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A robust method for the rapid generation of recombinant Zika virus expressing the GFP reporter gene

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ABSTRACT

Zika virus (ZIKV) infection is a major public health problem with severe human congenital and neurological anomalies. The screening of anti-ZIKV compounds and neutralizing antibodies needs reliable and rapid virus-based assays. Here, we described a convenient method leading to the rapid production of molecular clones of ZIKV. To generate a molecular clone of ZIKV strain MR766^{NIID}, the viral genome was directly assembled into Vero cells after introduction of four overlapping synthetic fragments that cover the full-length genomic RNA sequence. Such strategy has allowed the production of a recombinant ZIKV expressing the GFP reporter gene that is stable over two culturing rounds on Vero cells. Our data demonstrate that the ZIKV reporter virus is a very reliable GFP-based tool for analyzing viral growth and measuring the neutralizing antibody as well as rapid screening of antiviral effect of different classes of inhibitors.

1. Introduction

Zika virus (ZIKV) is a mosquito-borne, enveloped RNA virus first discovered in Uganda in 1947 (Dick et al., 1952). ZIKV belongs to the *Flaviviridae* family, *Flavivirus* genus, and is related to medically important flaviviruses such as dengue (DENV), West Nile (WNV), yellow fever (YFV), and Japanese encephalitis (JEV) (Plourde and Boch, 2016; Musso and Gubler, 2016; Weaver et al., 2016). Flavivirus genomic RNA contains a single open reading frame, which is translated into a single large polyprotein that is subsequently cleaved into three structural proteins (C, prM/M and E) and seven non structural proteins NS1 to NS5 by cellular and viral proteases.

Until recently, ZIKV outbreaks were sporadic and self-limiting and ZIKV infection remained relatively less studied in view of its low case numbers as well as low clinical impact relative to other mosquito-borne flaviviruses. The first large epidemic was reported from Yap Island in 2007 followed by a major outbreak of its related disease, Zika fever, in French Polynesia in 2013 (Duffy et al., 2009; Roth et al., 2014). Since 2015, Brazil has become the epicenter of the current ZIKV epidemic, which has rapidly spread across the Americas and Caribbean (Gatherer and Kohl, 2015). It is now considered as major public health issue with the clusters of severe congenital and neurological anomalies urging WHO to declare a Public Health Emergency of International Concern in 2016 (Sikka et al., 2016). Considering the significant increase in ZIKV threat, specific antiviral therapies and vaccines are urgently needed to control ZIKV-related diseases. The development of therapies for ZIKV requires both insights into the viral life cycle and strategies to identify potent antiviral inhibitors.

Virus reporter systems afford the ability to visualize and monitor viral expression inside the infected host cells (Ren et al., 2016). The development of flavivirus reverse technologies has allowed the construction of infectious full-length cDNA molecules containing reporter luciferase or green fluorescent protein (GFP) gene for both DENV and WNV (Pierson et al., 2005; Zou et al., 2011; Aubry et al., 2015). In the present study, we decided to generate a recombinant ZIKV expressing the reporter GFP gene using the reverse genetic method designed ISA (for Infectious-Subgenomic-Amplicons) (Aubry et al., 2014; De Wispelaere et al., 2015).

2. Results and discussion

To generate a molecular clone of ZIKV derived from Uganda 47 (MR766^{NIID}) strain, the full-length viral genome of 10,807 nucleotides was directly assembled into Vero cells after electroporation of the four overlapping synthetic fragments that encode

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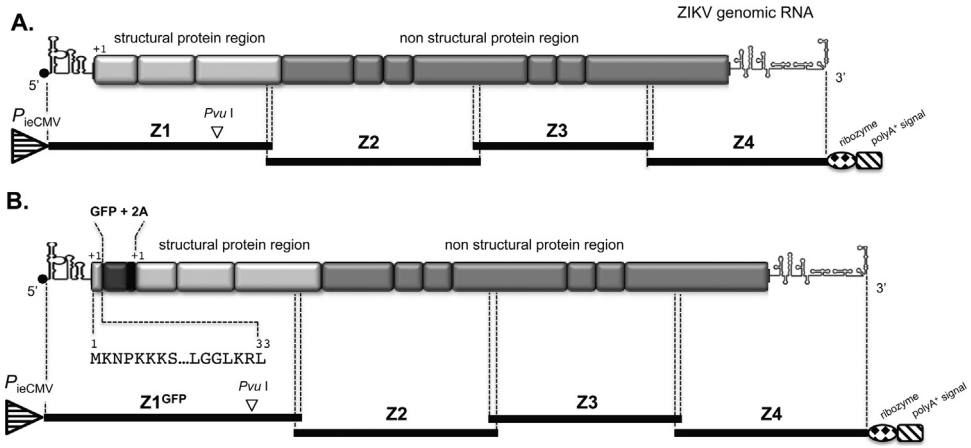

Fig. 1. Schematic representation of the strategy used to construct the molecular clone ZIKV-MR766$^{NIID\text{-}MC}$ and its derived mutant ZIKV$_{GFP}$. Description of overlapping fragments Z1 (or Z1^{GFP}) to Z4 covering the full-length viral genomic RNA. The position of the unique *Pvu* I cleavage site located in the viral E gene is indicated. *In* (A), Z1 contains the CMV promoter upstream of the viral 5' NTR followed by the structural protein region. Z4 contains a ribozyme followed by a polyadenylation signal downstream of the viral 3' NTR. *In* (B), the Z1^{GFP} derived from Z1 contains the eGFP gene fused in frame to the first 33 amino acids of the ZIKV C protein. The 2A protease is immediately adjacent to the reporter eGFP gene. The eGFP-2A cassette is juxtaposed to the viral RNA sequence coding for the structural proteins starting with the first amino acid of C (+1) followed by the nonstructural proteins and ended by the 3′NTR.

the different parts of genomic RNA, according to the ISA method. The cDNA sequences have been previously amplified by PCR from plasmids in which synthetic fragments, quoted Z1 to Z4, were inserted (Fig. 1). Primers were designed so that the Z1 to Z4 fragments shared a sequence homology of 41 (Z1 and Z2), 39 (Z2 and Z3), and 34 (Z3 and Z4) nucleotides. The Z1 fragment contains the CMV promoter, which allows transcriptional initiation, immediately adjacent of the 5' non-translated region (NTR) followed by the structural protein region of MR766^{NIID} (Fig. 1A). A silent mutation at positions 1145–1150 located in the E gene that creates the unique restriction site *Pvu* I was introduced into the Z1 fragment. The Z4 fragment contains the hepatitis delta virus ribozyme followed by an SV40 poly(A) signal, which are juxtaposed to ZIKV 3′ NTR (Fig. 1A). Five days post-electroporation, cell culture supernatant was collected and used to infect Vero cells in a first round of amplification (P1). Molecular clone of ZIKV-MR766^{NIID} (ZIKV-MR766$^{NIID\text{-}MC}$) was recovered 6 days later and amplified another 3 days on fresh Vero cells in order to grow final virus stocks P2 for further studies. A PCR fragment spanning the engineered *Pvu* I site into the E gene was amplified using RT-PCR from viral RNA (vRNA) extracted from ZIKV-MR766$^{NIID\text{-}MC}$. A similar PCR fragment derived from ZIKV strain PF-2013–18, which should be not cleavable by *Pvu* I, served as a negative control (Frumence et al., 2016). As shown in Fig. 2A, the RT-PCR product obtained from ZIKV-MR766$^{NIID\text{-}}$MC, but not ZIKV strain PF-2013-18, was readily cleaved by *Pvu* I indicating that it is a suitable genetic marker for the molecular clone of ZIKV strain MR-766. The infectivity of ZIKV-MR766$^{NIID\text{-}MC}$ was determined by using a standard plaque-forming assay on Vero cells (Frumence et al., 2016). We noted that a majority of virus plaques was of large size (Fig. 2B). The P2 stock of ZIKV-MR766$^{NIID\text{-}MC}$ grown on Vero cells reached an infectious titre up to 7.7 log PFU mL^{-1}. Viral growth was next studied on Vero cells infected at multiplicity of infection (MOI) of 1 (Fig. 2C). Virus progeny production increased by 1 log between 24 h and 48 h post-infection (p.i.) to reach almost 8 log PFU.mL^{-1} showing that the replication of molecular clone ZIKV-MR766$^{NIID\text{-}MC}$ was greatly efficient in Vero cells.

We decided to produce a GFP-expressing ZIKV derived from molecular clone ZIKV-MR766^{NIID} (Fig. 1B). We used a strategy in which a reporter protein is expressed as an additional part of the structural protein region of flavivirus and then excised from the viral polyprotein by the viral 2A protease factor (Zou et al., 2011; Zhang et al., 2016). To generate a viable reporter ZIKV, the plasmid pZ1^{GFP} was derived from pZ1 in which the eGFP reporter gene followed by the sequence encoding the protease 2A factor were fused in frame with the amino acids C-1 to C-33 of ZIKV (Fig. 1B) (Zhang et al., 2016). The N-terminal region of the C protein from ZIKV is thought to comprise a potential circularization sequence that is essential for flavivirus RNA replication (Clyde et al., 2008). In the pZ1^{GFP}, the complete coding region of ZIKV-MR766$^{NIID\text{-}MC}$ was immediately adjacent to the C-terminus of 2A protease that generates the authentic N-terminus of the C protein.

To generate the GFP-expressing mutant of ZIKV-MR766$^{NIID\text{-}MC}$ named ZIKV$_{GFP}$, the recombinant viral genome of 11,587 nucleotides was directly assembled in Vero cells after electroporation of fragments Z1^{GFP}, Z2, Z3, and Z4. The production of P1 and P2 stocks of ZIKV$_{GFP}$ was made as previously. The PCR fragment spanning the *Pvu* I site into the E gene of ZIKV$_{GFP}$ was readily cleaved by *Pvu* I showing that GFP-expressing mutant of ZIKV-MR766$^{NIID\text{-}MC}$ retained the genetic marker (Fig. 2A). We found that the infectious titre of ZIKV$_{GFP}$ stock P2 (6 log PFU mL^{-1}) was 1.5 log lower as compared to ZIKV-MR766$^{NIID\text{-}MC}$. The plaque morphology of ZIKV$_{GFP}$ was different to that of the parent with a phenotype of small plaques (Fig. 2B). We noted that infection of Vero cells with the P2 of ZIKV$_{GFP}$ or ZIKV-MR766$^{NIID\text{-}MC}$ at the MOI of 1 resulted in similar virus progeny production at 24 h and 48 h p.i. (Fig. 2C). A rapid generation of wild-type variants could explain the high viral titers of ZIKV$_{GFP}$ observed at P3. The issue regarding the genetic stability of the GFP-expressing mutant of ZIKV-MR766$^{NIID\text{-}MC}$ is addressed later in our study.

The expression of GFP in Vero cells infected 48 h with ZIKV$_{GFP}$ at the MOI of 1 was first analyzed by immunofluorescence (IF) assays (Fig. 2D). Infected cells were treated with a low concentration of Triton X-100 (0.01%) leading to a permeabilization of the plasma membrane alone and then incubated with anti-flavivirus E mAb 4G2 (Mertens et al., 2010). There was a marked immunostaining of the E protein in the vicinity of the surface of ZIKV-infected cells (Fig. 2D, ZIKV E). The GFP expression was clearly visualized in Vero cells positive for the ZIKV E protein (Fig. 2D). To study the subcellular distribution of GFP, cells were treated with 0.1% Triton X-100 and then incubated with a specific antibody directed against the ER-resident protein calnexin (CNX). At 48 h p.i., the GFP signal was mainly visualized within a distinct, large dot-like structure in the vicinity of the ER compartment (Fig. 2D, CNX). In the context of viral polyprotein expressed by ZIKV$_{GFP}$, the 2A protease ensures the release of the adjacent fusion

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