RNA interference acts as a natural antiviral response to O'nyong-nyong virus (*Alphavirus*; Togaviridae) infection of *Anopheles gambiae*

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RNA interference (RNAi) is triggered in eukaryotic organisms by double-stranded RNA (dsRNA), and it destroys any mRNA that has sequence identity with the dsRNA trigger. The RNAi pathway in *Anopheles gambiae* can be silenced by transfecting cells with dsRNA derived from exon sequence of the *A. gambiae Argonaute2* (*AgAgo2*) gene. We hypothesized that RNAi may also act as an antagonist to alphavirus replication in *A. gambiae* because RNA viruses form dsRNA during replication. Silencing *AgAgo2* expression would make *A. gambiae* mosquitoes more permissive to virus infection. To determine whether RNAi conditions the vector competence of *A. gambiae* for O'nyong-nyong virus (ONNV), we engineered a genetically modified ONNV that expresses enhanced GFP (eGFP) as a marker. After intrathoracic injection, ONNV-eGFP slowly spread to other *A. gambiae* tissues over a 9-day incubation period. Mosquitoes were then coinjected with virus and either control *β-galactosidase* dsRNA (ds*β*gal; note that "ds" is used as a prefix to indicate the dsRNA derived from a given gene throughout) or ONNV dsnsP3. Treatment with dsnsP3 inhibited virus spread significantly, as determined by eGFP expression patterns. ONNV-eGFP titers from mosquitoes coinjected with dsnsP3 were significantly lower at 3 and 6 days after injection than in mosquitoes coinjected with ds*β*gal. Mosquitoes were then coinjected with ONNV-eGFP and dsAgAgo2. Mosquitoes coinjected with virus and *AgAgo2* dsRNA displayed widespread eGFP expression and virus titers 16-fold higher than ds*β*gal controls after 3 or 6 days after injection. These observations provide direct evidence that RNAi is an antagonist of ONNV replication in *A. gambiae*, and they suggest that the innate immune response conditions vector competence.

innate immunity | mosquito | vector competence

Arboviruses (arthropod-borne viruses) continue to impact human and animal health worldwide. Mosquito-borne arboviruses replicate and disseminate within susceptible vector tissues before transmission to vertebrate hosts. We know little about how mosquitoes cope with arbovirus infections. However, we expect that mosquitoes have defense mechanisms to counteract or modulate arbovirus infections that could impair host functions. RNA interference (RNAi) may be an important pathway that mosquitoes use to modulate arbovirus replication (1).

RNAi is a potent intracellular response activated by double-stranded RNA (dsRNA) and results in a reduced steady-state level of specific RNA molecules with sequence similarity to the dsRNA (2, 3). The mechanism of RNAi has been studied in some detail in *Drosophila melanogaster*. In fruit flies, the RNase III enzyme Dicer is responsible for digesting dsRNA into 21- to 23-bp small interfering RNAs (siRNAs). The siRNAs are then unwound into single-stranded siRNAs in an ATP-dependent step and incorporated into an enzyme complex termed the RNA-induced silencing complex (RISC). The single-stranded siRNAs guide RISC to the target mRNA, and the complex cleaves the message or inhibits its translation (4). An essential component of RISC is Argonaute2 (AGO2), a member of the Argonaute family of proteins. AGO2 has been coimmunoprecipitated with Dicer from Dicer-transfected *Drosophila* S2 cells (5). A proposed interaction between AGO2 and Dicer2 facilitates the incorporation of siRNAs into RISC, which can target cognate mRNAs for destruction (5, 6).

There are several reasons to suspect that RNAi is an antagonist of arbovirus replication in mosquitoes. First, the RNAi-like posttranscriptional gene silencing (PTGS) pathway in plants is a potent antiviral response triggered by dsRNA generated by some plant viruses (7). Second, many plant RNA viruses encode suppressors of PTGS, supporting the observations that PTGS acts as a viral defense system (8). For example, the tombusvirus p19 protein suppresses PTGS in plants by binding siRNAs produced after virus infection (9). Third, Li *et al*. (10) demonstrated that the *B2* gene of the insect nodavirus, flock house virus (FHV), can suppress PTGS activity in plants and RNAi in *Drosophila* S2 cells, emphasizing that an evolutionarily conserved RNAi pathway plays a natural antiviral role. Also, this research group demonstrated that vaccinia and human influenza A, B, and C viruses each encode a protein that suppresses RNAi in mammalian and insect cells (11).

Studies have shown that RNAi is active in anopheline and culicine mosquitoes (1, 12–19). Recently, Hoa *et al*. (20) demonstrated that expression of Dicer2, AGO2, and AGO3 proteins are essential for RNAi activity in *Anopheles gambiae* Sua1B cells. Transient expression of luciferase in the Sua1B cell line was silenced ≈4,000-fold after transfection with dsRNA derived from the luciferase reporter gene. Pretreatment of the cells with dsRNA derived from cDNA sequence of *A. gambiae dcr2* (*AgDcr2*), *A. gambiae Ago2* (*AgAgo2*), or *AgAgo3* consistently yielded recovery of luciferase activity, demonstrating that RNAi can be used to silence genes involved in its own pathway and implying that these genes have important roles in RNAi within mosquito cells. The recovery phenomenon was not observed when the cells were treated with *β-galactosidase* (*β-gal*) dsRNA (ds*β*gal; note that "ds" is used as a prefix to indicate the dsRNA derived from a given gene throughout), or dsAgAgo1, dsAgAgo4, and dsAgAgo5 (20).

A logical progression from these studies would be to determine whether RNAi can act as an antagonist to arbovirus replication in *A. gambiae*. However, anopheline mosquitoes transmit few arboviruses. An exception is O'nyong-nyong virus (ONNV) (Togaviridae; *Alphavirus*), which is the etiological agent of a large outbreak of human disease in East Africa from 1959–1962 (21–23) and again in 1996 (24). In epidemics, *Anopheles* spp. are almost certainly the vectors. Wild-caught *Anopheles*

Abbreviations: RNAi, RNA interference; dsRNA, double-stranded RNA; eGFP, enhanced GFP; dpi, days postinjection; *β-gal*, *β-galactosidase*; ONNV, O'nyong-nyong virus; RISC, RNA-induced silencing complex; AgAgo, *Anopheles gambiae* Ago; AGO2/3, Argonaute2/3; pfu, plaque-forming units; siRNA, small interfering RNA.

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Table 1. Primer pairs for amplification of dsRNA template from *A. gambiae*

Target gene	Forward primer, 5′–3′	Reverse primer, 5′–3′
Ago1	GCAGGTGTCCCTGTTCAACCT	GGTTTGGCCGTTCTCTAGCTG
Ago2	GCATGAGCACGCTCAACAAC	GTTCGAGTCGTCGTACAGCA
Ago3	GTGTGGCATTGACACGTACC	GCTCAGCTGCTGCAGAATGTC
Ago4	GCGACTTCCTCAACTGCATGA	GTGTTGAGCGGCAGATAGTTG
Ago5	GACAAGTCGCTCTCGTACGGT	GTCTCGTCGAAGATCACGTTG
ONNV nsp3	CATGTGGCCAAAACAAACTG	CGAATTTGCGTACATTGGTG
β-gal	GGTCGCCAGCGGCACCGCGCCTTC	GCCGGTAGCCAGCGCGGATCATCGG

funestus and *A. gambiae* mosquitoes held alive for up to 20 days after capture have been found to be infected with ONNV (21).

ONNV is a small, enveloped RNA virus that replicates exclusively in the cytoplasm of infected cells (25). The genome is a positive-sense, single-stranded, nonsegmented RNA of ≈11.7 kb (25, 26). The 5′ two-thirds of the alphavirus genome is translated to form polyproteins that are posttranslationally processed into nsP1–nsP4 proteins to form replicase complexes that synthesize positive or negative RNAs (25, 27). Replication of alphavirus RNA occurs at intracellular membranes in infected cells and leads to formation of dsRNA forms called replicative intermediates (28). The subgenomic (26S) mRNA, colinear with the 3′ one-third of the genome, is translated into a structural polyprotein from which capsid, the envelope glycoproteins (E1 and E2), and two smaller polypeptides (E3 and 6K) are produced as cleavage products during glycoprotein processing. Alphavirus RNA genomes are readily manipulated as full-length cDNA infectious clones, and recombinant double subgenomic ONNV has been generated that expresses enhanced GFP (eGFP) as a marker of infection (29).

In this article, we describe the replication of a recombinant ONNV-eGFP after intrathoracic injection into *A. gambiae*. When this virus was coinjected with dsRNA derived from the viral genome, virus replication in the mosquito was compromised significantly. In contrast, mosquitoes coinjected with ONN-eGFP and dsRNA derived from *AgAgo2* were more permissive to virus replication and dissemination. These experiments demonstrate that RNAi acts as an antiviral response to ONNV-eGFP infections in *A. gambiae*.

Materials and Methods

Cell Lines and Virus Production and Generation of ONNV-eGFP. The infectious cDNA clone, pONN.30a, derived from the Uganda SG-650 strain of ONNV was provided by Ann Powers (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO). The infectious clone was further modified to a recombinant double subgenomic infectious clone expressing eGFP (p5′dsONNic-Foy/eGFP, but for simplicity, it is referred to here as ONNV-eGFP), constructed essentially as described (30) and linearized to form a template for *in vitro* transcription by using T7 polymerase. RNA from the transcription reaction was electroporated into BHK-21 cells. At 36 h later, the supernatant containing virus was collected, and the virus titer was determined by plaque assay. C6/36 cells (*Aedes albopictus*) were then infected at an multiplicity of infection of 0.01, and 60 h later, supernatant containing the virus was collected and titrated. The virus stock contained 2.1×10^7 plaque-forming units (pfu)/ml.

Mosquitoes and Intrathoracic Inoculation of Virus and/or dsRNAs. *A. gambiae* (G3 strain) were reared on an artificial diet of ground fish food at 30°C with a 14 h light/10 h dark photoperiod. Adult female *A. gambiae* (2–4 days after emergence) were injected with 0.5 μl of inoculum. For viral characterization studies, viral stock was diluted to 1×10^7 pfu/ml before injection, and thus, each mosquito received $\approx 5 \times 10^3$ pfu of virus. For coinjections of virus and dsRNA, dsRNA was diluted to a concentration of 1 μg/μl in PBS and mixed 1:1 (vol/vol) with the undiluted stock (2.1×10^7 pfu/ml). Thus, each mosquito was inoculated with $\approx 5.3 \times 10^3$ pfu of virus and 250 ng of dsRNA.

Characterization of ONNV Infection in *A. gambiae* Mosquitoes. After inoculation, *A. gambiae* adult female mosquitoes were assayed at 3, 6, and 9 days postinjection (dpi). Mosquitoes were killed by brief submersion in 70% ethanol, washed in saline, and assayed initially for eGFP expression under UV light. The heads were then removed, squashed on glass slides, fixed in acetone, and assayed for viral antigen by immunofluorescence assay using an mAb (30.11a) that was developed against Sindbis virus E2 protein but cross-reacts with ONNV E2 protein. The thorax and abdomen of each mosquito were frozen individually at −70°C and later titrated by plaque assay (described below).

Generation of dsRNA. cDNAs were generated from total mosquito RNA [extracted from fourth-instar larvae by using the RNeasy kit (Qiagen, Valencia, CA)] by RT-PCR using an oligo(dT) primer. Oligonucleotide primers were designed to amplify ≈500-bp regions of *A. gambiae Ago1–5* cDNA, and they incorporated T7 promoter sequences at the 5′ ends (Table 1). Control ≈500-bp cDNA templates were generated by PCR using primers specific for portions of the *Escherichia coli β-gal* cDNA clone and for the *nsP3* gene from ONNV-eGFP. To generate dsRNA, PCR products were purified by gel extraction and used as templates for *in vitro* transcription using the MegaScript kit (Ambion, Austin, TX), and dsRNAs were purified according to the manufacturer's instructions. The quality of dsRNA was checked by agarose gel electrophoresis and quantified by using a spectrophotometer.

Determination of Virus Titer. Mosquitoes were triturated in 270 μl of DMEM by using a mortar and pestle, and large particulates were pelleted by centrifugation. The supernatant was passed through a 0.22-μm filter and then titrated by standard plaque assay by using Vero cells (31). We counted pfu, and the data were $\log_{10}$ transformed. Differences in viral titers were first analyzed by ANOVA, and the titers in the treatment groups were found to be significantly different ($P < 0.01$). Pairwise *t* tests were then performed. Highly significant differences ($P < 0.0001$) between treatments groups and controls are marked with two asterisks, and significant differences ($P < 0.05$) are marked with a single asterisk.

Northern Blot Analysis. Total RNA was isolated at 2–3 dpi from 50–60 mosquitoes for each treatment group by using the guanidine isothiocyanate/phenol–chloroform method of extraction. mRNA was purified from total RNA by using the MicroPoly(A) Purist kit (Ambion). We separated 10 μg of mRNA on a 1% agarose-formaldehyde gel and transferred to a BrightStar Plus nylon membrane (Ambion). The blots were hybridized with ^{32}P-labeled probe complementary to the ONNV *E2* gene (Fig.

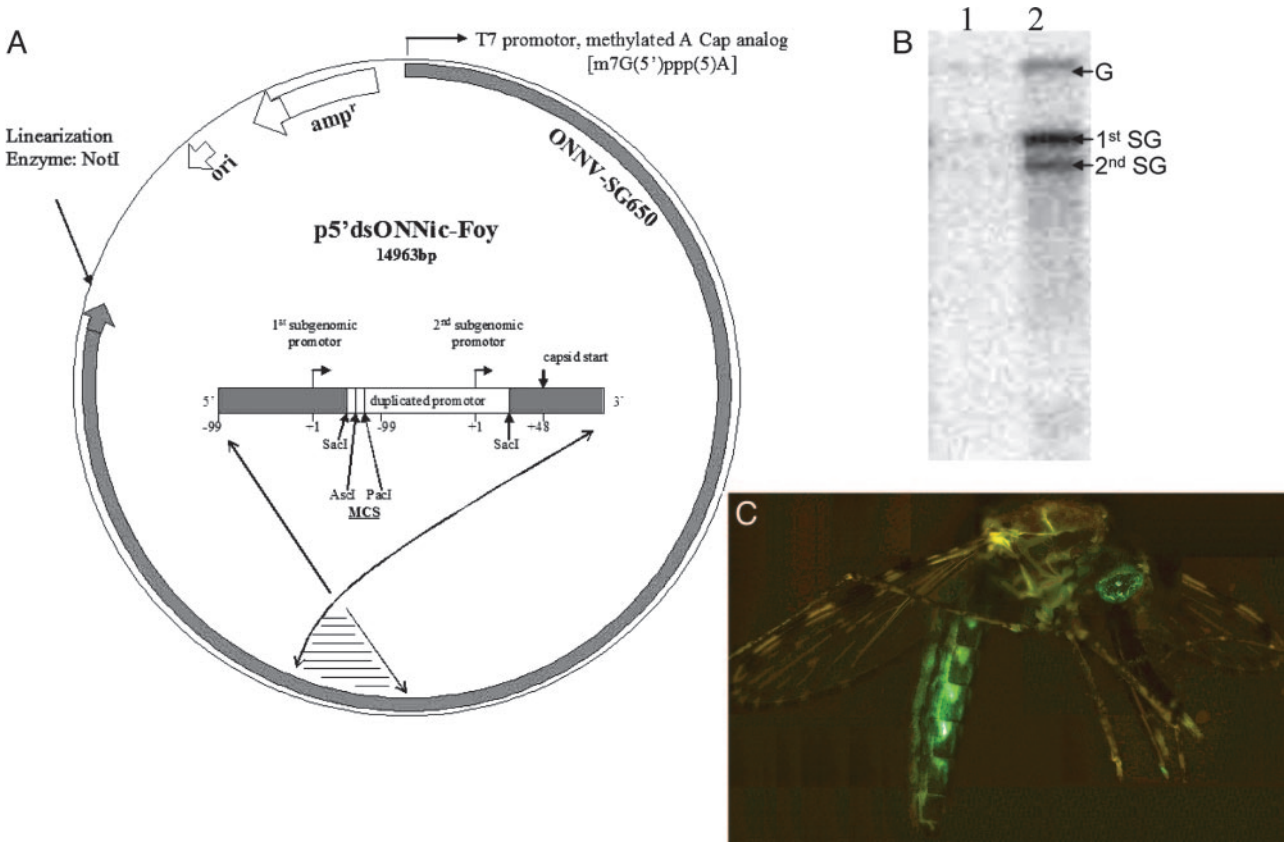

Fig. 1. Design and characterization of recombinant ONNV-eGFP. (*A*) p5′dsONNVic/Foy contains a full-length cDNA of the ONNV genome with a second subgenomic promoter inserted 3′ of the original subgenomic promoter. eGFP was cloned into the MCS and is transcribed under control of the first subgenomic promoter. (*B*) Characterization of viral transcripts of ONNV after injection into adult female *A. gambiae*. Lanes 1 and 2 show the ONNV-eGFP transcript profile at 48 and 72 h after infection, respectively. The blot was hybridized by using a radiolabeled ONNV E2 gene as probe, and it shows the production of the full-length genomic and two subgenomic transcripts. (*C*) eGFP expression at 9 dpi occurs throughout the body.

1*B*) or *AgAgo2* (Fig. 4). The blots were then washed at 68°C, and radioactivity was detected by using a Storm PhosphoImager (Molecular Dynamics, Amersham Biosciences).

Results

Construction and *in Vivo* Characterization of Intrathoracically Injected ONNV-eGFP. The pONNV.30a infectious clone was engineered to contain a duplicated subgenomic promoter, 3′ to the original subgenomic promoter (Fig. 1*A*). eGFP was inserted immediately downstream of the first subgenomic promoter into a multiple cloning site, generating ONNV-eGFP plasmid. Recombinant virus was produced from this plasmid and injected into *A. gambiae* mosquitoes. The three RNA species predicted from virus transcription were not detectable by Northern blotting of mosquito RNA after 48 h, but they were apparent after 72 h (Fig. 1*B*), indicating a relatively slow rate of viral replication in *A. gambiae* tissue. Viral titers in the mosquito at 3 dpi were lower than the input titer by >1.5 log pfu (Table 2), but they then slowly increased over a 9-day incubation period. Virus was detected in the head tissues of individual mosquitoes both by eGFP expression and by immunofluorescence analysis of the E1 glycoprotein (Table 2). The data indicate that these methods of detection are equivalent and that eGFP serves as a readily detectable marker of infection during this time period. eGFP expression in injected mosquitoes at 9 dpi revealed temporal and spatial infection patterns of the virus (Fig. 1*C*). Virus typically infected nervous, muscle, and fat body tissues. When ONNV-eGFP dissemination to the head occurred, ommatidia and cells in the maxillary palps, antennae, and in the mouth parts expressed eGFP, but the marker was not apparent in salivary glands (data not shown). In the abdomen, eGFP was most often sporadically associated with circular muscle fibers wrapping the alimentary canal and with fat body.

Inhibition of Virus Replication After Coinjection with ONNV and dsRNA Targeting ONNV nsP3 Gene Sequence. Initially, we tested whether the RNAi pathway in *A. gambiae* could inhibit virus replication and dissemination by introduction of *nsP3* gene dsRNA (dsnsP3) to target the virus genome. Mosquitoes were coinjected with 5.3×10^3 pfu of virus and 250 ng of ONNV dsnsP3. Mosquitoes were also coinjected with the same dose of ONNV-eGFP virus and dsβgal. Primers for generating dsRNAs are given in Table 1. In mosquitoes receiving dsnsP3, eGFP was usually restricted to thoracic tissue surrounding the site of injection (Fig. 2*A*). Mosquitoes coinjected with virus and dsβgal control usually had

Table 2. Dissemination of 5′ dsONNic-Foy/eGFP after injection in *A. gambiae*

	Head		Thorax and abdomen
dpi	GFP, %	IFA, %	pfu (log_{10})
3	24 (12/50)	24 (12/50)	2.1
6	33 (16/49)	30 (15/50)	1.7
9	52 (26/50)	68 (34/50)	2.3

IFA, immunofluorescence assay.

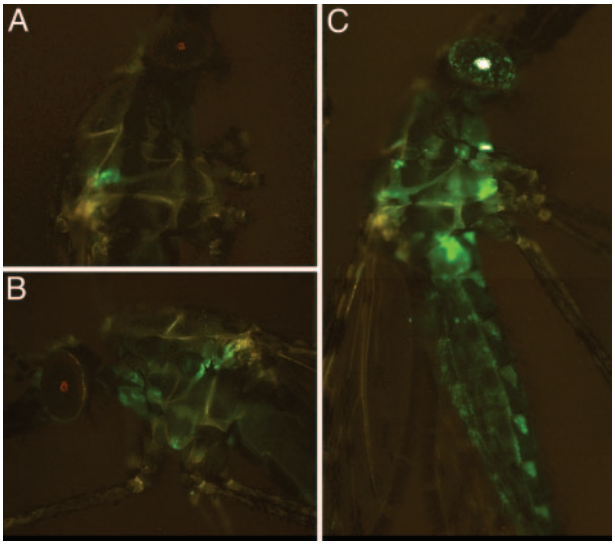

Fig. 2. eGFP expression at 3 dpi in mosquitoes coinjected with dsRNAs and ONNV-eGFP. (*A*) Coinjection of ONNV-eGFP and dsnsP3. (*B*) Coinjection of ONNV-eGFP and dsβgal. (*C*) Coinjection of ONNV-eGFP and dsAgAgo2. Mosquitoes injected with dsnsP3 show a dramatic reduction in eGFP expression when compared with dsβgal-injected controls, whereas mosquitoes injected with dsAgAgo2 show an increase in eGFP expression over controls.

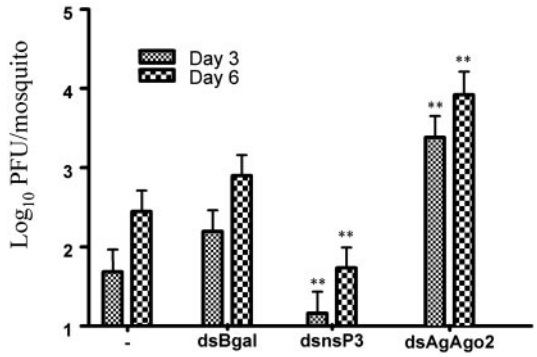

Fig. 3. Viral titers of ONNV-eGFP in mosquitoes coinjected with dsRNA homologous to ONNV *nsP3* and *AgAgo2*. Compared with the nonspecific dsβgal, mosquitoes coinjected with virus and dsnsP3 had statistically significant decreases in infection at both 3 and 6 dpi ($P < 0.0001$). Viral titers of ONNV-eGFP increased significantly in mosquitoes at 3 and 6 dpi after coinjection with dsRNAs homologous with *AgAgo2* ($P < 0.0001$ and $P = 0.0006$ at 3 and 6 dpi, respectively).

more extensive expression of eGFP in the thorax (Fig. 2*B*). At 3 dpi, 86% ($n = 58$) of mosquitoes receiving dsβgal expressed eGFP in thoracic tissues, but only 38% ($n = 52$) expressed eGFP in thoracic tissues when dsnsP3 was coinjected (Table 3). At 3 dpi, 23% ($n = 52$) of mosquitoes receiving dsnsP3 showed eGFP expression in head tissues and none of the same mosquitoes had eGFP in abdominal tissue (Table 3). In contrast, 34% ($n = 58$) of mosquitoes receiving dsβgal had eGFP expression in their head and abdominal tissues (Table 3). At 6 dpi, 57% ($n = 53$) of mosquitoes injected with dsβgal had eGFP in their head and abdominal tissues; however, eGFP could only be seen in 36% of the head tissue and 16% of the abdominal tissue ($n = 52$) of mosquitoes coinjected with virus and dsnsP3 (Table 3).

To quantitate virus abundance, mosquitoes were triturated and virus titer was determined by plaque assay from these treatment groups. No significant difference in the number of plaques was observed between the mosquitoes injected only with ONNV-eGFP and mosquitoes coinjected with dsβgal ($P > 0.08$). However, mosquitoes coinjected with ONNV-eGFP and dsnsP3 had significantly fewer plaques than those mosquitoes coinjected with ONNV-eGFP and dsβgal ($P < 0.0001$) at both 3 and 6 dpi (Fig. 3).

Coinjection of ONNV and dsRNAs Homologous to *AgAgo2*. We then coinjected mosquitoes with dsAgAgo2 to observe whether silencing of *AgAgo2* expression would make mosquitoes more permissive to ONNV. At 3 dpi, these mosquitoes usually displayed dramatic increases in eGFP expression with eGFP detected in tissues in all body segments (Fig. 2*C*). At 3 dpi, only 34% ($n = 58$) of mosquitoes treated with ONNV-eGFP and dsβgal showed eGFP in their heads and abdomens, but $\geq$97% ($n = 63$) of mosquitoes treated with dsAgAgo2 showed eGFP in the same tissues (Table 3). At 6 dpi, 57% ($n = 53$) of mosquitoes injected with virus and dsβgal displayed eGFP in head and abdominal tissues; 100% ($n = 36$) injected with virus and dsAgAgo2 displayed eGFP in those tissues (Fig. 3).

We examined mRNA accumulation of *AgAgo2* in mosquitoes after injection with dsAgAgo2 to determine whether the dsRNA specifically silenced *AgAgo2* mRNA. Injection of nonspecific dsβgal failed to silence *AgAgo2* mRNA, but injection of dsAgAgo2 silenced accumulation of *Ago2* mRNA in mosquitoes (Fig. 5, lane 3). Interestingly, we detected partial recovery of *AgAgo2* mRNA when ONNV was coinjected with dsAgAgo2 (Fig. 5, lane 4). *AgAgo2* mRNA levels in mosquitoes injected with ONNV alone were similar to noninjected and dsβgal-injected controls.

Finally, virus titers were recovered on the same mosquitoes used for determining the eGFP expression profiles. Mosquitoes treated with dsAgAgo2 had significantly more infectious virus per mosquito at 3 dpi than mosquitoes treated with dsβgal (16-fold increase; $P < 0.0001$, Fig. 4). At 6 dpi, viral titers increased in all tested mosquitoes, but dsAgAgo2-treated mosquitoes still had significantly more virus per mosquito than dsβgal-treated controls ($P = 0.0006$, Fig. 4).

Coinjection of ONNV and dsRNAs Homologous to *AgAgo1*, *AgAgo3*, *AgAgo4*, and *AgAgo5*. Mosquitoes were injected with virus and dsRNAs derived from *AgAgo1*, *AgAgo3*, *AgAgo4*, and *AgAgo5* (20). These studies were performed to observe whether silencing of other *AgAgo* genes also could increase mosquito permissive-

Table 3. Percentage of injected mosquitoes displaying eGFP expression in body tissues after coinjection of ONNV-eGFP and dsnsP3 or dsAgAgo2 at 3 and 6 dpi

	3 dpi			6 dpi		
Treatment	Head	Thorax	Abdomen	Head	Thorax	Abdomen
ONNV-eGFP + no dsRNA	10% (4/40)	85% (34/40)	0% (0/40)	36% (14/44)	93% (41/44)	20% (9/44)
ONNV-eGFP + dsβgal	34% (20/58)	86% (50/58)	34% (20/58)	57% (30/53)	89% (47/53)	57% (30/53)
ONNV-eGFP + dsnsP3	23% (12/52)	38% (20/52)	0% (0/52)	36% (20/55)	71% (39/55)	16% (9/55)
ONNV-eGFP + dsAgAgo2	98% (62/63)	100% (63/63)	97% (61/63)	100% (36/36)	100% (36/36)	100% (36/36)

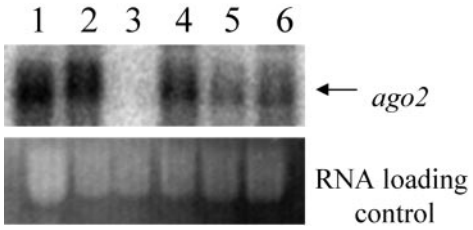

Fig. 4. Northern blot analysis of *AgAgo2* mRNA after injection of mosquitoes with ONNV-eGFP, dsRNA, or dsRNA and ONNV-eGFP. Injection of dsAgAgo2 results in the reduction of *Ago2* transcript levels. Mosquitoes injected with dsAgAgo2 and virus showed partial recovery of the *Ago2* mRNA accumulation. (*Upper*) Lane 1, mock injected; lane 2, dsβgal at 3 dpi; lane 3, dsAgAgo2 at 3 dpi; lane 4, dsAgAgo2 + ONNV-eGFP 3 at dpi; lane 5, ONNV-eGFP 2 dpi; and lane 6, ONNV-eGFP 3 dpi. (*Lower*) Ethidium bromide stain of Northern blot showing ribosomal RNA in each lane and verifying that equivalent amounts of total RNA were added to each lane.

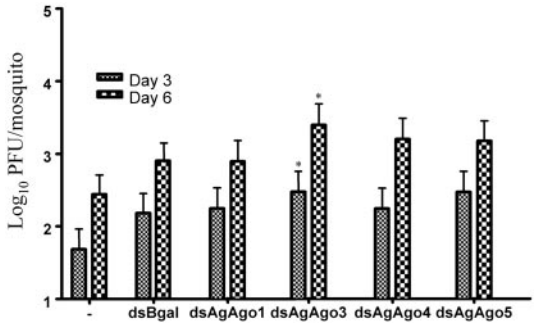

Fig. 5. Viral titers of ONNV-eGFP in mosquitoes at 3 and 6 dpi after coinjection of dsRNA homologous to *AgAgo1*, *AgAgo3*, *AgAgo4*, and *AgAgo5*. Compared with the nonspecific dsβgal, only mosquitoes coinjected with virus and dsAgAgo3 had statistically significant increases at 3 and 6 dpi ($P \leq 0.0141$).

ness to ONNV replication, possibly implicating them in RNAi modulation of ONNV-eGFP replication. At 3 and 6 dpi, mosquitoes coinjected with virus and *AgAgo*-derived dsRNAs (dsAgAgo1, dsAgAgo3, dsAgAgo4, and dsAgAgo5) usually displayed similar eGFP expression patterns to that seen with the dsβgal control mosquitoes (Table 4). The only exception was that mosquitoes injected with dsAgAgo3 consistently had greater dissemination of virus in all tissues than those injected with either dsβgal or dsAgAgo1, dsAgAgo4, and dsAgAgo5 (Table 4). These observations were confirmed by virus titration. Virus titers in mosquitoes injected with dsAgAgo3 differed significantly from those mosquitoes injected with dsβgal at 3 dpi ($P = 0.0067$) and at 6 dpi ($P = 0.0141$) (Fig. 5). Virus titers in mosquitoes injected with dsAgAgo1, dsAgAgo4, and dsAgAgo5 did not differ statistically from those mosquitoes injected with dsβgal at 3 and 6 dpi.

Discussion

In this study, we demonstrated that RNAi can act as an antagonist to arbovirus replication in mosquitoes. We describe an alphavirus transducing system based on ONNV and follow the course of infection after injection of recombinant virus into the *A. gambiae* hemocoel. After injection into the hemocoel, ONNV-eGFP replicated slowly in *A. gambiae.* Injection of dsRNA cognate to a portion of the ONNV genome and stimulation of RNAi further slowed ONNV replication in this mosquito species. Injection of dsRNA cognate to *AgAgo2*, which is a gene known to function in the *A. gambiae* RNAi pathway (20), silenced RNAi, thereby permitting ONNV-eGFP to replicate and disseminate quickly in mosquitoes. In addition, dsRNA derived from *AgAgo3* also made mosquitoes more permissive to ONNV-eGFP replication at 3 and 6 dpi. In contrast, dsRNAs derived from *AgAgo1*, *AgAgo4*, and *AgAgo5* did not alter virus replication significantly at either 3 or 6 dpi. These data suggest a regulatory role for RNAi in controlling arbovirus infections in mosquitoes.

The pathogenesis of ONNV in *A. gambiae* is unusual when compared with typical alphavirus–vector models. When injected into culicine mosquitoes, ONNV, like other alphaviruses (Sindbis virus and Venezuelan equine encephalitis virus), replicates efficiently and rapidly spreads throughout the mosquito (32–34; B.D.F., unpublished data). However, ONNV replication in *A. gambiae* is relatively slow after intrathoracic injection. ONNV also infects *A. gambiae* midgut tissues after *per os* infection, but it has an unusual tropism for the anterior midgut epithelium and is limited in its ability to escape from the alimentary canal (29). *A. gambiae* may not be the ideal vector for ONNV. *A. funestus* has been implicated as a potentially better vector of ONNV in Africa (21, 23). It would be interesting to observe whether *A. funestus* can modulate ONNV-eGFP infection as readily as *A. gambiae*.

Our results point to the AGO2, and possibly AGO3, proteins as critical components of a mosquito RNAi pathway involved in the inhibition of alphavirus replication. ONNV replication was affected most by silencing of *Ago2*, which has been shown to be an important RISC component in *Drosophila* (5). Northern blot analysis showed that the presence of dsAgAgo2 leads to reduced amounts of *AgAgo2* mRNA *in vivo*, but the analysis also suggests that infection with ONNV stimulates recovery of this transcript accumulation. A possible explanation is that ONNV infection induced transcription of *AgAgo2*, although there is no supporting evidence for this hypothesis. The other, more likely, explanation is that ONNV encodes a repressor of RNAi in the mosquito, which has been shown with other RNA viruses (8). The ONNV suppressor might counteract the silencing from dsAgAgo2 injection. Transcription of ONNV-eGFP was first detected at 72 h after infection (Fig. 1*B*), which is when an ONNV suppressor protein may be translated in sufficient quantities to have an

Table 4. Percentage of injected mosquitoes displaying eGFP expression in body tissues after coinjection of ONNV-eGFP and dsRNAs from *AgAgo1, AgAgo3, AgAgo4,* and *AgAgo5* at 3 and 6 dpi

	3 dpi			6 dpi		
Treatment	Head	Thorax	Abdomen	Head	Thorax	Abdomen
ONNV-eGFP + no dsRNA	10% (4/40)	85% (34/40)	0% (0/40)	36% (14/44)	93% (41/44)	20% (9/44)
ONNV-eGFP + dsβgal	34% (20/58)	86% (50/58)	34% (20/58)	57% (30/53)	89% (47/53)	57% (30/53)
ONNV-eGFP + dsAgAgo1	16% (9/55)	98% (54/55)	32% (18/55)	81% (26/32)	97% (31/32)	94% (30/32)
ONNV-eGFP + dsAgAgo3	55% (22/40)	98% (39/40)	78% (31/40)	75% (29/39)	100% (39/39)	80% (31/39)
ONNV-eGFP + dsAgAgo4	38% (20/52)	94% (49/52)	56% (29/52)	73% (25/34)	100% (34/34)	82% (28/34)
ONNV-eGFP + dsAgAgo5	46% (24/52)	98% (51/52)	48% (25/52)	62% (22/36)	94% (34/36)	42% (15/36)

effect on RNAi. Even if one or both of these possibilities is correct, it is clear that the injection of *dsAgAgo2* has a strong biological effect that resulted in increased ONNV titers in the mosquito that lasted at least 6 days after treatment.

The Argonaute family comprises a group of proteins, some of which are required for RNAi and others of which have roles in regulating development. We have demonstrated that *AgAgo2* and *AgAgo3* are involved in RNAi both in cell culture (20) and in mosquitoes. Whereas the *Drosophila* ortholog of *Ago2* has been characterized, *Ago3* has not (5, 35). *DmAgo1* is required for efficient RNAi in *Drosophila*, functioning in the pathway after the production of siRNA (36). We hypothesized previously that the function of *AgAgo3* may be analogous to that of *DmAgo1* (20). The *Drosophila* paralogues of *AgAgo4* and *AgAgo5*, *piwi* and *aubergine*, have known functions in development (35).

Several innate immune pathways in mosquitoes have been elucidated for defense against bacterial and macroparasite infections (18, 37–40); however, no antiviral mechanisms or pathways have been described. In vertebrate species, there are innate immune mechanisms that recognize and mount responses to dsRNA, including the interferon and protein kinase R pathways, but neither has been detected in the mosquito. The data presented in this article support the idea that RNAi is a mechanism to protect mosquitoes from viral infection. We hypothesize that vector competence for an alphavirus is partly due to the balance struck between the opposing forces of vector and arbovirus evolution. Some alphaviruses may replicate and disseminate so quickly as to avoid induction of an RNAi defense, or they may quickly disseminate from tissues with a strong RNAi response and into mosquito tissues with a weak RNAi response (30, 41, 42). For example, the *C. elegans* nervous system has been shown to be refractory to silencing of mRNA by RNAi (43). Also, many plant viruses encode suppressors of RNAi that may also be present in alphaviruses (8). Different vector species are also likely to show differences in their RNAi responses. Some mosquitoes may preferentially express negative regulators of RNAi, such as the ERI-1 protein that has been identified in *C. elegans* (44). Data demonstrating dsRNA effectiveness against myriad targets in *A. gambiae* (12, 18, 19) may indicate that these mosquitoes generally have a robust RNAi response, which could partly explain why anopholine mosquitoes are such poor vectors of arboviruses. Mosquitoes such as *Aedes aegypti* readily transmit both alphaviruses (Chikungunya and Sindbis), and flaviviruses (yellow fever and dengue), possibly indicating that these mosquitoes have a weaker RNAi response. In support of this hypothesis, we have observed that ONNV readily disseminates in *A. aegypti* tissues after injection but that the Sindbis virus MRE16 strain (30), which disseminates very efficiently in all of the culicines that we have injected, could not replicate in *A. gambiae* tissues (data not shown).

This study is the first step in understanding how RNAi naturally modulates arboviral infection in the mosquito. An understanding of whether the RNAi response can modulate arboviral infection and dissemination in other vector mosquitoes and by other families of arboviruses would provide a better understanding of how mosquitoes respond to virus infection, and it would possibly provide researchers with information on how to design strategies that enhance mosquito refractoriness to arboviruses.

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