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DNA forms of arboviral RNA genomes are generated following infection in mosquito cell cultures

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ABSTRACT

Although infections of vertebrate hosts by arthropod-borne viruses may lead to pathogenic outcomes, infections of vector mosquitoes result in persistent infections, where the virus replicates in the host without causing apparent pathological effects. It is unclear how persistent infections are established and maintained in mosquitoes. Several reports revealed the presence of flavivirus-like DNA sequences in the mosquito genome, and recent studies have shown that DNA forms of RNA viruses restrict virus replication in *Drosophila*, suggesting that DNA forms may have a role in developing persistent infections. Here, we sought to investigate whether arboviruses generate DNA forms following infection in mosquitoes. Our results with West Nile, Dengue, and La Crosse viruses demonstrate that DNA forms of the viral RNA genome are generated in mosquito cells; however, not the entire viral genome, but patches of viral RNA in DNA forms can be detected 24 h post infection.

1. Introduction

Arthropod-borne viruses or arboviruses are responsible for serious human infectious diseases nearly worldwide (Weaver and Reisen, 2010). For example, alphaviruses like Chikungunya virus (CHIKV) and Eastern equine encephalitis virus (EEEV) of the *Togaviridae* family, and flaviviruses such as Dengue (DEN), West Nile (WN), Yellow Fever (YF), Japanese Encephalitis (JE), and Zika (ZIK) viruses of the *Flaviviridae* family are highly pathogenic; infections with these viruses bear huge medical and economic consequences (Bhatt et al., 2013; Higgs, 2016; Petersen et al., 2016; Weaver and Reisen, 2010). Although arbovirus infections cause serious human diseases, including hemorrhagic fevers, encephalitides, and microcephaly, infections of arthropod vectors are non-pathogenic and persistent throughout the life of the vector mosquitoes or ticks. In mosquito cell cultures, a persistent phase with low levels of virus production follows an acute phase with efficient virus production (Davey and Dalgarno, 1974). However, the mechanisms by which persistent infections are established and maintained in mosquitoes are poorly understood. Several ideas, including the generation of defective-interfering particles, mutations that affect the cytopathogenic pathway or that affect post-translational proteolytic cleavage, and the presence of extracellular virus have been proposed as the mechanism for establishment and maintenance of persistent infections (Chen et al., 1994, 1996; Ebner et al., 2008; Juarez-Martinez et al., 2013; Riedel and Brown, 1977; Tsai et al., 2007). The results of several investigations, however, have suggested that different outcomes of arbovirus infections in vertebrates and insects are due to the differences in their immediate or innate-immune responses to viral infections (Blair, 2011; Blair and Olson, 2015; Fragkoudis et al., 2009; Kingsolver et al., 2013; Prasad et al., 2013).

Most arboviruses contain RNA as their genetic material (Weaver and Reisen, 2010). The robust response following virus infection in mosquitoes is the RNA-interference (RNAi) pathway (Blair, 2011; Blair and Olson, 2015; Gammon and Mello, 2015). There are three well-characterized RNAi pathways, where three RNA classes differ in their size and mode of biosynthesis: (I) small-interfering RNA (siRNA) pathway, in which siRNAs are generated from exogenous double-stranded RNA (dsRNA), such as viral dsRNA (also known as *exo*-siRNA), or are generated from cellular transcripts (*endo*-siRNA). (II) microRNA pathway (miRNA) in which miRNAs are generated from cellular transcripts and normally function at the translational level, and (III) PIWI-interacting RNA (piRNA) pathway, where Dicer-independent piRNAs are transcribed from the cellular genome, and function in the epigenetic control of transposable elements in the germ line. In mosquitoes, it is the

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siRNA pathway that is primarily responsible for antiviral activity (Blair, 2011; Blair and Olson, 2015; Campbell et al., 2008; Cirimotich et al., 2009; Fragkoudis et al., 2009; Hoa et al., 2003; Keene et al., 2004; Kingsolver et al., 2013; Olson et al., 1996; Prasad et al., 2013; Sanchez-Vergas et al., 2009). The role of viral RNA-derived miRNAs in vector-virus interactions is uncertain (Hussain and Asgari, 2014; Miesen et al., 2016; Skalsky et al., 2014). Generation of virus-derived piRNAs has been demonstrated following infection with various arboviruses (Hess et al., 2011; Miesen et al., 2016; Morazzani et al., 2012; Leger et al., 2013; Schnettler et al., 2013; Scott et al., 2010; Vodovar et al., 2012); the population of piRNA increases in siRNA-defective cells following infections with several arboviruses, suggesting a cross talk among different RNA-silencing pathways (Brackney et al., 2010).

In mosquitoes, *exo*-siRNA is initiated by intracellular detection of exogenous long dsRNA, which are cleaved by a cytoplasmic RNase III enzyme Dcr2 into predominantly 21 nucleotide siRNA duplexes. These siRNAs are loaded into the Argonaute-2 (Ago2)-containing RNA-induced silencing complex (RISC). The RISC degrades one of the siRNA strands and retains the other strand as a guide strand for recognition and annealing to the target RNA, leading to degradation of the target RNA.

The presence of the strong RNAi antiviral defense mechanisms cannot protect mosquitoes from developing persistent infections by arboviruses. Several plant viruses and insect pathogenic viruses express virus-encoded suppressors of RNAi (VSR), to evade RNA-interference pathways (Blair, 2011). However, no such VSR have been identified for arboviruses. All flaviviruses studied thus far produce subgenomic RNAs, which result from incomplete digestion of the genomic RNA by the cellular 5′–3′ *exo*ribonuclease Xrn1 (Pijlman et al., 2008). Previous reports have suggested that the subgenomic RNA may act as a VSR during flaviviral infection.

Several studies have indicated the presence of genomic sequences from non-retroviral RNA viruses in the DNA form within the plant, mammalian, and insect genomes (Chiba et al., 2011; Goic et al., 2013; Horie et al., 2010; Klenerman et al., 1997; Tanne and Sela, 2004; Zhdanov, 1975). The presence of flavivirus-related sequences in the DNA form, originally termed *cell silent agents*, were detected in both laboratory-reared and wild-caught uninfected mosquitoes, as well as in uninfected and infected *Aedes albopictus* cell cultures (Chen et al., 2015; Cook et al., 2006; Crochu et al., 2004; Rizzo et al., 2014; Roiz et al., 2009; Sanchez-Seco et al., 2010; Vazquez et al., 2012). These DNA sequences in uninfected cells likely originated following infection by the corresponding RNA viruses or defective-interfering particles, which were subsequently cleared. Although conversion of flaviviral RNA into the DNA form following infection has been established from these studies, it is not known whether these DNA sequences play any role in virus replication. These DNA sequences, however, have the potential to generate dsRNA during host genomic transcription. For example, dsRNAs can be generated from transcription of overlapping regions on opposite strands of the genome, and from genomic regions that generate transcripts with secondary structures. It has been observed that in *Drosophilla* and *Aedes aegypti*, during persistent infections, Dcr2-dependent viral interfering RNAs (viRNAs) are generated from preferred regions in the viral genome (Flynt et al., 2009; Scott et al., 2010), and the bulk of viral sRNAs is not loaded into any Argonaute proteins (Flynt et al., 2009), raising doubts as to the source of the viRNA molecules. In addition, the DNA form of the viral RNA has been shown to regulate viral replication in *Drosophila* (Goic et al., 2013). Thus, the viral DNA (vDNA) may provide the siRNAs required for establishment and maintenance of persistent infections. We therefore set out to determine the roles of DNA forms of the arboviral RNA genomes in mosquitoes. In this report, we present evidence that all arboviruses tested here (WNV, La Crosse virus, and DENV2) generate DNA forms following infection of mosquito cell cultures.

2. Material and methods

2.1. Cell lines and viruses

Ae. aegypti (Aag) cells (ATCC, CCL-125) were cultured in Eagle's Minimum Essential Medium (MEM; Sigma-Aldrich, M0268) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 1.5 g/L sodium bicarbonate, at 28 °C with 5% CO_2. *Culex tarsalis* cells (a gift from A. Brault, CDC, Fort Collins) were grown in Schneider's media (Sigma-Aldrich, S9895) supplemented with 10% FBS, 0.6 g/L calcium chloride, 0.4 g/L sodium bicarbonate, and maintained at 28 °C in 5% CO_2. *Ae. albopictus* cells (C6/36; ATCC, CRL-1660) were grown in MEM supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and maintained at 28 °C in 5% CO_2. All media had 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. Viral titers were determined by plaque assay in African green monkey kidney (Vero; ATCC CCL-81) cells, which were grown in MEM (Sigma-Aldrich, M0268) supplemented with 10% FBS, 2.2 g/L sodium bicarbonate, and maintained at 37 °C in 5% CO_2. DENV2 (New Guinea C strain), WNV (NY99), and LACV (LAC74-32813) were used in this study. All stock viruses were generated in C6/36 cells.

2.2. Cell passaging

Mosquito cells were infected with the virus in six-well plates. After one hour of incubation at 28 °C, 3 ml of maintenance medium containing 2% FBS were added to the infected cell and incubated again at 28 °C. For Aag cells, 3 ml of complete growth medium were added following one hour of incubation at 28 °C. After one week, the supernatant was removed, cells were washed twice with phosphate-buffered saline, trypsinized, centrifuged and suspended again after 1:4 dilution with 5 ml of fresh growth media in a T25 flask, and incubated at 28 °C. Similar passaging was done once every week. Finally, passage 10 (P10) genomic DNA from persistently infected mosquito cells was isolated using DNeasy spin columns (Qiagen) in the presence of RNase.

2.3. PCR conditions

PCR conditions were the same for all three viruses. Each PCR reaction contained 50 ng of template DNA, 10 pM of each primer, and the annealing temperature was at least 5 °C below the Tm of the primer sets. Primer extension was carried out at 72 °C for 30 s for a total of 40 cycles. The primer-sequence information is provided in Table 1.

3. Results

3.1. Detection of the WNV genome in the DNA form in the infected mosquito cell cultures

To determine whether the WNV RNA genome exists in the DNA form, we infected *Culex tarsalis* mosquito cells with WNV at 50 multiplicity of infection (moi), and then incubated at 28 °C, after adding maintenance media containing 2% FBS. A high moi was used to ensure that nearly all cells are infected with the virus. The infected cells were passaged after a week in fresh growth media and incubated at 28 °C. Cell passaging was continued until passage 10 (P10), when the cellular genomic DNA was isolated in the presence of RNase. The presence of the WNV genome in the DNA