Patchy DNA forms of the Zika virus RNA genome are generated following infection in mosquito cell cultures and in mosquitoes

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Abstract

Zika virus (ZIKV) is a mosquito-borne flavivirus and has historically been reported to cause mild symptomatic diseases during human infections. More recently, the explosion of microcephaly among infants born to ZIKV-infected women has made ZIKV a global public health concern. While ZIKV causes acute human diseases, infections of vector mosquitoes are basically non-pathogenic, allowing persistent infections and conferring lifelong ability to transmit the virus. Recent studies have revealed that DNA forms of arboviral RNA genomes play a significant role in viral persistence in mosquitoes. We have initiated experiments to determine whether ZIKV generates viral DNA (vDNA) forms following infection in mosquitoes. Here we show that vDNAs are generated following ZIKV infection both in mosquito cell cultures and in its primary vector \textit{Aedes aegypti}. vDNA formation is more extensive in RNA interference (RNAi)-deficient \textit{Aedes albopictus}-derived \textit{C6/36} cells compared to RNAi-proficient mosquito cells. In addition, vDNAs are generated via multiple template-switching events.

Zika virus (ZIKV; family, \textit{Flaviviridae}; genus, \textit{Flavivirus}) is an enveloped virus with a positive-strand RNA genome and is closely related to dengue, West Nile, and yellow fever viruses [1]. Apart from reports of sexual and congenital transmission, ZIKV is largely transmitted by certain species of infected \textit{Aedes} mosquitoes, such as \textit{Aedes aegypti} and \textit{Aedes albopictus} [2–6]. After its arrival in the western hemisphere, ZIKV caused extensive outbreaks in central and South America [7] and such outbreaks are often associated with serious birth defects and neurological syndromes [8, 9]. The marked increase in the number of infants born with microcephaly as a result of their mothers being infected with ZIKV during pregnancy has made infection with ZIKV a serious public health crisis across the globe. In the absence of a vaccine or an antiviral drug, vector-control strategies remain the only mechanism to limit the spread of ZIKV infections. Consequently, gaining a detailed knowledge of viral replication in mosquitoes may identify new strategies for vector control.

Although ZIKV infections cause human disease and birth defects, infections of arthropod vectors are basically non-pathogenic and persistent throughout the life of mosquitoes. The development of persistent infections, also known as viral tolerance [10], allows the virus to replicate in mosquito hosts to produce high pathogen loads, but low virulence, and without significant negative impacts on host fitness. In mosquito cell cultures, a persistent phase with low levels of virus production follows an acute phase with efficient virus production [11]. Little is known about how mosquitoes establish and maintain persistent infections.

Several mechanisms, such as the formation of defective-interfering (DI) particles, the presence of extracellular virus and alterations in post-translational processing, have been proposed to explain persistent infections in mosquitoes [12–15]. A number of studies, however, have suggested that different outcomes in mosquitoes and humans are mainly due to their differences in innate immune responses to viral infection [16–21]. The robust response following viral infection in mosquitoes is the RNA-interference (RNAi) pathway that uses virus-derived double-stranded RNA (dsRNA) to produce small-interfering RNA (siRNA) molecules, which target viral RNA for degradation, causing inhibition of viral replication. RNAi consists of three pathways: (i) the siRNA pathway, in which siRNAs are generated from exogenous dsRNA, such as viral dsRNA (also known as exo-siRNA), or are generated from cellular transcripts (endo-siRNA); (ii) the microRNA (miRNA) pathway in which miRNAs are generated from cellular transcripts and normally functions at the translational level; and (iii) the PIWI-interacting RNA (piRNA) pathway, where piRNAs are transcribed...
from the cellular genome, and maintain genome integrity by suppressing mobilization of transposable elements. In mosquitoes, it is the siRNA pathway that is primarily responsible for antiviral activity [16, 22–25]. The role of viral RNA-derived miRNA in vector–virus interactions is unclear [26–28]. Virus-derived piRNA has been demonstrated in mosquito somatic cells following infection with various arboviruses [27, 29–31]. Previous reports suggested that there is a cross talk among various RNA-silencing pathways [32].

In mosquitoes, the exo-siRNA pathway is triggered by the detection of exogenous long dsRNA, in the form of viral replication intermediates or as secondary structures within the viral RNA genome. Endonuclease Dcr2-mediated cleavage of these dsRNAs generates predominantly 21-nucleotide viral siRNA (vsiRNA) duplexes. These vsiRNAs are loaded into the Argonaute-2 (Ago2)-containing RNA-induced silencing complex (RISC). The RISC retains one strand as a guide strand for recognition and eventual cleavage of viral RNA by the Ago2 endonuclease. Dcr2-deficient mosquitoes and Ae. albopictus (C6/36) cells propagate arboviruses to higher titres than Dcr2-proficient mosquitoes and mosquito cell lines [33–35]. The presence of strong RNAi antiviral defense mechanisms, however, cannot shield mosquitoes from developing persistent infections by arboviruses. Several plant viruses and insect-pathogenic viruses express a virus-encoded suppressor of RNAi (VSR), to escape RNA-interference pathways [22]. All flaviviruses studied thus far produce subgenomic RNAs with extensive secondary structures [36]. There is evidence that the sub-genomic RNA as well as the capsid protein may serve as suppressors of RNAi during flavivirus replication [37, 38].

Various investigations have described the presence of genomic sequences from non-retroviral RNA viruses in the DNA form, called endogenous viral elements (EVEs), within the plant, mammalian and insect genomes [39–43]. Additionally, flavivirus-related sequences in the DNA form, initially termed cell silent agents, have been observed in both laboratory-reared and wild-caught uninfected mosquitoes, and in uninfected and infected Ae. albopictus cell cultures [44–49]. These EVEs or viral DNA (vDNA) sequences found in uninfected cells and mosquitoes likely originated following infection by the corresponding RNA viruses or DI particles, which were subsequently cleared [44]. Recently, our laboratory and Goic et al. have separately shown that DNA forms of several arboviruses, such as dengue, chikungunya, West Nile and La Crosse viruses are generated following infections in mosquito cell cultures and in mosquitoes [50, 51]. These studies have shown that vDNA reinforces the siRNA-mediated antiviral defense mechanism in mosquitoes. Inhibition of vDNA formation causes reduction in chikungunya virus-derived small RNAs, extreme susceptibilities to viral infections, and loss of viral tolerance and reduced survival [50]. These observations underscore the importance of vDNA in RNA virus replication and development of persistent infections.

To determine whether vDNAs play a role in ZIKV replication, we conducted studies to detect the appearance of ZIKV DNA forms following infections of both mosquito cell cultures and its primary vector Ae. aegypti. vDNAs are believed to form when the cellular reverse transcriptase switches from the parental RNA template to a heterologous virus RNA molecule present nearby during reverse transcription [42, 43, 52]. The probability of template-switching events increases as the copy number of heterologous RNA increases. Since Ae. albopictus-derived C6/36 cells are RNAi-deficient [32], viral RNA replicates more efficiently in C6/36 cells compared to RNAi-proficient Ae. aegypti-derived Aag or Ae. albopictus-derived U4.4 cells [33]. As a result, the probability of the generation of vDNA forms in C6/36 cells is likely to be higher than in Aag or U4.4 cells, due to the presence of a large number of viral RNA. For this reason, we initiated the detection of vDNAs in ZIKV-infected C6/36 cells.

C6/36 cells (ATCC, CRL-1660) were infected with the ZIKV (strain: PRVABC59, KU501215.1) at 0.1 m.o.i. Our previous studies with several arboviruses indicated that vDNA could be detected as early as 24 h post-infection (p.i.) [51]. For a better signal generation, genomic DNA was isolated from infected cells 6 days p.i. in the presence of RNase. The genomic DNA was used as a template in conventional PCR assays, as described in [51], to detect DNA forms of the ZIKV RNA genome. As a control, we used the DNA from uninfected cells. Primers were designed covering the entire viral genome, and the PCR reactions were expected to generate a fragment size between 200–400 bp. The PCR products were analysed after running them in agarose gels. Our results showed that DNA forms of the ZIKV RNA genome were generated in infected cells (Fig. 1a). PCR products were generated only with genomic DNA from infected cells, not with DNA from uninfected cells. We repeated these experiments and obtained similar results.

To demonstrate that the PCR products were not generated due to non-specific primer binding, DNA fragments were gel purified and analysed by DNA sequencing. All PCR products had the correct sequence, indicating that the PCR products were generated from ZIKV DNA present in the infected C6/36 cells. In addition, we treated the genomic DNA with RNase or DNase; only the RNase treatment generated PCR products (Fig. 1b). Furthermore, vDNA was not generated in a PCR assay when DNA-free RNA, isolated from infected cells, was used as a template (data not shown), suggesting that the PCR products were produced from DNA and not from any leftover viral RNA present in the genomic DNA preparations.

Since C6/36 cells are RNAi-deficient, it is possible that ZIKV DNA forms are generated due to enhanced RNA replication, so it may not be the case in RNAi-proficient cells. To investigate that possibility, we performed similar experiments in RNAi-proficient Ae. albopictus-derived U4.4 cells (a gift from Raquel Hernandez and Dennis Brown, North Carolina State University, Raleigh, NC) and Ae. aegypti-derived Aag cells (ATCC, CCL-125). ZIKV
infection and genomic DNA preparation were carried out as described for C6/36 cells. Our results indicated that both U4.4 and Aag cells generated vDNAs following infection with ZIKV (Fig. 1a), suggesting that the generation of DNA forms is not specific to a particular cell type. In C6/36 cells, 95% of the primer combinations, covering nearly the entire viral genome was converted into DNA forms, except that a primer combination near the 3’ end failed to generate PCR products. In U4.4 and Aag cells, on the other hand, vDNA formations were patchy, as most of the primer combinations failed to generate PCR products (Fig. 1c). Similar differences in PCR results between Aag and C6/36 cells were also observed for the well-characterized dengue 2 strain [51].

Since most of the RNA genome is converted into the DNA forms in C6/36 cells, we were interested in determining whether such DNA forms were derived from a single reverse-transcription reaction. If vDNA formation in C6/36 cells was a one-step process, then a primer combination, one from the 5’ end and one from the 3’ end would generate a long PCR product. As shown in Fig. 2(a), we used several primer combinations to determine the longest DNA fragment that can be detected in C6/36 cells. As a positive control, we used a cDNA of the ZIKV RNA genome in each PCR reaction (Fig. 2b). A primer combination of ZK3420F/ZK7674R would generate a PCR product of 4.2 kb. Although the positive control sample had a PCR product, no such bands were observed either with DNA from uninfected or infected samples.

**Fig. 1.** Generation of DNA forms of the ZIKV RNA genome in mosquito cell cultures. (a) Examples of the presence of vDNAs in various mosquito cell cultures. Conventional PCR was carried out with genomic DNA preparations as described in [51]. Lane C had ZIKV cDNA as a positive control; U had the DNA from uninfected cells; I had the DNA from infected cells shown on the left. Actin primers were used to ensure the presence of DNA in each sample. (b) PCR products were generated from DNA not RNA. RT-PCR was carried out using viral RNA; the rest of the lanes had genomic DNA preparations from ZIKV-infected cells, except the DNase-I lane, which had DNA from uninfected cells. DNase was inactivated by heat treatment. In the DNase-DNA lane, genomic DNA was treated with DNase, heat treated, then genomic DNA was added again before PCR. (c) Regions of the ZIKV RNA genome in the DNA form in various mosquito cell cultures. Numbers above and below the line represent the nucleotide position of primers in the RNA genome. Primer sequences are available upon request. Green rectangles represent primers that produced PCR-positive results.
combinations between ZK3420F and ZK7674R primers, only ZK3420F/ZK4953R primers produced a PCR product (Fig. 2a). However, primers ZK4661F/ZK6900R also yielded positive results, suggesting that these two positive results were due to separate template-switching events. Similarly, primers ZK2951F/ZK6900R, ZK2951F/ZK4953R and ZK2290F/ZK4275R failed to generate PCR products, but ZK2951F/ZK4275R did produce a PCR product, again suggesting that the 1.3 kb vDNA was generated in an independent template-switching event.

It is possible that ZIKV DNA forms are generated in mosquito cell cultures, as described above, but not in mosquitoes. To test this possibility, a laboratory established colony of *Ae. aegypti* (kindly supplied by G. D. Ebel following collection in Poza Rica, Mexico) were fed on an infectious blood meal containing $1 \times 10^9$ p.f.u. ml$^{-1}$ of ZIKV, and then maintained with 10 % sucrose for 21 days to allow infections to develop. Control mosquitoes were given a non-infectious blood meal and reared similarly. Each mosquito was then suspended in 250 µl PBS and stored at $-80 \, ^\circ\text{C}$ for later studies. After homogenizing mosquito samples in a homogenizer, 50 µl of the sample was used for total nucleic acid isolation, and infection was monitored by quantitative RT-PCR. With this method, we obtained a 52 % infection rate. Genomic DNA was isolated from the remaining sample in the presence of RNase and then tested for the presence of vDNA forms as described above, except that PCR amplifications were carried out for 46 cycles. We tested three mosquitoes that were given non-infectious blood meals as negative controls and six infected mosquitoes. All six infected mosquito samples, but not the uninfected controls, generated PCR products with primers ZK9075F/ZK9358R (Fig. 3a). Similar results were also obtained with mosquitoes collected 14 days post feeding (data not shown). These results suggest that vDNAs are produced in both cell cultures and in mosquitoes following infection with ZIKV. We have also used other primer sets with the mosquito DNA preparations, to detect the presence of the corresponding regions in the DNA form. The PCR products were subjected to DNA sequence analysis to make sure that they originated from the correct region. More primer combinations generated PCR products in mosquitoes than in Aag cells (Fig. 3b).

In Aag cells, several primer combinations produced PCR products. However, most of these PCR products were either also present in the control uninfected samples, or upon DNA sequencing, exhibited no significant similarities to the viral genome. PCR products that were confirmed by DNA sequencing are shown in Fig. 1(c). This difference in the magnitude of vDNA formation between Aag cells and *Ae. aegypti* mosquitoes suggests that either cell cultures and mosquitoes do not share identical environments for viral replication, or that in Aag cells, vDNA signals are below the detection level of our assay system. It is also possible

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![Figure 2](https://www.microbiologyresearch.org/)

**Fig. 2.** ZIKV DNA forms in *C6/36* cells were generated by multiple events. (a) Several primer combinations were used to generate long PCR products. Black and green lines under the ZIKV genetic map represent primers that generated negative and positive results, respectively. (b) Examples of PCR-positive and PCR-negative results are shown. Lane C had cDNA as a template; lanes U and I had genomic DNA preparations from uninfected and ZIKV-infected cells, respectively.
that the time of sample collection (6 days for Aag cells vs 21 days for mosquitoes) is a contributing factor for differences in the magnitude of vDNA formation between Aag cells and Ae. aegypti mosquitoes. In mosquitoes, 21 days may provide more time for vDNA formation than 6 days in cell cultures. The intensity of PCR products on agarose gels varied from primer-to-primer combinations both in cell cultures and in mosquitoes. It is possible that certain regions of the viral RNA generate more vDNAs than the rest of the genome and these vDNAs provide a large number of templates for some primer combinations, resulting in strong signals after gel electrophoresis. For example, long sequence homology between the dissociated parental cDNA and the heterologous viral RNA would increase the probability of template switching (thus vDNA formation) at sites bearing sequence homology. Similarly, frequent polymerase dissociation from the parental template at a particular site and repeated reassociation with the viral RNA at the same site would enhance the probability of vDNA formation close to the annealing site.

In C6/36 cells, most of the viral RNA genome was converted into DNA forms following infection. As mentioned before, extensive vDNA formation in C6/36 cells is likely due to the high level of viral RNA replication in the absence of an efficient siRNA mechanism. We were unable to detect long PCR fragments covering the entire genome, and the above results suggest that vDNAs were generated by multiple independent template-switching events. It is also possible that not all cells in the population contain the same vDNA; different segments of the RNA genome may have converted into DNA forms in separate cells in the population, depending on where on the RNA genome template switching had occurred.

The above results showed that DNA forms of the ZIKV RNA genome are generated following infection in cultured cells and in Ae. aegypti mosquitoes. PCR products were generated from DNA and not from any leftover RNA in the DNA preparation. DNA forms covered nearly the entire RNA genome in RNAi-deficient cells, while in RNAi-proficient cells, vDNA formation was sparse. These results agree with previous observations that flavivirus-like sequences were detected in both laboratory reared and field-collected mosquito genomes [44–49]. It is at present unclear whether vDNAs are present as extrachromosomal elements or integrated within the host genome, although the presence of EVEs in uninfected Ae. albopictus and Ae. aegypti genomes [48] suggests that the vDNA may have integrated into the host genome at a certain frequency, and may also enter germ-line cells.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


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