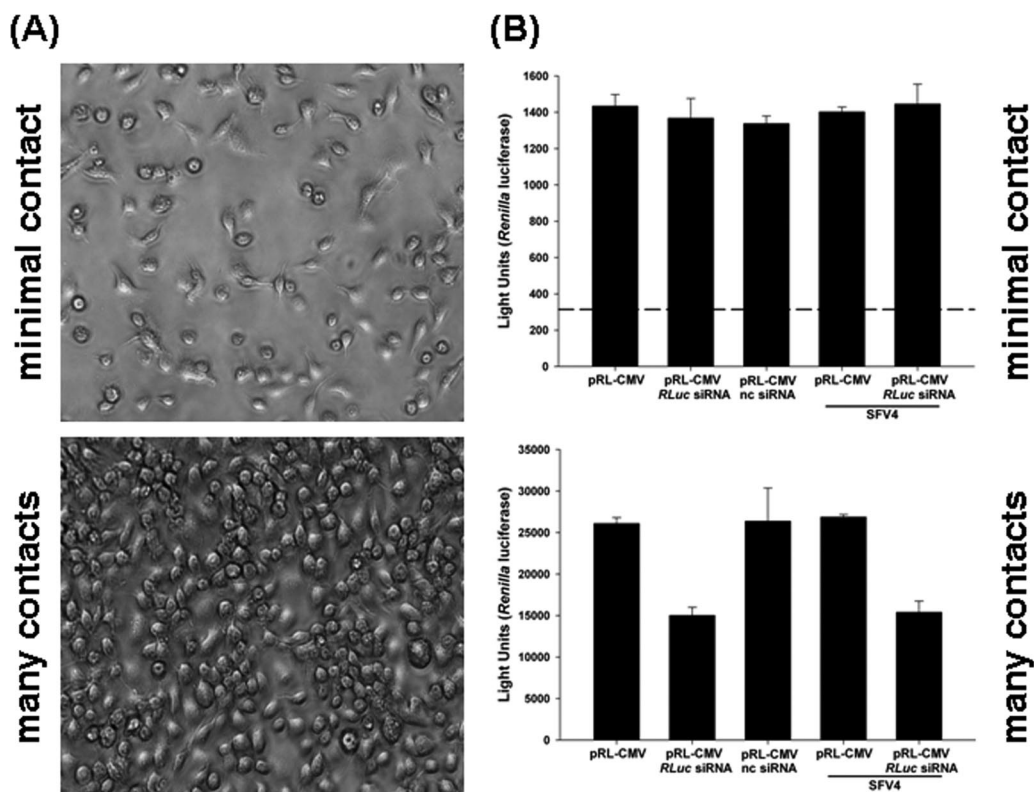


FIG. 4. Spread of RNAi signal between mosquito cells. U4.4 mosquito cells were transfected with pRL-CMV (expressing *RLuc*) or transfected with siRNAs (*RLuc* or negative control [nc]) (5 nM) or first infected with SFV4 (MOI of 10) and then transfected with siRNAs. This was followed by cell mixing. (A) Phase-contrast microscopy showing U4.4 cells mixed at low density (minimal contact [top]) or high density (many contacts [bottom]). (B) RNAi in cultures with minimal (top) or many (bottom) cell-to-cell contacts and effect of SFV4 infection on spread of RNAi signal to U4.4 cells transfected with reporter plasmid pRL-CMV. *RLuc* activities were determined 24 h after cell mixing. Dotted line, background. Each bar represents the mean of three replicates; error bars indicate standard deviations. Every experiment was repeated at least twice.

bers of fluorescent cells per field were determined (Fig. 7A). While cell numbers remained the same at 1 and 6 h postmixing, there was a significant ($P < 0.0001$ by paired t test) increase of 40 to 60% in fluorescent cells (Fig. 7A). A similar experiment was performed to quantify the increase in fluorescent cells from 1 to 6 h post-scrape loading by FACS (described in Materials and Methods). As shown in Fig. 7B, a similar increase in the number of fluorescent cells was observed. This shows that siRNA can spread between U4.4 cells.

Spread of dsRNA/siRNA between mosquito cells inhibits SFV replication. Next, we determined whether cell-to-cell spread of the RNAi signal in mosquito cells was able to affect virus replication. RNAi donor cells were prepared by infecting U4.4 cells with SFV(nseGFP/H)1 VLPs (MOI of 10; control). VLPs were not found to induce the synthesis of secreted antiviral activities. After 24 h to allow SFV replicon-derived dsRNAs and siRNAs to accumulate (and when SFV superinfection exclusion is established) (our observations), VLP-infected or noninfected donor cells were mixed with U4.4 cells freshly infected (1 h p.i.) with SFV4(3H)-*RLuc* (Fig. 3A) (reporter cells) at low or high density (as described above). As shown in Fig. 8A, at 24 h postmixing, in low-density cultures with minimal or no cell-to-cell contact, relative to the noninfected control (NIC) donor cells, the VLP-infected donor cells did not suppress virus replication, as determined by *RLuc*

activity in the reporter cells. In contrast (Fig. 8A), virus replication was strongly inhibited (relative to that of the NIC) when the reporter cells were in close contact (“many contacts”) with siRNA donor cells. An additional experiment was performed to verify that the suppression signal generated from the donor cells involved RNAi. Donor cells were infected with SFV VLPs in which the replicons also expressed the influenza virus NS1 protein under the control of the SFV subgenomic promoter [SFV(nseGFP/H)1-NS1]. The NS1 protein functions as an RNAi inhibitor in arthropod cells, binding both siRNA and longer dsRNA (10, 33, 56). After 24 h (to allow SFV replicon-derived dsRNAs and siRNAs to accumulate), NS1-expressing VLP-infected donor cells were mixed with U4.4 cells infected 1 h previously with SFV4(3H)-*RLuc* (reporter cells) at low or high density (as described above). As shown in Fig. 8A, at 24 h postmixing, in low-density cultures with minimal cell-to-cell contact relative to noninfected donor cells (NIC), the NS1-expressing VLP-infected dsRNA/siRNA donor cells did not affect replication of the reporter virus in the reporter cells. In contrast, virus replication was rescued by about 50% relative to that of donor cells infected with VLPs not expressing NS1 (control), when the reporter cells were in close contact (“many contacts”) with donor cells infected with the NS1-expressing SFV replicon (Fig. 8A).

The NS1 protein is multifunctional in vertebrate cells, and in

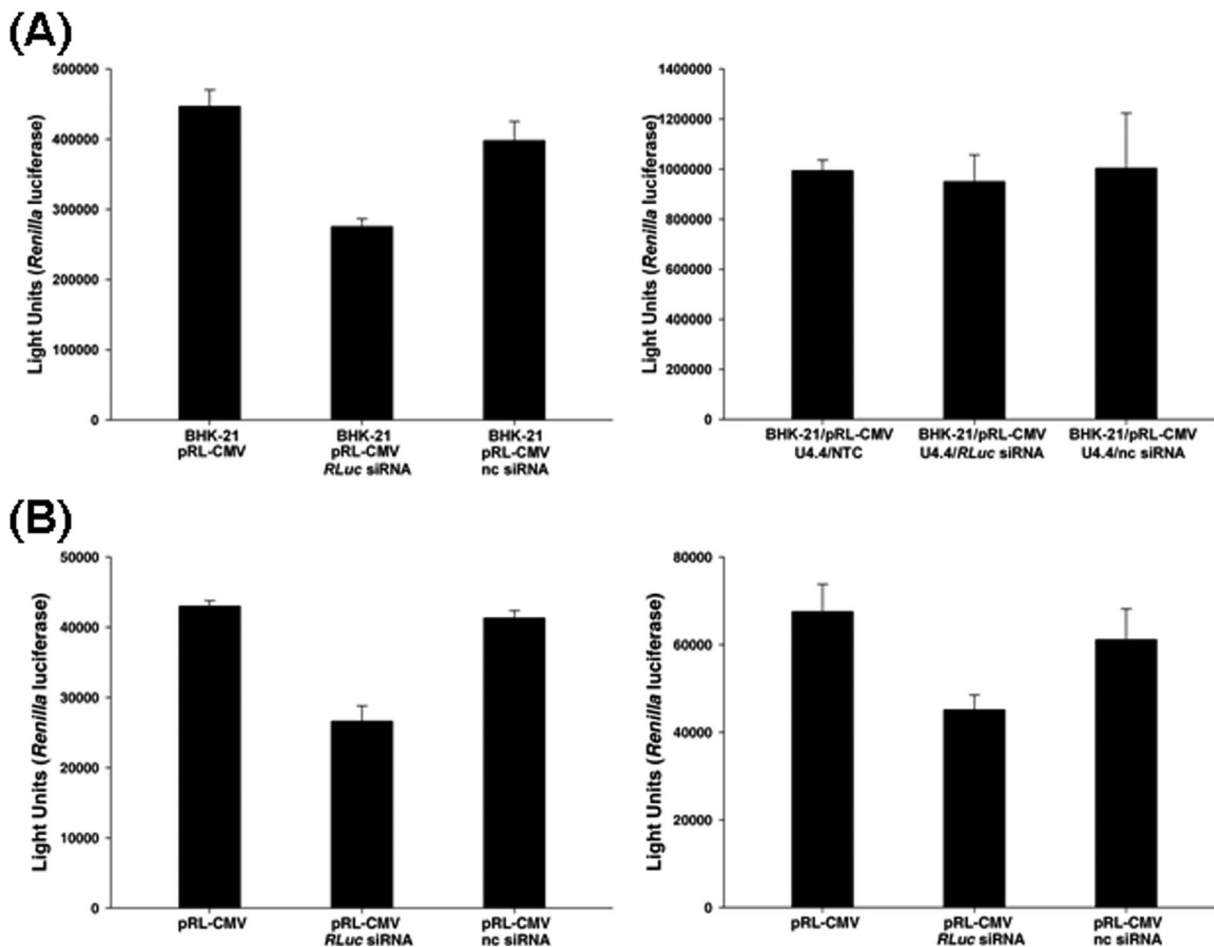


FIG. 5. Spread of RNAi signal is specific and does not require membrane damage. (A, left) Silencing of *RLuc* activity in BHK-21 cells (incubated at 28°C) transfected with 20 ng pRL-CMV and siRNAs (5 nM). (Right) Absence of *RLuc* silencing by RNAi in BHK-21 cells (incubated as described above) transfected with 20 ng pRL-CMV and mixed with U4.4 cells previously transfected with siRNAs (*RLuc* or negative control [nc]) (5 nM). NTC, nontransfected control. (B) U4.4 mosquito cells transfected with pRL-CMV (20 ng) (left) or siRNA (*RLuc* or negative control [nc]) (5 nM) (right) were scraped and then seeded at high density onto U4.4 cells attached to the dish surface and previously transfected with siRNA (left) or pRL-CMV (right), respectively. *RLuc* activities were determined 24 h after cell mixing. Each bar represents the mean of three replicates; error bars indicate standard deviations. Every experiment was repeated at least twice.

the above-described system, reporter cells are infected with virus capable of spreading between reporter cells. While we have not detected an induction of mosquito signaling pathways by dsRNA (our unpublished observations) or SFV itself (28), we had to exclude any secondary effects of NS1 or replicating virus. A similar experiment was therefore performed with SFV VLPs containing control SFV(nseGFP/H)1 or SFV(nseGFP/H)1-p19 replicons as an siRNA donor. Twenty-four hours p.i. (to let replicon-derived siRNAs accumulate), siRNA donor U4.4 cells were mixed with U4.4 reporter cells freshly infected (1 h p.i.) with SFV(*RLuc*/H)1 VLPs (SFV replicon expressing *RLuc* instead of eGFP, as described in the legend of Fig. 6A). At 24 h postmixing, in low-density U4.4 cultures with minimal cell-to-cell contact relative to NIC, the VLP-infected dsRNA/siRNA donor cells did not suppress virus replication, as determined by *RLuc* activity in the reporter cells. However, virus replication in reporter cells was rescued again by about 50% relative to that in donor cells infected with a replicon not expressing p19 when the reporter cells were in close contact (“many

contacts”) with donor cells enriched in dsRNAs and siRNAs derived from the p19-expressing SFV replicon (Fig. 8B).

To directly analyze the effect of replicon-induced, spreading siRNA on SFV production, we carried out an experiment similar to that described in the legend of Fig. 8B, when cells were in close contact (“many contacts”). U4.4 mosquito cells were noninfected (NIC) or infected (MOI of 10) with SFV VLPs containing control SFV(nseGFP/H)1 or SFV(nseGFP/H)1-p19 replicons as siRNA donors. At 24 h p.i. (to let replicon-derived siRNAs accumulate), siRNA donor U4.4 cells were mixed with U4.4 reporter cells freshly infected (1 h p.i.) with SFV4 (MOI of 1). Virus production from SFV4-infected reporter cells was completely rescued (more than a 10-fold increase, similar to NIC donor cell levels) relative to donor cells infected with a replicon not expressing p19 when the reporter cells were in close contact (“many contacts”) with donor cells enriched in dsRNAs and siRNAs derived from the p19-expressing SFV replicon (Fig. 8C).

Together, these studies show that the donor cell-derived

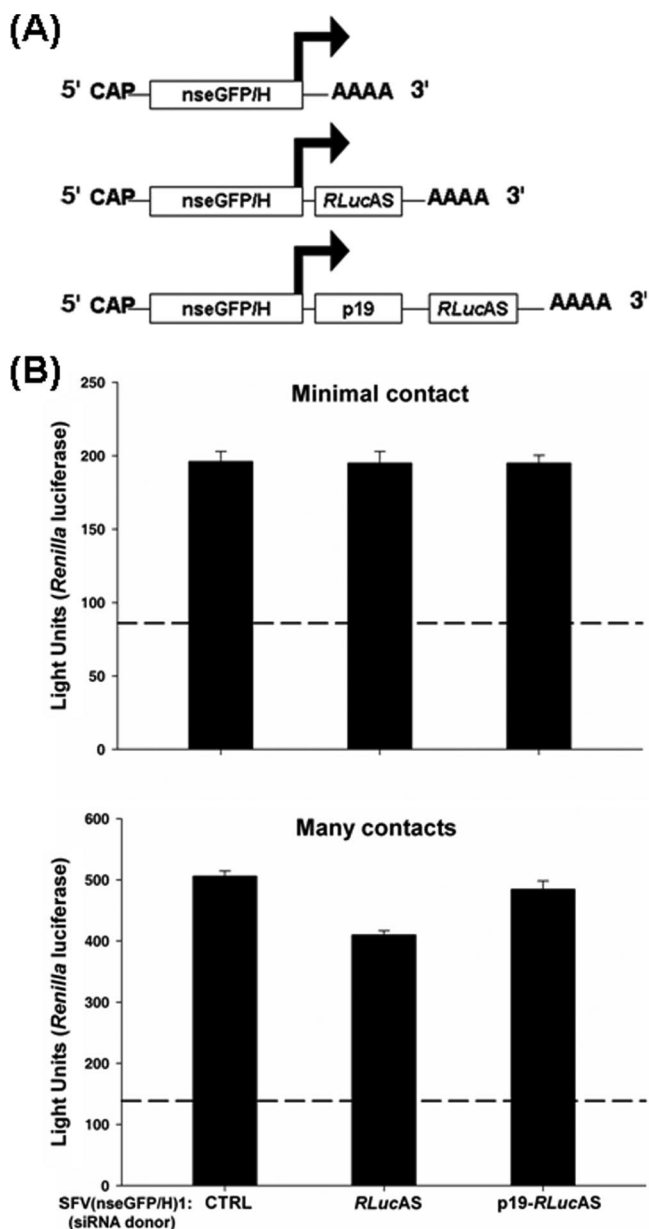


FIG. 6. SFV-induced siRNA spread between U4.4 mosquito cells. (A) Replicons used to produce VLPs. eGFP was inserted between duplicated nsP2 cleavage sites in the nsP3/4 junction region of the nonstructural open reading frame. (B) *RLuc* expression plasmid pRL-CMV was electroporated into U4.4 cells (reporter cells), which were then mixed at a low density (minimal contact [top]) or high density (many contacts [bottom]) with U4.4 cells infected for 24 h with the VLPs SFV(nseGFP/H)1 (control replicon [CTRL]), SFV(nseGFP/H)1-*RLuc*AS, or SFV(nseGFP/H)1-p19-*RLuc*AS to allow siRNA accumulation (donor cells). *RLuc* activities were determined at 24 h postmixing. Dotted line, background. Each bar represents the mean of three replicates; error bars indicate standard deviations. Every experiment was repeated at least twice.

signal is RNAi based and that it can spread from cell to cell to inhibit virus replication.

Viral expression of an siRNA-binding protein strongly enhances virus spread through cultured cells. Given the above-described findings, we wanted to know whether the spread of

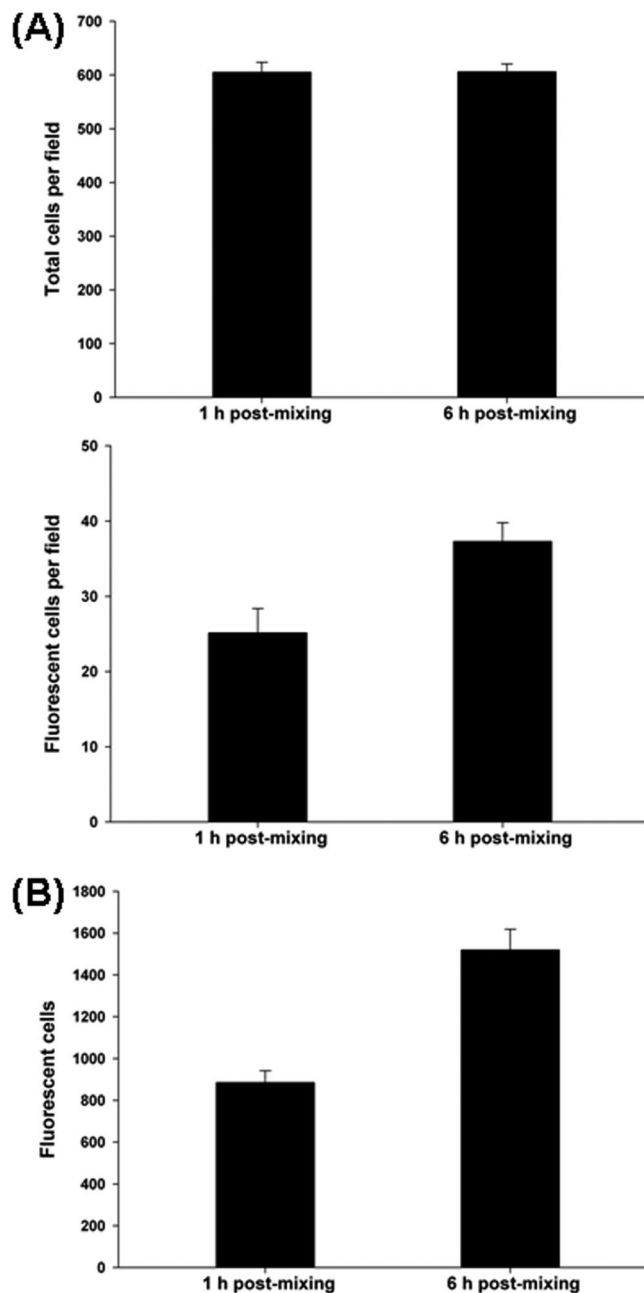


FIG. 7. Scrape loading of siRNA and spread of fluorescein-labeled siRNA (Block-iT fluorescent oligonucleotide; Invitrogen). Scraping of cells from the surface using a cell scraper briefly results in damaged membranes through which RNA can be taken up (see Materials and Methods). U4.4 mosquito cells were scraped from the dish surface and immediately incubated with fluorescein-labeled siRNA for 5 h; scrape-loaded cells were then washed, counted, mixed with fresh U4.4 cells at a ratio of 1:10, and plated onto coverslips (A) or into wells (B). (A) Cells were fixed at 1 h (to allow cell attachment and recovery of normal morphology) and 6 h postplating; total cell numbers (top) and numbers of fluorescent cells (bottom) per field were counted (at magnifications of $\times 40$ [10 fields] and $\times 20$ [15 fields], respectively). (B) Alternatively, cells seeded into wells (in triplicate for each time point) were scraped and immediately analyzed by FACS; for each data point, 20,000 cells were gated, and fluorescent cells were counted. Numbers of fluorescent cells at 1 and 6 h postmixing are indicated. Bars represent the means, and error bars indicate standard deviations. Every experiment was repeated at least twice.

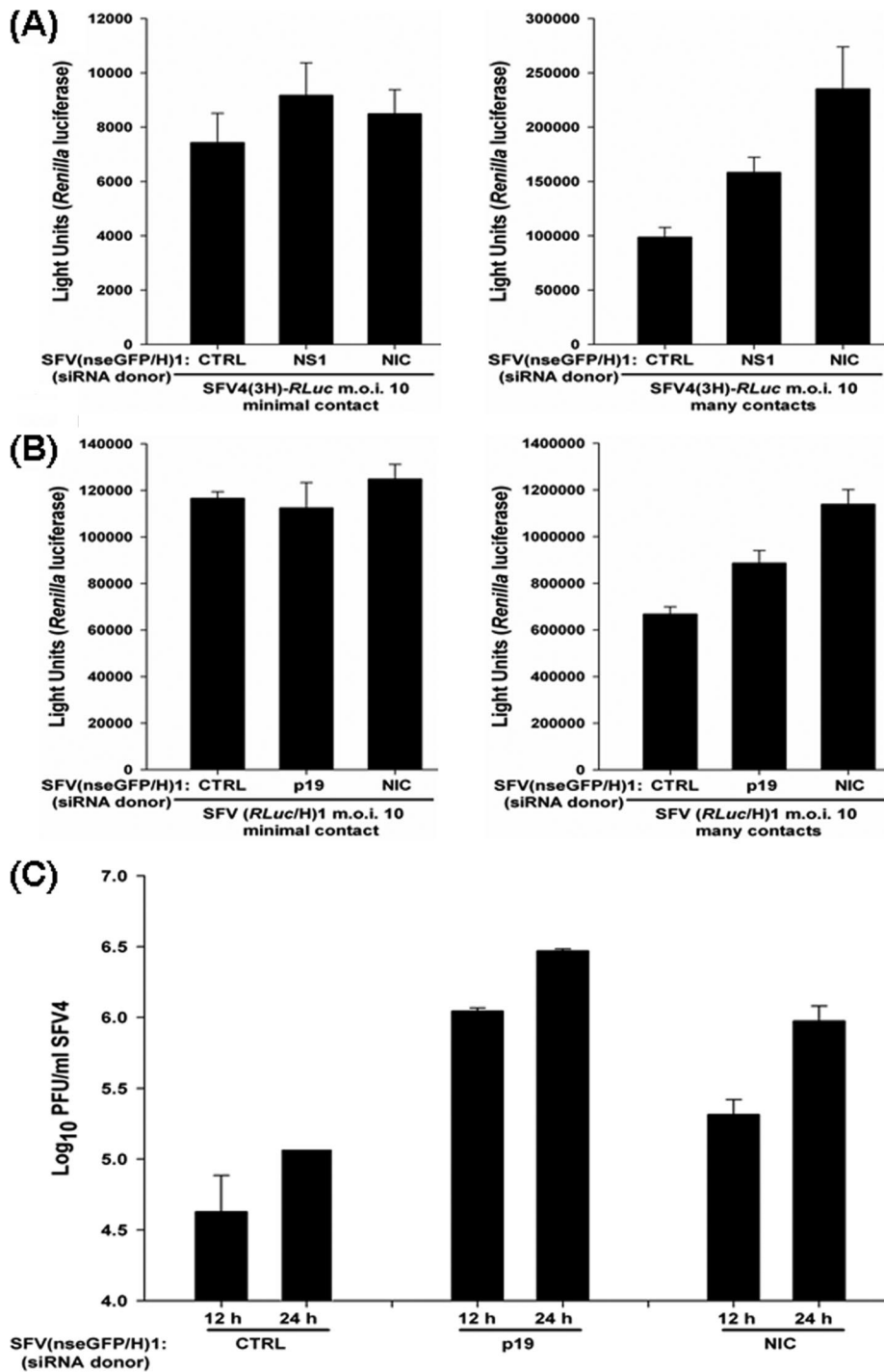


FIG. 8. Spread of the RNAi signal between cells results in antiviral activity. Parallel cultures of U4.4 mosquito cells were noninfected (NIC) (A and B) or infected (MOI of 10) with VLPs containing the SFV replicons SFV(nseGFP/H)1 (control replicon [CTRL]) (A and B), SFV(nseGFP/H)1-NS1 (A), or SFV(nseGFP/H)1-p19 (B) and then incubated for 24 h to allow replicon-derived dsRNA/siRNA accumulation in donor cells. Replicons express eGFP from the nonstructural region (as described in the legend of Fig. 6) and no additional protein (control), influenza virus NS1 (dsRNA/siRNA-binding RNAi inhibitor in arthropod cells), or tombusvirus p19 (siRNA-binding protein) from the subgenomic promoter. After 24 h, replicon-containing or noninfected cells were mixed with U4.4 cells freshly infected (1 h p.i.) with *RLuc*-encoding SFV4(3H)-*RLuc* virus (A) or SFV(*RLuc/H*)1 VLPs (B) (MOI of 10) (reporter cells) at low (minimal contact) or high (many contacts) density. *RLuc* activity (indicating replication of virus) was measured 24 h postmixing. (C) Cell-to-cell spread of siRNA inhibits SFV4 production. U4.4 mosquito cells were noninfected (NIC) or infected (MOI of 10) with VLPs containing SFV replicons (as described above), SFV(nseGFP/H)1 (control replicon), or SFV(nseGFP/H)1-p19 and then incubated for 24 h to allow replicon-derived dsRNA/siRNA accumulation in donor cells. After 24 h, replicon-containing or noninfected donor cells were mixed with U4.4 cells freshly infected (1 h p.i.) with SFV4 (MOI of 1) at high density (many contacts). SFV4 production at 12 and 24 h postmixing is shown; virus titers (PFU/ml) were determined by plaque assay (as described in Materials and Methods). Each bar represents the mean of three replicates; error bars indicate standard deviations. Every experiment was repeated at least twice.

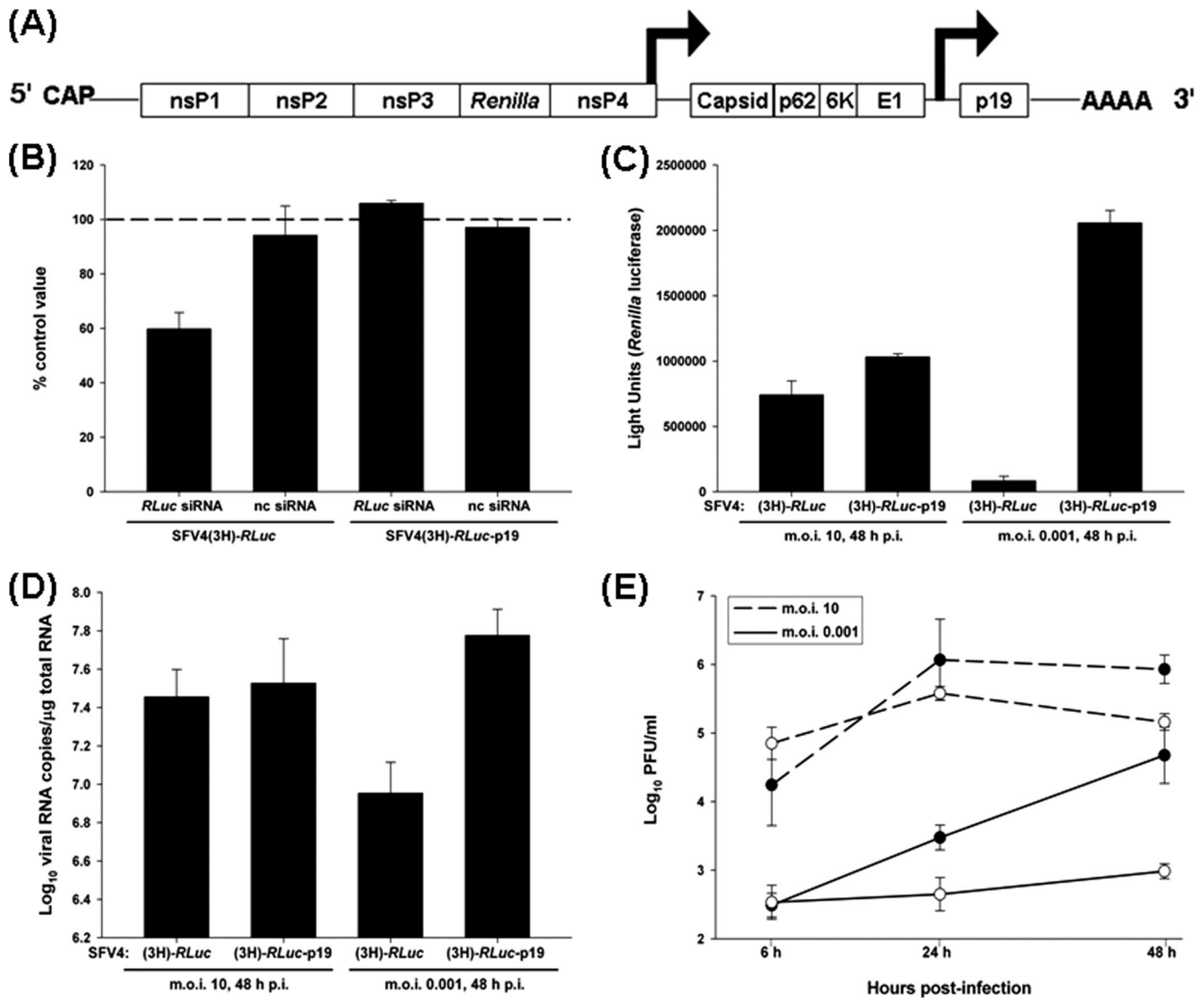


FIG. 9. Replication of SFV encoding an RNAi inhibitor. (A) *RLuc* reporter SFV expressing tobusvirus siRNA-binding protein p19 from a duplicated subgenomic promoter [SFV4(3H)-*RLuc*-p19]. (B) Confluent U4.4 cells were infected at an MOI of 1 with SFV4(3H)-*RLuc* (Fig. 3) or SFV4(3H)-*RLuc*-p19 and transfected with *RLuc* or negative control (nc) siRNAs (concentration of 10 nM) for 5 h at 8 h p.i. *RLuc* activities were measured 24 h posttransfection. Results are expressed as a percentage of the control value (nontransfected, infected U4.4 cells [dotted line]). (C) Confluent U4.4 mosquito cells were infected at a high MOI (MOI of 10) or low MOI (MOI of 0.001) with SFV4(3H)-*RLuc*-p19 or SFV4(3H)-*RLuc*. Cells were lysed at 48 h p.i., and *RLuc* activities were determined. High MOI mimics initial infection as all cells are infected, while low MOI allows the virus to spread through the monolayer and undergo multiple rounds of infection. (D) Infection of U4.4 mosquito cells with SFV4(3H)-*RLuc* or SFV4(3H)-*RLuc*-p19 at high MOI (MOI of 10) or low MOI (MOI of 0.001). RNA was extracted at 48 h p.i., and virus genome copy numbers were determined by real-time qPCR (targeting a region of nsP3 as described in Materials and Methods). (E) U4.4 mosquito cells were infected with SFV4(3H)-*RLuc* or SFV4(3H)-*RLuc*-p19 at high MOI (MOI of 10) or low MOI (MOI of 0.001). Virus production (PFU/ml) was measured at 6, 24, and 48 h p.i. by plaque assay titration of supernatants (as described in Materials and Methods). ●, SFV4(3H)-*RLuc*-p19; ○, SFV4(3H)-*RLuc*. Each bar represents the mean of three replicates (with the exception of real-time qPCR, where more replicates were analyzed) (see Materials and Methods); error bars indicate standard deviations. Every experiment was repeated at least twice.

the RNAi signal is important in limiting virus dissemination in mosquito cells. Studies with arbovirus-derived replicons show that the expression of RNAi inhibitors had weak enhancing effects on replication (5, 33). Previous work has also shown that antiviral RNAi restricts the spread of O'nyong-nyong and Sindbis alphaviruses into mosquito tissues (14, 45); however, the mechanisms involved remain unclear: a secondary effect of enhanced replication/virus production in initially infected cells,

enhanced dissemination of virus, differential RNAi responses in tissues, or a combination of these factors is a possibility. To analyze the link between virus dissemination and cell-to-cell spread of the RNAi signal, we studied SFV4 expressing the siRNA-binding tobusvirus protein p19.

p19 was inserted into an *RLuc*-encoding SFV after a duplicated subgenomic promoter, placed after the structural region to create SFV4(3H)-*RLuc*-p19 (Fig. 9A). The replication of

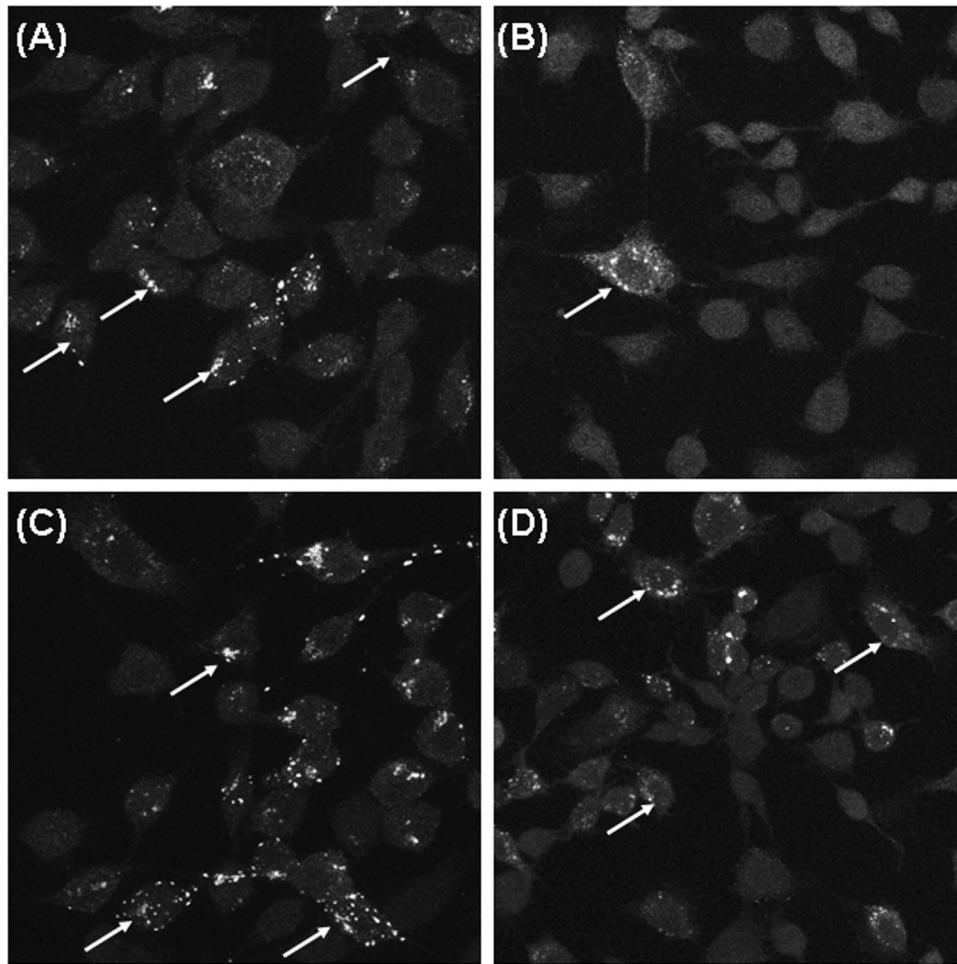


FIG. 10. Visualization of SFV spread. U4.4 mosquito cells were infected with SFV4(3H)-*RLuc* (A and B) or SFV4(3H)-*RLuc*-p19 (C and D) at a high MOI (MOI of 10) (A and C) or low MOI (MOI of 0.001) (B and D). Cells were fixed at 24 h p.i. and stained with an anti-nsP3 antibody (as described in Materials and Methods). Representative sections of cells are shown; selected replication complexes (bright focal staining) are indicated by arrows.

SFV4(3H)-*RLuc* but not of SFV4(3H)-*RLuc*-p19 was inhibited by antiviral siRNAs (Fig. 9B). In subsequent experiments, confluent cultures of U4.4 mosquito cells were infected with SFV4(3H)-*RLuc*-p19 or SFV4(3H)-*RLuc* at a high MOI (MOI of 10) or a low MOI (MOI of 0.001) and lysed at 48 h p.i., and *RLuc* activities (replication readout) were determined (Fig. 9C). Following high-MOI infection, in which all cells were infected synchronously, the level of virus replication was only slightly increased with p19 expression. Following low-MOI infection, however, which allows rounds of virus infection and spread, p19-encoding virus replicated far more efficiently than control virus (20-fold increase); similar results were also found with the *A. albopictus*-derived cell line C7-10 (not shown). No cytopathic effect or change in cell numbers was observed at either high- or low-MOI infection (not shown).

Real-time qPCR was used to quantify viral genomes. At 48 h p.i., as shown in Fig. 9D, at a high MOI (MOI of 10), genome levels in SFV4(3H)-*RLuc* or SFV4(3H)-*RLuc*-p19 are similar, while infection at a low MOI (0.001) leads to an accumulation of SFV4(3H)-*RLuc*-p19 genome RNA (approximately a sixfold increase). In addition, in U4.4 cells

infected at a high MOI (MOI of 10), virus production by SFV4(3H)-*RLuc*-p19 was similar or approximately eightfold increased (at 48 h p.i.) in comparison to that of SFV4(3H)-*RLuc*, while low-MOI (MOI of 0.001) infection leads to an important production of SFV4(3H)-*RLuc*-p19 [50-fold increase at 48 h p.i. compared to SFV4(3H)-*RLuc*, which is rapidly inhibited] (Fig. 9E). These results also demonstrate the accuracy of *RLuc* as a readout for virus replication and genome RNA levels.

To visualize virus spread directly by immunofluorescence, U4.4 cells were infected at a high MOI (MOI of 10) or low MOI (MOI of 0.001) with SFV4(3H)-*RLuc* or SFV4(3H)-*RLuc*-p19, and cells were fixed at 24 p.i. SFV replication complexes were stained using an anti-nsP3 antibody (28). As shown in Fig. 10, low-MOI infection with SFV4(3H)-*RLuc*-p19 leads to the rapid infection of many cells within the cell monolayer at 24 h p.i. In contrast, very few cells are infected by spreading SFV4(3H)-*RLuc*. As expected, all cells are infected following high-MOI infection by either virus. These data confirm results obtained with *RLuc* as a replication readout.

DISCUSSION

In this paper, we show that SFV cannot avoid or efficiently inhibit the establishment of nonviral or antiviral RNAi responses and that the generation of an antiviral RNAi response effectively suppresses virus replication. Even though RNAi is known to limit arbovirus replication, observations of Sindbis virus-infected *A. aegypti* mosquitoes suggest that the knock-down of genes involved in RNAi did not result in increased mortality rates (14). However, it has recently been shown that infection of *A. aegypti* mosquitoes with Sindbis and O'nyong-nyong alphaviruses encoding flock house virus B2 protein (an RNAi suppressor which binds long dsRNA as well as siRNA) led to a decreased rate of survival of mosquitoes (62). This suggests that RNAi is crucial for maintaining the balance between vector survival and virus replication and transmission.

Previous findings by others and by us showed that SFV replicon replication in arthropod cells is inhibited if RNAi is induced before infection and induces the production of virus-derived siRNAs and that replication is weakly enhanced by the expression of RNAi inhibitors (such as the dsRNA-binding protein NS1) (5, 15, 32, 33). This study extends these findings and, as the virus is sensitive to RNAi, supports the hypothesis that arboviruses do not encode generally active RNAi inhibitors (55), with the possible exception of plant-infecting arboviruses (41). We also show that SFV cannot, or cannot efficiently, interfere with antiviral RNAi induced p.i.; this is different from siRNA resistance reported previously after West Nile flavivirus infection of mammalian cells (36).

While virus strain-specific differences have been observed in mosquitoes infected with the closely related Sindbis virus (14), virus-derived siRNAs seem to be a common feature of alphavirus infection of arthropod cells (32, 73), and our results show that SFV fails to inhibit the cleavage of long dsRNA into biologically active siRNAs (Fig. 3C). This is unlike pathogenic insect viruses, such as flock house virus or *Drosophila C* virus, which encode RNAi suppressors (39, 53). Importantly, our results show that the RNAi signal spreads between mosquito cells and that this can inhibit the replication of incoming SFV. This effect can be inhibited by expressing dsRNA/siRNA-binding proteins, confirming the nature of the spreading RNAi signal. In the mosquito, spread of the RNAi signal is likely to curtail virus dissemination. Some plant viruses specifically inhibit systemic RNAi (67). Short-distance cell-to-cell spread would not require cellular RNA-dependent RNA polymerase, which is absent in mosquito cells (43, 85). Similar cell-to-cell spread seems to be absent in *Drosophila melanogaster*, although short-distance cell-to-cell spread of the RNAi signal might not be easily observed in an entire organism (69). Interestingly, experiments with p19-expressing SFV show that while there is little enhancing effect of the siRNA-binding protein on replication at a high MOI (mimicking initially infected cells, as all cells in the culture are infected), the enhancing effects of p19 (derived from plant-infecting tombusvirus) are strong at a low MOI, where the virus is allowed rounds of infection and spreads through the U4.4 cell monolayer. In plants, the main role of *Cymbidium ringspot tombusvirus* p19 is not to prevent siRNA/RISC complex assembly by siRNA binding in initially infected cells but to facilitate virus spread into surrounding

tissues by inhibiting the cell-to-cell spread of siRNAs (40, 77, 80). Our cell contact experiments as well as infections at high and low MOIs with p19-expressing SFV indicate that cell-to-cell spread of antiviral siRNAs can inhibit the replication of incoming virus and slows down the spread of SFV through the mosquito cell monolayer. This defines at least one of the mechanisms resulting in altered alphavirus dissemination patterns in mosquitoes when RNAi is not functional (14, 45). Whether the cell-to-cell spread of siRNA in mosquito cell cultures involves gap junctions or other mechanisms is currently under investigation.

A recent publication suggested that efficient antiviral immunity in *Drosophila* requires the systemic spread of the RNAi response, based mainly on genetic data (70). Those authors suggested a model by which long dsRNA (not siRNA) is released from infected cells and taken up by other cells in the fly by a known receptor; mutation of this system leads to hypersensitivity to virus infection. The lineages containing *Drosophila* and *Aedes* diverged 250 million years ago, are distant from an evolutionary point of view, and show extensive diversification in their respective immune repertoires (18, 76, 86). Unlike *Drosophila* cells, *Aedes* cells in culture do not (to our knowledge or in our experience) take up free dsRNA from medium. We show that in cultured mosquito cells, siRNA can move between cells that are in contact, the secretion or subsequent uptake of dsRNA is not taking place, and spreading siRNA has direct antiviral activity on incoming SFV. Despite functional differences, this shows that the systemic aspect of antiviral RNAi is important in insects.

Presumably, replication rates before the inhibition of virus replication by RNAi are high enough to ensure virus spread through the mosquito even in the absence of a generally active, efficient RNAi inhibitor. Alternatively, cells within mosquito tissues with weaker (or completely absent) RNAi responses might continuously produce virus or at least be more permissive to arbovirus replication; differential dissemination patterns, as described previously by Keene et al. (45), after silencing of the RNAi machinery suggest that this could be the case. It would be interesting to know if alphaviruses encoding RNAi inhibitors also disseminate differently in mosquitoes (as our results suggest spreading patterns that are different from those of wild-type viruses), whether localized pathologies are responsible for the rapid death of infected mosquitoes by cell death, for example (as results from a recent study suggest) (62), or whether a combination of both exists. Arboviruses such as SFV are relatively efficiently passed on by biting of vertebrate hosts, and high levels of virus amplification take place within one or more vertebrate hosts. In the arthropod vector, virus levels need only be sufficient to ensure transmission. An early phase of high-level virus production and dissemination followed by conversion to a low-level productive persistent infection may achieve a sufficient level of virus without compromising vector viability and transmission to the amplifying vertebrate host. Indeed, a reduction in the level of virus transmission as a result of reduced vector survival as a result of high virus titers could act as a negative selective pressure, preventing the evolution in arboviruses of highly efficient mechanisms to suppress host responses, such as RNAi.

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