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Infection, Dissemination, and Transmission of a West Nile Virus Green Fluorescent Protein Infectious Clone by *Culex pipiens quinquefasciatus* Mosquitoes

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Abstract

We report the construction and comparative characterization of a full-length West Nile virus (WNV) cDNA infectious clone (ic) that contains a green fluorescent protein (GFP) expression cassette fused within the viral open reading frame. Virus derived from WNV-GFP ic stably infected *Culex pipiens quinquefasciatus* mosquitoes at comparable rates to virus derived from the parental (non-GFP) ic. However, insertion of this GFP cassette resulted in a temporal delay in *in vivo* replication kinetics and significantly decreased dissemination to head tissue. Consistent with previous reports of WNV-infected mosquito midguts, focal GFP expression was observed at 3 days post-infection (dpi), with the majority of posterior midgut epithelial cells being positive by 7 dpi. GFP foci were observed in one pair of salivary glands (1/15) dissected 14 dpi. Mice exposed to WNV-GFP–infected mosquitoes developed viremia, and GFP was detected in lymph node homogenates. These data demonstrate the effectiveness of our strategy to generate a replication competent construct with increased reporter gene stability that may be used to study early events in infection.

Key Words: Culex—Flaviviridae—Mosquito(es)—Transmission—West Nile.

Introduction

A number of flavivirus reverse genetic systems have been developed and utilized for the study of basic viral genetics, *in vivo* interactions, and the advancement of vaccine technology (Rice et al. 1989, Lai et al. 1991, Sumiyoshi et al. 1995). Genes of interest (GOI) have been cloned into these recombinant genomes in proximity to the genomic 3′-end under the control of exogenous translation control elements, such as internal ribosomal entry sequences (Scholle et al. 2004, Pierson et al. 2005, Rossi et al. 2005). Further random mutagenesis studies have identified regions of the flavivirus genome that tolerate small GOI insertions. As a result, small epitopes derived from T cells, Rift Valley fever virus, Lassa fever virus, and Influenza A virus have been expressed via direct insertion into the flaviviral open reading frame (ORF) (Rice 1990, Tao et al. 2005, Bredenbeek et al. 2006, Pugachev et al. 2008). However, previous attempts to generate a full-length West Nile virus (WNV) construct with a fluorescent reporter gene using similar manipulations have been confounded by genetic instability and the rapid emergence of mutants with deleted GOI sequences (Pierson et al. 2005). In a recent study, Shustov et al. (2007) reported the development of a pseudoinfectious bipartite genome yellow fever virus 17D reverse genetic system that stably expressed GOI via insertion of a 5′ expression cassette into the genome-encoded polyprotein.

WNV is a mosquito-borne flavivirus that can infect the central nervous system of vertebrate hosts with potential for subsequent development of encephalitis, meningitis, meningoencephalitis, and poliomyelitis-like disease (www.cdc.gov/ncidod/dvbid/westnile). WNV is principally maintained in nature through transmission by ornithophilic *Culex* mosquitoes with amplification in susceptible avian hosts (Apperson et al. 2004). However, WNV has been recovered from numerous mosquito and tick species (Whitman and Aitken 1960, Karabatsos 1985, Abbassy et al. 1994, Anderson et al. 2003), and a number of nonconventional modes of infection have been identified (Granwehr et al. 2004, Higgs et al. 2005, McGee et al. 2007, Reisen et al. 2007). Like all of the members of the genus *flavivirus*, WNV possesses a positive-sense, single-stranded RNA genome containing a single ORF, which encodes three structural gene proteins (capsid,

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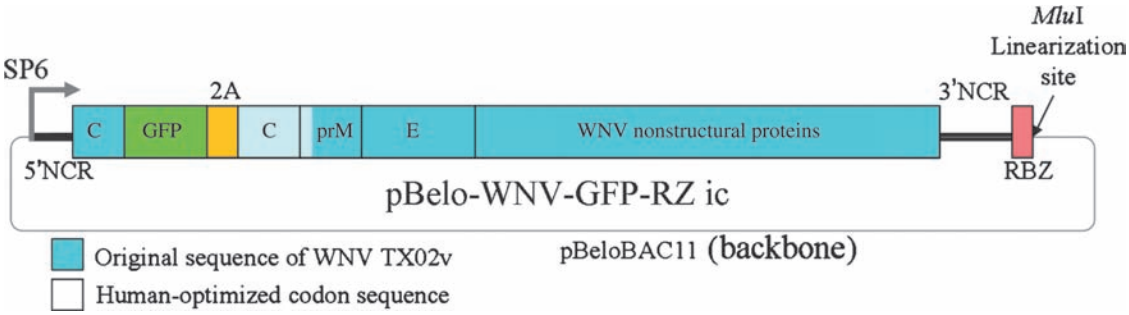

FIG. 1. pBelo-WNV-GFP-RZ ic plasmid map. WNV, West Nile virus; GFP, green fluorescent protein; ic, infectious clone; RB2, rybozyme.

pre-membrane (prM/M), and envelope (E) and seven nonstructural gene proteins in a 5′–3′ orientation, flanked by terminal 5′ and 3′ untranslated regions (Brinton 2002).

The aims of this study were to generate a full-length WNV infectious clone (ic) stably expressing the green fluorescent protein (GFP) reporter gene; to determine the effects of insertion of this heterologous gene on the replication of recombinant virus *in vivo*; and to utilize the GFP-expressing WNV in the studies of the natural viral life cycle. In our study, the recent results of extensive characterization of WNV intravector replication and tropism (Girard et al. 2004, 2005, 2007) were expanded through the utilization of a full-length WNV ic (Rossi et al. 2005) and a highly susceptible strain of *Culex pipiens quinquefasciatus* mosquitoes.

Materials and Methods

Cell lines, plasmid constructs, and viruses

Cells. *Aedes albopictus* (C6/36) cells were grown in Liebovitz L-15 media supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin (pen-strep), and 1% L-glutamine (L-glu) (Cellgro®; Mediatech, Herndon, VA) and maintained at 28°C. Green monkey kidney (Vero) cells were grown in either Lebovitz L-15 medium supplemented as above and maintained at 37°C or in minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 2% bovine growth serum (Hyclone, Logan, UT), 1% pen-strep, 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO), and 0.3% L-glu and maintained at 37°C in the presence of 5% CO_2. BHK-21 (baby hamster kidney) cells were grown in Alpha minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 1% pen-strep, 1% L-glu, and vitamins and maintained at 37°C in the presence of 5% CO_2.

Plasmid constructs. The *E. coli* plasmid (bacterial artificial chromosome) that contains a full-length cDNA clone for recombinant WNV-GFP virus was created and designated pBelo-WNV-GFP-RZ ic (Fig. 1). The genetic backbone of WNV in this construct originates from a human isolate of WNV obtained in 2002 (isolate WNV-TX02, published in Rossi et al. 2005). In the pBelo-WNV-GFP-RZ ic sequence the viral ORF is modified by insertion of additional genes, namely, the gene encoding GFP, a 17 codon-long sequence for foot-and-mouth disease virus protease 2A, and an artificially created second copy of WNV capsid gene (Copt). All the mentioned additional sequences are placed in the above order between the natural WNV capsid gene (Cnat) and prM gene.

In the pBelo-WNV-GFP-RZ ic construct the recombinant viral cDNA is positioned under control of T7 promoter and equipped with herpes delta virus ribozyme downstream of the genomic 3′-terminus to ensure proper processing of terminal nucleotides. Detailed genetic map and sequence of the construct are available from the authors upon request.

The above-described genetic design is similar to that previously described for recombinant GFP-expressing yellow fever virus (Shustov et al. 2007). Cloning was done utilizing PCR-amplified fragments and unique sites of the WNV genome using standard molecular cloning methodologies (Sambrook et al. 1989). Bacterial artificial chromosomes bearing WNV cDNA were shown to be genetically stable during preparative amplification of plasmid DNA in *E.coli* (R. Suzuki and P.W.M., unpublished).

Generation of virus stocks. WNV-GFP infectious virus stocks, 6.95 $\log_{10}$ tissue culture infectious dose $(TCID)_{50}$/mL, were generated from recombinant plasmid pBelo-WNV-GFP-RZ ic as follows. Plasmid DNA was linearized by digestion with *Mlu*I restriction endonuclease and subjected to *in vitro* transcription as described elsewhere (Rossi et al. 2005). The *in vitro* transcription reaction mixture (containing $\sim$8–10 μg RNA) was used to electroporate BHK-21 cells ($\sim 1–2\times10^7$ cells) without additional purification. Electroporation efficiency was estimated as previously described (Tsetsarkin et al. 2006). Electroporation-derived stock (titer: 1×10^7 plaque forming units (pfu)/mL) collected from pBelo-WNV ic-transfected BHK-21 cells was used to infect naïve Vero cells. Virus collected from Vero cell cultures at 2 days post-infection (dpi) was designated WNV-TX02 (with titer 7.52 $\log_{10}$ $TCID_{50}$/mL).

Mosquito maintenance and infection

The well-characterized Sebring strain of *Cx. p. quinquefasciatus* mosquitoes generation >F30, originally isolated in Sebring County, Florida, 1998, were chosen for these experiments due to their proven susceptibility to WNV (Girard et al. 2004, 2005, 2007, Vanlandingham et al. 2004). Mosquitoes were maintained as previously described (Vanlandingham et al. 2004), and all mosquito infections were conducted in a arthropod containment level 3 insectary.

Mosquito infection. Mosquitoes were orally infected by combining fresh virus inoculum (infected cell culture supernatant harvested 2 to 4 dpi) 1:1 with defibrinated sheep

blood (Colorado Serum Company, Denver, CO). The infectious bloodmeal was warmed to 37°C and presented to mosquitoes. Engorged females, >4+ (Pilitt and Jones 1972), were selected and maintained at standard conditions as previously described (McGee et al. 2007). A 1.0 mL aliquot of infectious bloodmeal and three engorged mosquitoes (day = 0) per infectious feed were sampled. Mosquitoes were sampled ($n = 3$) at 1, 2, and 3 and ($n = 8$) at 5, 7, and 14 dpi to assess whole-body titer, and on days 3, 5, 7, and 14 for midgut and salivary gland dissection (WNV-GFP infected groups only). GFP expression in infected tissues was visually assessed using a Olympus IX51 compound fluorescent microscope. All remaining mosquitoes were collected at 14 dpi and decapitated, and bodies and corresponding heads were stored at −80°C before titration to determine infection and dissemination rates, respectively. Mosquitoes were also infected via intrathoracic (IT) inoculation of undiluted filter-sterilized virus stock as previously described (McGee et al. 2007).

Analysis of mosquito and murine infectivity

To determine if WNV-GFP could be transmitted by mosquitoes, and to evaluate the infectivity and stability of WNV-GFP in a vertebrate, mice were exposed to the feeding of WNV-GFP–infected mosquitoes. Briefly, mosquitoes 7–10 days post IT-inoculation with WNV-GFP (≤10 per mouse) were allowed to probe and feed on the left ear of anesthetized three-week-old Swiss Webster mouse (Harlan, Indianapolis, IN) for 1 h. All mosquitoes observed to have imbibed blood were removed and stored at −80°C for virus titration. Mosquitoes from eight mouse feeds were pooled, homogenized, and titered, and mosquitoes from the remaining one mouse feed were individually assayed to determine infection rates for IT-infected mosquitoes used in this study. Mice were bled and then euthanized at 2, 4, and 7 dpi ($n = 3$ per day) and the lymph node, liver, spleen, and brain from each animal was surgically removed. Whole organs were examined macroscopically for GFP expression using an Olympus SZX12 fluorescent stereo microscope. Serum was separated and whole organs homogenized and stored at −80°C for virus titration. All animal manipulations were performed in accordance with National Institutes of Health and UTMB humane laboratory animal use standards.

Virus titration and stability. Ten-fold serial dilutions of infectious bloodmeals, serum samples, individual whole mosquitoe homogenates, mosquito body and head homogenates, and whole mouse organ homogenates were titrated on Vero cell culture as previously described (Higgs et al. 1997). Endpoints were determined for infectious bloodmeals, whole mosquitoes, murine organ homogenates, and serum and expressed as $\log_{10}$ $TCID_{50}$/mL, mosquito, or organ, respectively. Day 14 bodies and heads were only assayed for the presence/absence of virus to determine infection and dissemination rates. All wells were examined visually for GFP expression and cytopathic effects (cpe) at 3–5 and 7 dpi, respectively.

Occasionally, high-concentration mosquito homogenates can induce changes in Vero cell morphology similar to cpe. Therefore, in those samples where cpe was observed in the absence of GFP expression, quantitative real-time reverse transcriptase polymerase chain reaction was used to confirm the presence of WNV (McGee et al. 2007). Samples verified to be WNV positive but GFP negative were further analyzed. Briefly, >80% confluent Vero monolayers seeded into 35 mm dishes were infected with a 100 μL undiluted sample followed by Trizol® (Invitrogen) extraction of total cellular RNA 24–48 h postinfection in accordance with manufacturer's protocols. Nonplaque-purified viral RNA was reverse transcribed, cloned, and sequenced to determine the nature of the expression impairment.

Statistical analysis

Differences in infection and dissemination rates and whole-body titers were tested for significance using Fisher's exact test in SPSS version 14.0 and Student's *t*-test in SigmaPlot 9.0, respectively.

Results

Electroporation of RNA *in vitro* transcribed from linearized pBelo-WNV-GFP ic (efficiency $\sim 10^6$ ffu/μg RNA) resulted in the generation of relatively high-titer 6.95 $\log_{10}$ $TCID_{50}$/mL WNV-GFP stock. The passage of WNV-TX02 postelectroporation supernatant (1×10^7 pfu/mL) in Vero cells resulted in the generation of 7.52 $\log_{10}$ $TCID_{50}$/mL virus stock. Since it was unknown which cell type would generate the most stable, highest titer WNV-GFP inoculum, mosquitoes were fed virus passaged once more in either Vero or C6/36 cells harvested once >95% of cells were observed to be expressing GFP. This strategy resulted in generation of WNV-GFP bloodmeals 100-fold disparate in titer (Vero: 4.95 $\log_{10}$ $TCID_{50}$/mL vs. C6/36; 6.95 $\log_{10}$ $TCID_{50}$/mL). The infectious WNV-TX02 bloodmeal was only harvested following propagation in Vero cells 2 dpi (7.52 $\log_{10}$ $TCID_{50}$/mL).

Viral titers for mosquito whole-body homogenates are shown in Figure 2 as mean titer + standard deviation calculated from all positive mosquitoes per time point. There was no significant difference ($p < 0.05$) in body titer for mosquitoes exposed to comparable titers of WNV-TX02 ($d = 0$; 5.33 + 0.73 $\log_{10}$ $TCID_{50}$/mL) and WNV-GFP ($d = 0$; 5.00 + 0.50 $\log_{10}$ $TCID_{50}$/mL) for any day except for 14 dpi. Although comparable titers were attained, insertion of the GFP expression cassette did affect the temporal kinetics of intra-mosquito replication. The whole-body titers of mosquitoes infected with WNV-GFP required an additional 24 h of *in vivo* replication to become comparable to those infected with WNV-TX02 (Fig. 2). Furthermore, when mosquitoes ingested a low-titer WNV-GFP bloodmeal ($d = 0$; 4.33 + 0.33 $\log_{10}$ $TCID_{50}$/mL) this temporal lag in replication kinetics became even more pronounced, with whole-body titers being significantly less ($p < 0.05$; Fig. 2) than for comparable time points of WNV-TX02 from 5 to 14 dpi. Although average whole-body titers of mosquitoes imbibing low-titer WNV-GFP were significantly less ($p < 0.05$) than the WNV-GFP high-titer group at 7 dpi, average whole-body titers from both of these groups were similar at 14 dpi (Fig. 2).

Similar day 14 infection rates were observed when WNV-TX02 (7.52 $\log_{10}$ $TCID_{50}$/mL; 88/96; 92%) and WNV-GFP (6.95 $\log_{10}$ $TCID_{50}$/mL; 47/56; 84%) were ingested at comparable titer (Table 1). As expected a significantly lower infection rate was observed after ingestion of low-titer WNV-GFP (4.95 $\log_{10}$ $TCID_{50}$/mL; 10/70; 14%). Within the low-titer experiment, significantly more mosquitoes were midgut GFP positive than were titration positive, limit of detection 1.06

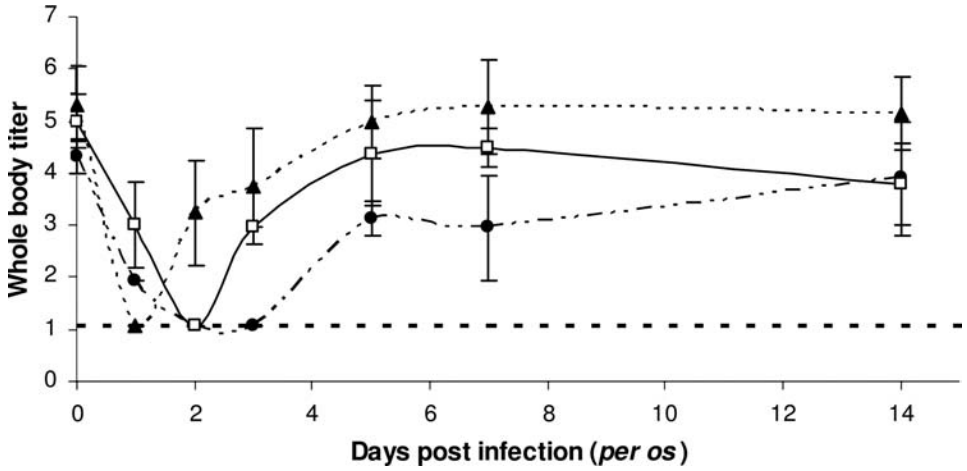

FIG. 2. Average whole-body titers (expressed as $\log_{10}$TCID$_{50}$/mosquito) of *Culex pipiens quinquefasciatus* mosquitoes infected (*per os*) with virus derived from either pBelo-WNV-RZ ic or pBelo-WNV-GFP ic. ▲, WNV-TX02 infectious bloodmeal titer 7.52 $\log_{10}$TCID$_{50}$/mL; □, WNV-GFP infectious bloodmeal titer 6.95 $\log_{10}$TCID$_{50}$/mL; •, WNV-GFP infectious bloodmeal titer 4.95 $\log_{10}$TCID$_{50}$/mL. Mean titers calculated only from virus-positive mosquitoes. Error bars represent standard deviation about the mean. TCID, tissue culture infectious dose.

$\log_{10}$ TCID$_{50}$/mosquito ($p = 0.01$). The most notable effect of insertion of the GFP cassette was a significant loss of dissemination potential at comparable input titer ($p < 0.001$; Table 1).

At 14 dpi, one non-GFP disseminated WNV infection was detected and confirmed by quantitative real-time reverse transcriptase polymerase chain reaction (data not shown) (4.95 $\log_{10}$ TCID$_{50}$/mL bloodmeal). An additional three non-GFP WNV infections were also detected and confirmed within this feed set, one at 5 dpi and two at 14 dpi. Sequence analysis from nonplaque-purified "loss-of-GFP" RNA samples was used to determine the nature of the reporter impairment. A mixed population containing multiple deletions in the expression cassette, classified as major or minor (based on prevalence), was identified. The major mutant species was characterized by a 330 nucleotide (nt) deletion (Δ) of the GFP coding sequence with a complete preservation of Cnat, while minor species included one sequence variant having a single 618 nt Δ in GFP, and a sequence variant with simultaneous presence of two deletions, one Δ is 198 nt missing from Cnat-GFP junction region, and second Δ is the 330 nt Δ within GFP sequence, same as was found in major mutants. Thus, a loss of heterologous insert by accumulation of deletions can occur during consecutive passaging of virus with the heterologous genetic cassette placed in the structrural region of genome. However, the rate of insert loss seems to be low compared to this rate in viruses with additional sequences engineered into 3′-terminal portion of genome (data not shown).

GFP-expressing foci of midgut epithelial cells were detected as early as 3 dpi and at all time points thereafter (Fig. 3). At 3 dpi, with a high-titer WNV-GFP bloodmeal, GFP-expressing foci were randomly distributed throughout the posterior midgut. In one mosquito, at 3 dpi, foci were also detected in anterior midgut and in the cardia/intussuscepted

Table 1. Infection and Dissemination of Green Fluorescent Protein–West Nile Virus in *Culex pipiens quinquefasciatus* After Ingestion of Low- and High-Titer Infectious Bloodmeals

Virus	*Bloodmeal titer*[a]	*dpi*	*Positive midguts/total (%)*	*Positive SG/positive midguts (%)*	*Positive bodies/total (%)*	*Positive heads/positive bodies (%)*
WNV-TX02	7.52	14	—	—	88/96[b] (92)	53/88[b] (60)
WNV-GFP	6.95	3	3/4 (75)	—	—	—
		5	3/6 (50)	—	—	—
		7	8/8 (100)	0/8 (0)	—	—
		14	15/19[c] (79)	1/15 (7)	47/56[b] (84)	2/47[b] (4)
WNV-GFP[d,e]	4.95	14	9/20[c] (45)	0/9 (0)	10/70[b] (14)	0/10[b] (0)

[a]Bloodmeal titer expressed as $\log_{10}$TCID$_{50}$/mL.
[b]$p < 0.001$.
[c]$p < 0.05$.
[d]Non-GFP disseminated infections detected.
[e]Non-GFP quantitative real-time reverse transcriptase polymerase chain reaction confirmed infections discussed in text but not included in infection and dissemination rate calculations.
dpi, days postinfection; WNV, West Nile virus; GFP, green fluorescent protein; TCID, tissue culture infectious dose.

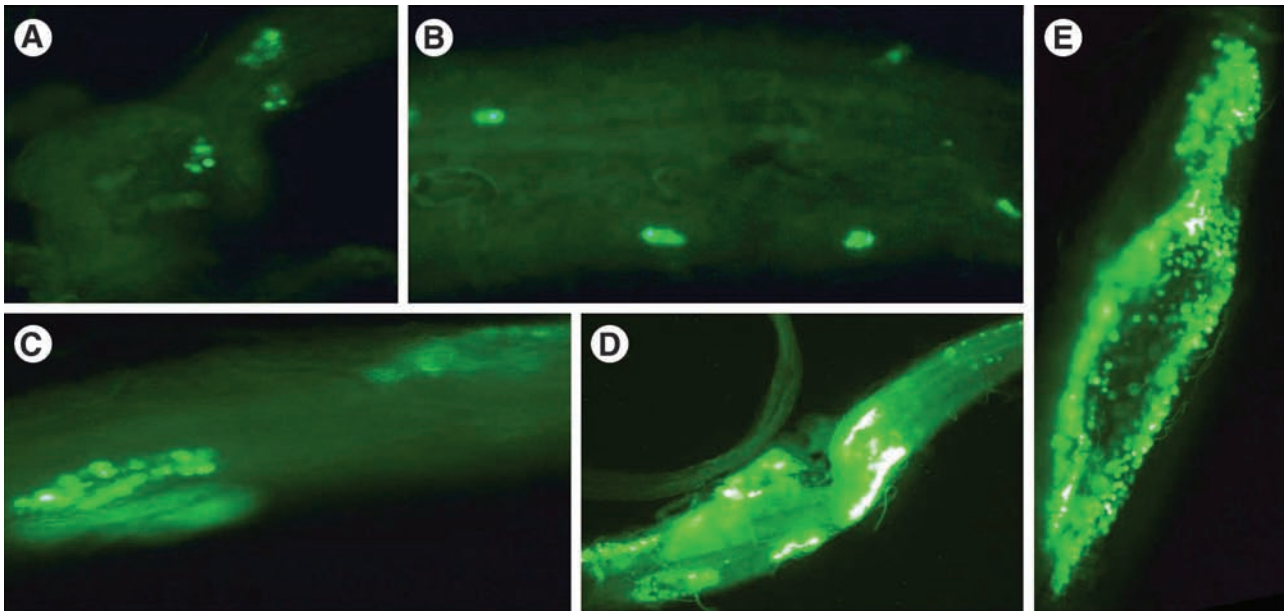

FIG. 3. WNV-GFP in selected segments of the *Cx. p. quinquefasciatus* digestive tract. (**A, B**) Green foci at 3 days postinfection (dpi) distributed throughout the cardia/intussuscepted foregut, anterior, and posterior midgut. (**C**) WNV-GFP infectious foci in the posterior midgut 5 dpi. (**D, E**) GFP expression posterior in midguts 7 dpi.

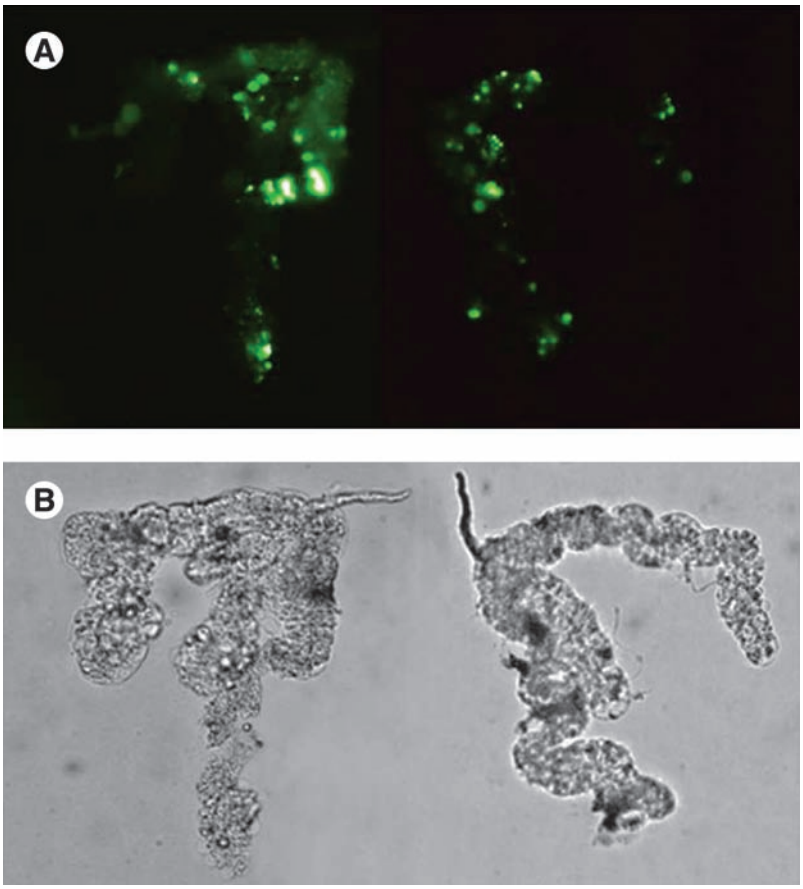

FIG. 4. Green fluorescent foci (**A**) in a *Cx. p. quinquefasciatus* salivary gland pair. (**B**) 14 days postingestion of a high-titer (6.95 $\log_{10}$ $TCID_{50}$/mL) WNV-GFP infectious bloodmeal.

foregut (Fig. 3A, B). The size of GFP foci increased over time (Fig. 3 C–E) with the majority of the posterior midgut being positive for GFP expression in some mosquitoes by 7 dpi (Fig. 3E). Foci size and distribution did not increase between 7 and 14 dpi (data not shown). Although cells along the entire gut appear to be susceptible to infection from the luminal direction, GFP expression was not observed in basally associated midgut tissues such as circular and longitudinal muscles as has been previously reported (Girard et al. 2004). GFP expression was detected in one pair of salivary glands (1/15) dissected 14 dpi with green foci being sporadically distributed throughout the lobes of each gland (Fig. 4). Transmission capability was verified in a pilot study in which mosquitoes were IT inoculated with WNV-GFP; green foci were observed in salivary gland pairs dissected at 7 dpi and in C6/36 cells infected with pooled saliva harvested 7 dpi ($n = 10$ WNV-GFP–infected mosquitoes; data not shown). After *in vitro* transmission confirmation, nine mice were exposed to the feeding of WNV-GFP IT–infected *Cx. p. quinquefasciatus* mosquitoes (10 ± 1 engorged mosquitoes per mouse). WNV-GFP was detected in all eight pools of mosquitoes examined ($6.56 + 0.31$ $\log_{10}$ $TCID_{50}$/pool) and in 10/10 individually titrated mosquitoes. WNV-GFP was detected in serum (3/3; $4.0 + 0.83$ $\log_{10}$ $TCID_{50}$/mL) and lymph node homogenates (2/3; $2.11 + 0.33$ $\log_{10}$ $TCID_{50}$/lymph node) at 2 dpi and in lymph node homogenates (2/3; 1.06 $\log_{10}$ $TCID_{50}$/lymph node) at 4 dpi. Green fluorescence was never detected in any macroscopically examined organ or in cells infected with homogenates prepared from spleen, liver, or brain.

Discussion

Historically, the study of intra-vector infection, replication, dissemination, tropism, and transmission has relied heavily on antigen detection and ultrastructural analysis to identify sites/tissues important for virus replication. These techniques, though quite effective, are labor intensive and may be confounded by staining and sample fixation/preparation artifacts and can lack the sensitivity to detect very low level infections. Reverse genetic technology, specifically the ability to express a visible marker from a recombinant viral genome, provides an excellent alternative for the study of *in vivo* replication kinetics. The major advantage of reporter gene expression is that it enables the researcher to easily identify sites of replication and subsequently follow the progression of infection through the living tissue. GFP was first used as a marker to detect viral replication in mosquitoes by Higgs et al. (1996) and has subsequently been adopted as the marker of choice to study arbovirus-vector systems and to identify genetically manipulated vectors (Pinkerton et al. 2000).

Indeed insertion of GFP into recombinant alphavirus genomes has facilitated the study of the following: Sindbis virus infection and dissemination (Rayms-Keller et al. 1995, Higgs et al. 1996, Olson et al. 2000, Pierro et al. 2003, Foy et al. 2004), o'nyong nyong virus tissue tropism (Brault et al. 2004), Venezuelan equine encephalitis midgut susceptibility (Smith et al. 2008) and escape (Romoser et al. 2004), and chikungunya virus infectivity (Vanlandingham et al. 2005) and vector adaptation (Tsetsarkin et al. 2006, 2007). Reporter gene expression in these studies was facilitated by the subgenomic organization of the alphaviruses being studied. Recent attempts to generate replication-competent GFP-expressing flavivirus ics suggest that different strategies are required (Pierson et al. 2005) (Higgs, Zhao, and Mason, unpublished data).

In an effort to increase flavivirus reporter stability we generated the WNV recombinant genome pBelo-WNV-GFP-RZ ic using the strategy recently described (Shustov et al. 2007). GFP-expressing virus was viable and competent for replication in both vertebrate (Vero and BHK) and arthropod (C6/36) cells. Additionally, changes in cellular morphologies (cpe) resulting from viral replication were similar to those observed for infections with virus derived from the parental WNV (non-GFP) ic derived from a North American human isolate of WNV. However, as expected, insertion of the expression cassette resulted in a slight temporal lag in replication.

The most profound effects of the GFP expression cassette insertion were observed with respect to *in vivo* temporal replication kinetics. After ingestion of comparable titers of WNV-GFP and WNV-TX02, no significant differences in infection rates were observed 14 dpi; however, a 24 h temporal delay in replication kinetics and significantly decreased whole-body titers were noted at 14 dpi. This temporal lag was likely the result of a combination of having to replicate a >1.0 kb larger genome and decreased codon usage efficiency. As expected, this temporal lag became even more pronounced after ingestion of lower titer WNV-GFP infectious bloodmeal as has been previously noted for various N. American mosquito species (Akhter et al. 1982, Tiawsirisup et al. 2004, 2005, Erickson et al. 2006).

Similar to previous reports of the number and distribution of WNV primary midgut infections that used immunofluorescently detected WNV-like particles (Scholle et al. 2004) and immunohistochemically detected WNV (Girard et al. 2004), small distinct GFP foci were observed distributed throughout the cardia/intussuscepted foregut and anterior and posterior midgut of WNV-GFP–infected mosquitoes as early as 3 dpi, with relatively few infectious foci per individual (Fig. 3A and 3B). This may indicate that a relatively small number of cells are susceptible to primary infection, as has been previously suggested for Venezuelan equine encephalitis virus (Smith et al. 2008) and WNV (Scholle et al. 2004), but also that cells capable of serving as primary sites of luminal infection are distributed throughout the entire gut. A temporal increase in the size of infectious GFP foci was observed by 5 and 7 dpi similar to previous IHC analysis of antigen distribution in infected mosquito midguts (Girard et al. 2004) with infection of the posterior midgut being widely distributed by 7 dpi (Fig. 3) and dissemination to the salivary glands (1/15) by 14 dpi (Fig. 4).

Girard et al. (2004) suggested a model for the sequential dissemination of WNV in *Cx. p. quinquefasciatus* where virus escaping the posterior midgut infects and amplifies in fat body, followed by infection of anterior midgut and midgut-associated muscles. It was also suggested that infection of midgut muscle tissue was a somewhat unique hallmark of WNV infection and that the most important tissue for facilitating midgut infection was the thoracic fat body. Our analysis did not include mosquito fat body; however, GFP expression was never observed in midgut-associated muscle cells. This could possibly result from an inability to release infectious virions from the posterior midgut epithelium or an inability of released virions to infect muscle cells. Since fluorescence distribution (number of GFP-positive cells) was observed to

temporally increase within the posterior midgut epithelium and infectious WNV-GFP could be harvested from mosquito homogenates, we do not believe this to be the case. Concurrently, the observation of efficient salivary gland infection after IT inoculation (mechanically bypassing the midgut) indicates that WNV-GFP virions are capable of infecting secondary tissues. However, the observation of significant differences in low-titer feed infection rates when measured by GFP detection versus virus titration indicates that abortive infections may occur. It is possible that in these individuals infectious mature virus progeny is not efficiently generated or released or that some mosquito cellular response such as RNA interference is effectively confining or resolving the midgut infection. Nevertheless, the near loss of salivary gland infection after oral infection indicates that GFP cassette insertion results in the introduction of a significant impediment to intravector dissemination.

IT-infected mosquitoes were observed to be capable of transmission of WNV-GFP with subsequent replication in murine lymph nodes and viremia by 2 dpi. These latter data are consistent with recent work showing that WNV-like particles injected subcutaneously into mice rapidly induce high level of IFNα and traffic to draining lymph nodes (Bourne et al. 2007). Based on previous experience we would expect WNV vector–borne murine infection to result in a pronounced infection involving multiple organ systems and a moderate level of mortality (Schneider et al. 2006, 2007). However, WNV-GFP viremia and lymph node infection were self-limiting and never associated with any clinical symptomology, again indicating that GFP expression cassette insertion results in *in vivo* attenuation.

It is generally accepted that the requirement for replication in two phylogenetically disparate hosts (arthropods and vertebrates) imposes negative selective pressure on the flavivirus genome evolution and that most manipulations should result in dysfunctional replication in at least one host system. To achieve GFP expression, $>$1.0 kb of coding sequence was inserted into the viral ORF, although the effect of $\sim$10% increase in length for the flaviviral genome in terms of molecular replication or packaging is questionable. We hypothesize that the biological nature of changes we made to viral genome, specifically the appearance of Cnat-GFP fusion protein, is to some extent detrimental to WNV growth (additional data to support this proposition available from authors). It should also be noted that some of the sequences in the utilized genetic cassette may have suboptimal codon usage for replication in mosquito cells. Thus, based on our results we propose that insertion of heterologous inserts within the context of WNV structural region increases reporter stability, but negatively impacts WNV *in vivo* replication kinetics, thereby favoring host response and a controlled infection.

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Disclosure Statement

No competing financial interests exist.

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