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Sub-genomic RNA of defective interfering (D.I.) dengue viral particles is replicated in the same manner as full length genomes

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ABSTRACT

The predicted secondary structure of sub-genomic RNA in dengue virus defective interfering (D.I.) particles from patients, or generated *in vitro*, resembled that of the 3′ and 5′ regions of wild type dengue virus (DENV) genomes. While these structures in the sub-genomic RNA were found to be essential for its replication, their nucleotide sequences were not, so long as any new sequences maintained wild type RNA secondary structure. These observations suggested that these sub-genomic fragments of RNA from dengue viruses were replicated in the same manner as the full length genomes of their wild type, "helper", viruses and that they probably represent the smallest fragments of DENV RNA that can be replicated during a natural infection. While D.I. particles containing sub-genomic RNA are completely parasitic, the relationship between wild type and D.I. DENV may be symbiotic, with the D.I. particles enhancing the transmission of infectious DENV.

Introduction

Dengue viruses (*Flaviridae)* are maintained in cycles of transmission involving, principally, humans and *Aedes* mosquitoes. Infections of humans with dengue viruses (DENV) may be inapparent or they may result in a spectrum of clinical symptoms varying in severity from uncomplicated dengue fever through to dengue shock syndrome (Bhatt et al., 2013). One of the great controversies in the field of dengue research over the past thirty years has centred on the relative roles of the host and the virus in determining the severity of an infection (Rosen, 1977). A wide range of defects in the genomes of DENV have been described (Aaskov et al., 2006; Wang et al., 2002) that might be expected to prevent replication of viruses containing these genomes and some of the defects appeared to be maintained through scores of cycles of transmission in nature (Aaskov et al., 2006) suggesting that their retention may aid virus transmission (Ke et al., 2013). More recently, some extreme forms of defective DENV were identified in sera from patients, and in isolates made from patients using the C6-36 *Aedes albopictus* cell line, which resembled defective interfering (D.I.) viral particles (Li et al., 2011; Barrett and Dimmock, 1986). Genomes from D.I. particles of all DENV serotypes contained large internal deletions while retaining intact 3′ and 5′ regions of the genome. The prevalence of these D.I. particles in patients suggested some biological role, perhaps in aiding virus transmission. Recently, Pesko et al., (2012) recovered West Nile viruses (WNV) that contained genomes with smaller (approximately 2 kb), but nonetheless significant, in-frame deletions, from dead lorikeets, suggesting that large deletions in the genomes of flaviviruses may be relatively common but that they are not being detected with the methodology in use or there has been no concerted effort to find them. WNV with these deletions behaved as D.I. particles *in vitro* and reduced replication and dissemination of wild type WNV in mosquitoes.

The open reading frames (ORF) of flavivirus genomes are flanked by a 5′-untranslated region (5′UTR) containing approximately 100 nucleotides (nt) and by a 3′ UTR consisting of 400–800 nt (reviewed in Lindenbach and Rice, 2003). The 5′- and 3′-termini of flavivirus genomes each form thermodynamically stable stem-loop structures, referred to as the 5′SL and 3′SL, respectively and these regions also contain other secondary structures (SLB, DBS1 and DBS2) and conserved sequences (5′UAR, 5′CS, 3′UAR, 3'CS, CS2, RCS2) that are critical for the production of sense and anti-sense RNA and for translation of the RNA. The complementary nt. sequences in the 5′ and 3′ CS and 5′ and 3′UARs enable the genome to form a panhandle structure that is essential for virus replication (reviewed in Gebhard et al., 2011).

The aim of this study was to determine if the retention of the nucleotide sequences and/or the secondary structures of any or all of these elements in DENV D.I. particles was essential for the replication of the sub-genomic RNA.

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Results and discussion

Effect of reducing the number of nucleotides between the 5′ and 3′ CS in DENV D.I. RNA

The shortest piece of RNA between the 5′ and 3′ CS observed in sub-genomic RNA in naturally occurring DENV 2 D.I.s was 40 nt (Fig. 1A, D2–SG). It was not clear whether this was the minimum required to separate functional elements at the 5′ and 3′ ends of the genome or whether the length of this intervening RNA was determined by the position of nt. sequences that would enable recombination to give rise to the sub-genomic RNA (Li et al., 2011).

Plasmids were constructed coding for the sub-genomic RNA from DENV 2 D.I. particles (Li et al., 2011; pD2-SG) or with the intervening 40 nt. reduced to 20 nt. (pD2-SG-20 nt) or to 3 nt. (pD2-SG-3 nt). Deletion of these nucleotides from between the CS elements had no effect on the predicted secondary structure of the conserved elements of 5′ and 3′ regions of the sub-genomic RNA (5′ SL, 3′ SL, UAR, CS. Fig. 1A) but the loop between the 5′ and 3′ CS became smaller and more ordered.

C6-36 cells were infected with infectious clone-derived DENV 2 prior to transfection with equivalent amounts of each RNA transcribed from the plasmids, pD2-SG, pD2-SG-20 nt and pD2-GS-3 nt. The replication of DENV 2 provided the viral proteins for sub-genomic RNA transcription and packaging. While the amounts of intracellular sub-genomic RNA generated in cells transfected with the various sub-genomic RNAs were similar 6 days post-transfection (Fig. 1B), there were noticeable differences in the amounts of each type of sub-genomic RNA in the culture supernatant at this time.

Culture supernatant from these three cultures were collected 6 days post-transfection and used to infect fresh C6-36 cells. Six days post-infection similar, intracellular, levels of the three sub-genomic RNAs, were detected again but the amounts of extracellular sub-genomic RNA appeared to decrease as the number of nucleotides between the 5′ and 3′ CS decreased. These experiments indicated that wild type DENV 2 was able to replicate subgenomic RNA with different numbers of nucleotides between 5′ and 3′ CS but that shortening the sub-genomic RNA, or the sequence between the 3′ and 5′CS from that in naturally occurring D.I. particles may have interfered with its packaging into DENV capsids prior to egress from host cells. No sub-genomic RNA was detected in cells infected with infectious clone-derived DENV 2 and not transfected.

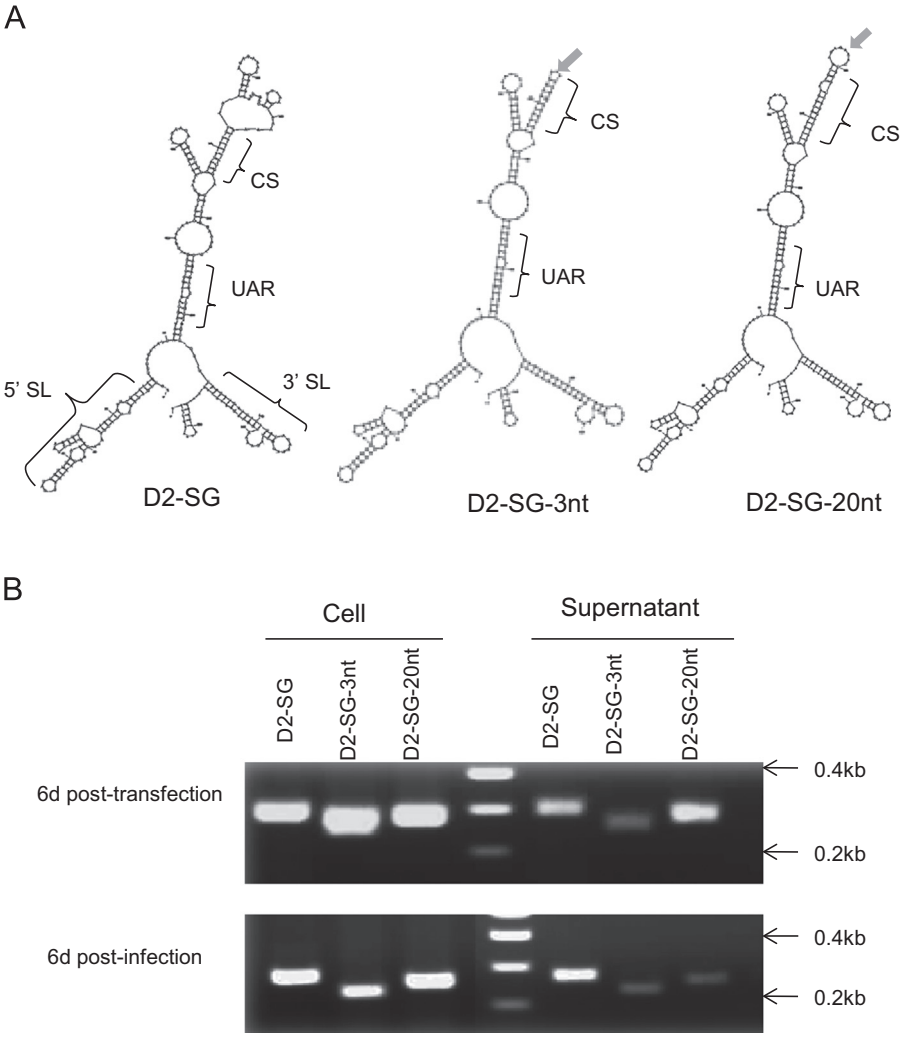

Fig. 1. Influence of the number of nucleotides between the 5′ CS and the 3′ CS on the replication of DENV 2 sub-genomic RNA. (A) Predicted secondary structure of sub-genomic RNA containing 40 intervening nucleotides (D2-SG), 3 intervening nucleotides (D2-SG-3 nt) or 20 intervening nucleotides (D2-SG-20 nt). The sites of the changes are indicated by arrows. (B) cDNA detected after RT-PCR of sub-genomic RNA from DENV 2 infected C6-36 cells, or the corresponding culture supernatant, 6 days after transfection with sub-genomic RNA or 6 days after cells were infected with culture supernatant from the infected-transfected cells.