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Development of a novel DNA-launched dengue virus type 2 infectious clone assembled in a bacterial artificial chromosome

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ABSTRACT

Major progress in Dengue virus (DENV) biology has resulted from the use of infectious clones obtained through reverse genetics. The construction of these clones is commonly based on high- or low-copy number plasmids, yeast artificial chromosomes, yeast-*Escherichia coli* shuttle vectors, and bacterial artificial chromosomes (BACs). Prokaryotic promoters have consistently been used for the transcription of these clones. The goal of this study was to develop a novel DENV infectious clone in a BAC under the control of the cytomegalovirus immediate-early promoter and to generate a virus with the fusion envelope-green fluorescent protein in an attempt to track virus infection. The transfection of Vero cells with a plasmid encoding the DENV infectious clone facilitated the recovery of infectious particles that increased in titer after serial passages in C6/36 cells. The plaque size and syncytia phenotypes of the recombinant virus were similar to those of the parental virus. Despite the observation of autonomous replication and the detection of low levels of viral genome after two passages, the insertion of green fluorescent protein and Renilla luciferase reporter genes negatively impacted virus rescue. To the best of our knowledge, this is the first study using a DENV infectious clone under the control of the cytomegalovirus promoter to facilitate the recovery of recombinant viruses without the need for *in vitro* transcription. This novel molecular clone will be useful for establishing the molecular basis of replication, assembly, and pathogenesis, evaluating potential antiviral drugs, and the development of vaccine candidates for attenuated recombinant viruses.

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1. Introduction

Dengue has become the main arthropod-borne viral disease in several tropical and subtropical countries, causing more than 50–100 million cases and 500,000 hospitalizations annually around the world (Guzman et al., 2010). Disease manifestations cover a wide spectrum ranging from dengue with or without warning signs to severe dengue, in which plasma leakage, hemorrhage and organ impairment can lead to death (TDR/WHO, 2009). The disease is caused by one of four *Dengue virus* (DENV) serotypes (DENV-1 to -4), which are enveloped, single-stranded and positive-sense RNA viruses belonging to the *Flavivirus* genus within the *Flaviviridae* family (Lindenbach et al., 2007). These viruses possess a short genome of approximately 10.7 kb with a type 1 cap structure ($m^7GpppAmpN_2$) at the 5′end, two highly structured untranslated regions (UTRs) at the 5′ and 3′ ends, a long open reading frame (ORF) encoding a single viral polyprotein and lacking a polyadenylate tail at the 3′ end. After proteolytic processing, three structural and seven non-structural proteins are generated from the viral polyprotein (Bartenschlager and Miller, 2008).

Several *cis*-acting elements at the 5′UTR [stem-loops SLA and SLB, upstream the AUG region (5′UAR)], at the capsid-coding region [downstream AUG region (5′DAR), capsid-hairpin (cHP), 5′ cyclization sequence (5′CS), downstream 5′CS (dCS), CCR1] and at the 3′UTR [highly variable region (HVR), semi-variable region (SVR), pseudoknots (PK2 and PK1), 3′CS, 3′DAR, 3′UAR and 3′ stem-loop (3′SL)] are critical for virus replication, translation or assembly (Friebe and Harris, 2010; Friebe et al., 2012; Groat-Carmona et al., 2012; Paranjape and Harris, 2010).

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The role of the DENV proteins and the UTRs of the RNA genome in viral genome replication (Lindenbach et al., 2007), post-translational cleavage (Falgout et al., 1991), virion morphogenesis [reviewed in (Murray et al., 2008)], regulation of the host immune response (Munoz-Jordan et al., 2003, 2005) and virus entry into susceptible cells through receptor-mediated endocytosis (Crill and Roehrig, 2001; Hsieh et al., 2011) and membrane fusion for the release of virions into the cytoplasm (Modis et al., 2004) have been partially clarified.

Although there is not an effective and available antiviral therapy or vaccine and many aspects of the DENV biology remain unclear, the major progress during the last two decades in the study of this virus, particularly in identifying *cis*-acting elements regulating translation and replication, has resulted from the use of infectious clones and replicons obtained through reverse genetics (Paranjape and Harris, 2010). This technology has also been important in identifying and evaluating adaptive mutations and virulence determinants, due to the possibility of generating mutations in specific viral genes or RNA elements, whose effect on viral RNA replication and pathogenesis can be assessed *in vitro* and *in vivo*, respectively (Clyde et al., 2008; Filomatori et al., 2011; Friebe and Harris, 2010; Grant et al., 2011; Iglesias et al., 2011; Leardkamolkarn et al., 2012; Lodeiro et al., 2009; Yu et al., 2008). Infectious clones and derived replicons lacking structural components necessary for virus assembly and propagation have also shown potential for the development and characterization of live-attenuated viruses as vaccine candidates (Huang et al., 2003; Kelly et al., 2010; Zhu et al., 2007), genetic vaccines (Pang et al., 2001a) and high-throughput platforms for screening of chemical antiviral compounds (Chao et al., 2012; Hsu et al., 2012; Leardkamolkarn and Sirigulpanit, 2012; Qing et al., 2010; Zou et al., 2011) and siRNAs (Ng et al., 2007). In addition, infectious clones with reporter genes fused to some of the structural proteins become a powerful tool in cell biology of viral infection for subcellular localization of viral proteins and for tracking the viral particles during the entry, maturation and exit steps of the vital cycle (Brandenburg and Zhuang, 2007).

Common strategies for the construction of infectious cDNAs from DENV genomes through reverse genetics have been based on standard high-copy and low-copy number plasmids (Gualano et al., 1998; Kinney et al., 1997; Lai et al., 1991; Sriburi et al., 2001). However, the high instability of the full-length clones leading to rearrangements and unsuccessful clone rescue has been a common problem when using some of these backbones (Suzuki et al., 2007). To overcome this problem, yeast artificial chromosomes and yeast-*Escherichia coli* shuttle vectors have been successfully used with higher stability (Kelly et al., 2010, 2011; Polo et al., 1997; Pu et al., 2011; Puri et al., 2000). More recently, DENV-1 and -2 infectious cDNAs have been cloned into bacterial artificial chromosomes (BACs) (de Borba et al., 2012; Pierro et al., 2006; Suzuki et al., 2007), a system facilitating the stable cloning of inserts up to 300 kb, as there are typically only one or two copies of the BAC per cell (Kim et al., 1996; Shizuya et al., 1992). However, these DENV infectious clones have been constructed under the control of the prokaryotic T7 promoter, requiring *in vitro* transcription (Run-off) and subsequent transfection of the synthesized RNAs. One decade ago, the first stable cloning of the transmissible gastroenteritis virus (TGEV) under the transcriptional control of the eukaryotic cytomegalovirus (CMV) immediate-early promoter was demonstrated (Almazan et al., 2000). TGEV, as a member of the coronavirus genus, has one of the largest RNA genomes of approximately 30 kb in length, thus demonstrating the functional stable cloning of that genome into infectious cDNAs (Lai, 2000) and the opportunity for DNA-launched transcription from an eukaryotic promoter, avoiding expensive *in vitro* transcription and the subsequent manipulation of transcribed RNAs for transfection.

In the present study, we developed a new infectious cDNA for DENV-2, stably cloned into the BAC system under the control of the CMV promoter. The generated infectious cDNA was successfully rescued and constitutes a useful tool for improving our knowledge of the role of structural and non-structural proteins during the life cycle of DENV, as well as the role of naturally occurring mutations generated in circulating strains during epidemic spread, because of the possibility of evaluating their direct effect on virus replication, assembly and pathogenesis, through the use of isogenic variants with punctual mutations. Additionally, *EGFP*-bearing clone of DENV-2 was constructed, which expanded our knowledge of the constrictions of the viral genome for the generation of reporter viruses.

2. Materials and methods

2.1. Cells and viruses

The sequence of the DENV type 2 New Guinea C prototype strain (DENV-2 NGC), originally isolated from a clinical case of dengue fever in New Guinea in 1944 (GenBank accession number: EU854293), was used to assemble the infectious clone. African Green Monkey kidney (Vero) cells were obtained from the American Type Culture Collection (ATCC number: CCL-81) and maintained as low passage cultures (up to passage 10) in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Carlsbad, CA, USA), supplemented with 2–10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and incubated at 37 °C, 5% CO_2 atmosphere and 95% relative humidity. C6/36 cells, derived from whole larvae of *Aedes albopictus* (ATCC number: CRL-1660), were cultured in Leibovitz's L-15 medium (Gibco BRL, Carlsbad, CA, USA), supplemented with 2–10% FBS and 20 mg/mL tryptose phosphate broth and incubated at 28 °C, 5% CO_2 atmosphere and 95% relative humidity. A viral stock of DENV-2 NGC M2 was produced by inoculating C6/36 cells at a multiplicity of infection (MOI) of 0.01 plaque-forming units (PFU)/cell. The supernatants were collected at 96 h postinfection (hpi), cleared from cell debris through low-speed centrifugation, aliquoted and stored at −80 °C. For virus titration using a plaque assay, Vero cells were infected with serially diluted virus supernatants, incubated at 37 °C with an overlay containing carboxymethylcellulose, fixed with paraformaldehyde and stained with Crystal Violet at 9 days post-infection (dpi), as previously described (Martinez-Gutierrez et al., 2011). Because of the small plaque size phenotype of the parental DENV-2 NGC M2 strain and the derived infectious clone, the plaques were counted and captured using an inverted light microscope with minimal magnification (4× objective). The ImagePro Plus 3.1 software (Media Cybernetics, Silver Springs, MD, USA) was used to delimitate the plaques, and their diameters were estimated by ruling the images with a scale obtained from the microscope configuration.

2.2. Plasmids and bacterial strains

The plasmids pBeloBAC11 (Kim et al., 1996; Shizuya et al., 1992) and pBAC-TGEV5′-3′ (St-Jean et al., 2006), kindly provided by Dr. Luis Enjuanes (Centro Nacional de Biotecnología CNB-CSIC, Madrid, Spain), were used for assembling the infectious DENV cDNA clone flanked by the cytomegalovirus (CMV) immediate-early promoter, the Hepatitis Delta Virus Ribozyme (HDV-RZ) and the bovine growth hormone (BGH) termination and polyadenylation signal sequences. BAC propagation and manipulation was performed in *E. coli* DH10B (Gibco BRL, Carlsbad, CA, USA) as previously described (Almazan et al., 2008). The enhanced green fluorescence protein (*EGFP*) and synthetic *Renilla* Luciferase (*hRLuc*)

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