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Identification and characterization of small sub-genomic RNAs in dengue 1–4 virus-infected cell cultures and tissues

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ABSTRACT

Dengue virus (DV) contains a single-stranded, positive-sense RNA genome, and the 3′ non-coding regions (NCRs) have been demonstrated to play crucial roles in viral RNA replication and translation initiation. In this study, we report the presence of a species of small sub-genomic RNA (sgRNA) derived from the 3′ NCR in DV-infected cell cultures and animal tissues. Further experiments demonstrate that these sgRNAs range from 390 to 430 nt in length and that they accumulate together with viral genomic RNA during replication. Sequence analysis and secondary structure prediction indicate that the stem–loop structures within the 3′ NCR are responsible for the production of DV sgRNAs. Unexpectedly, a unique DV isolate produced three species of sgRNAs, compared to only one in the other isolates. Collectively, the structural and biochemical properties of DV sgRNAs warrant further study of their biological functions during the viral life cycle and pathogenesis.

Introduction

Dengue has emerged as one of the most prevalent arthropod-borne viral diseases in humans, and its incidence worldwide has increased sharply in recent decades [1]. Annually, around 100 million people in the world suffer from dengue fever, including up to 50,000 cases of life-threatening dengue hemorrhagic fever and dengue shock syndrome [2]. Dengue is transmitted by mosquitoes infected with any one of the four serotypes of dengue virus (DV1–DV4). Currently, no vaccines or antiviral drugs against dengue are commercially available, and extensive efforts are underway to understand the life cycle and pathogenesis of DV.

DV belongs to the genus *Flavivirus*, family *Flaviviridae*, and contains a single-stranded, positive-sense RNA genome. The RNA genome is approximately 11 kb in length and is capped by a 5′-type 1 structure (m^7GpppA), but is not polyadenylated at the 3′-terminus. A single, long open reading frame, flanked by the 5′ and 3′ non-coding regions (NCRs), encodes a single polypeptide that is co-translationally processed by cellular and viral proteases, yielding three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The 5′ NCRs of DV are approximately 100 nucleotides in length, and the 3′ NCRs range from 380 to 460 nucleotides in length [3]. Although the detailed mechanism of DV genomic RNA replication remains unclear, it has been well documented that both the 5′ and 3′ NCRs are vital to viral genomic RNA (gRNA) translation and replication [4,5]. Previous reports have revealed that a small species of sub-genomic RNAs (sgRNAs) derived from the 3′ NCR are present in cells infected with mosquito-borne flaviviruses, including Japanese encephalitis virus (JEV) [6] and West Nile virus (WNV) [7]. It has been proposed that these sgRNAs have a marked impact on the fate of infected cells and virulence *in vivo* [8,9].

In this study, we identified and characterized sgRNAs generated by all four serotypes of DV *in vitro* and *in vivo*. Unexpectedly, a unique domestic isolate of the dengue 2 virus produced three species of sgRNAs simultaneously. Considering the structural and biochemical properties of the sgRNAs, potential biological roles can be postulated for these sgRNAs during the DV life cycle, and further investigation is warranted.

Materials and methods

Cells and viruses. Baby hamster kidney (BHK-21), African green monkey kidney (Vero), human lung epithelial carcinoma (A549), and hepatoma cells (HepG-2) were cultured at 37 °C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 50 U/ml penicillin, and 50 μg/ml streptomycin. Mosquito C6/36

Abbreviations: DV, dengue virus; gRNA, genomic RNA; JEV, Japanese encephalitis virus; NCR, non-coding regions; RACE, rapid amplification of cDNA ends; sgRNA, sub-genomic RNA; WNV, West Nile virus.

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cells derived from *Aedes albopictus* were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum at 28 °C with 5% CO_2. Except for the prototype strain NGC (DV2-NGC), all of the DV strains used in study, including DV1 strain 128 (DV1-128), DV2 strain 43 (DV2-43), DV2 strain FJ10 (DV2-FJ10), DV2 strain FJ14 (DV2-FJ14), DV3 strain 80 (DV3-80), and DV4 strain B5 (DV4-B5), were isolated from mainland China and kept in our laboratory.

Virus infection and viron purification. Virus stocks were titered using a standard plaque forming assay on BHK-21 cells as previously described [10]. Culture cells were seeded in six-well plates, infected at the appropriate multiplicities of infection at 37 °C for 1 h, and then refreshed with DMEM or RPMI 1640 medium supplemented with 2% fetal bovine serum. Suckling mice were inoculated in the brain and then sacrificed when neuropathogenesis syndrome manifested 6–8 d.p.i. To harvest dengue virions, culture supernatants were clarified by centrifugation at 3000 rpm for 10 min followed by filtration through a 0.22-μm pore membrane (Millipore). Ultracentrifugation was then performed over a 20% sucrose cushion in phosphate buffer saline (PBS, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4) at 35,000 rpm (SW41 rotor, Beckman) at 10 °C for 2 h. The pellets were resuspended in PBS and subjected to RNA extraction.

Preparation of DIG-labeled RNA probes. Probes were designed to target the last 130 nt of the 3′ NCR, which is highly conserved among the four known serotypes of DV. Probe cDNA was amplified by standard RT-PCR from DV-infected cultures. The forward primer is 5′-TCTACAGCATCATTCCAGGCACAG-3′, and the reverse primer containing the T7 promoter (shown in italics) is 5′-*TAATACGACTC ACTATAGG*GAGAACCTGTTGATTCAACAGCAC-3′, located at nucleotide positions 10670–10693 and 10713–10735 (DV1-128), respectively. cDNA templates were purified using a gel extraction kit (Qiagen) and then subjected to *in vitro* transcription using T7 RNA polymerase (NEB). The transcription reaction was incubated in the presence of DIG-labeled rUTP (Roche) at 37 °C for 2 h to synthesize the DIG-labeled RNA probes. The labeled RNA probes were then treated with RNase-free DNase (Promega) and purified using an RNeasy kit (Qiagen). Aliquots were stored at −70 °C until use.

RNA extraction and Northern blotting assay. Total RNAs from the DV-infected cultures and mouse brain tissues were extracted with Trizol reagent (Invitrogen) following the manufacturer's recommendations and were then quantified using a NanoDrop spectrophotometer (NanoDrop). About 5 μg of RNA was subjected to denaturing gel electrophoresis in 1.5% agarose and 2% formaldehyde. RNA was then transferred onto Immobilon-Ny membranes (Millipore) in 20× SSC buffer (3.0 M sodium chloride, 0.3 M sodium citrate, pH 7.0), followed by UV cross-linking for 40 s. Hybridization and visualization were performed by using the DIG Northern Starter Kit with the prepared DIG-labeled probes (Roche) following the manufacturer's instructions.

Rapid amplification of cDNA ends (RACE). Standard 5′ RACE was carried out with a 5′ RACE kit (Roche) according to the manufacturer's protocol. Briefly, small RNAs (<500 nt) were extracted using the miRcute RNA isolation kit (Tiangen) and quantified using a NanoDrop spectrophotometer. A total of 1 μg of RNA was used for the first strand cDNA synthesis with the 3′ NCR reverse primer mentioned above, and the cDNA products had a poly-A tail added using dATP and terminal deoxynucleotidyl transferase. The A-tailed cDNA was then used as a template for PCR (30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by extension at 72 °C for 7 min) with the anchor primer provided with the RACE kit. PCR products of 300–500 bp in size were gel-purified, cloned into a pMD18-T vector (Takara), and subsequently sequenced by the Beijing AuGCT Biotechnology Company.

RNA secondary structure prediction. Genomic sequences of DV were collected from GenBank. Multiple alignment of 3′ NCR sequences was performed using the DNAstar software package with the clustal W method. The secondary structure formed by the DV 3′ NCR was predicted using the Alifold and RNAfold programs in the Vienna RNA package (web server: http://rna.tbi.univie.ac.at/).

Results and discussion

To identify whether sgRNAs derived from the 3′ NCR were present in DV-infected cells, Northern blotting analysis was performed using the corresponding 3′ NCR-specific RNA probes. The results showed that small RNA species were visualized in DV-infected cells using all four serotypes together with the full-length gRNA (Fig. 1A). The lengths of the sgRNAs produced by DV1–DV4 were estimated to be ~500 nt in length. Since no such small RNAs were detected in mock-infected cells, these sgRNAs were hypothesized to be of viral origin. Total RNAs derived from DV-infected mouse brain tissue were subjected to Northern blotting, and similar RNA species were also detected *in vivo* (Fig. 1B).

To further characterize these sgRNAs, the kinetics of the appearance of sgRNA and gRNA in BHK-21 cells were monitored at the indicated times post-infection. The results show that in DV-infected BHK-21 cells, sgRNAs accumulated along with viral gRNA, while the abundance of sgRNA was much greater than that of viral gRNA (Fig. 1C). Furthermore, these sgRNAs were not detected in purified virions (Fig. 1C), indicating that the production of these sgRNAs was associated with viral replication, and no sgRNAs were packaged into mature virions.

Further infection experiments with different cell types showed that these sgRNAs were generated in all of the DV permissive cell types, including those of vertebrate (human, primate, and rodent) and invertebrate (mosquito) origin, although the relative abundances of sgRNA and gRNA were not identical in the distinct cell types. In the case of DV-susceptible BHK-21 and C6/36 cells, the amount of sgRNA was much greater than that of gRNA. In contrast, an inverse pattern was observed in the other cell lines (Fig. 1D). These data indicate the possible involvement of host factors in DV sgRNA biogenesis.

Taking strain-specificity into consideration, the production of sgRNAs by DV2 isolates, including DV2-NGC, DV2-FJ-10, DV2-FJ-14, and DV2-43, was further investigated. As expected, most DV2 isolates produced sgRNAs with similar sizes (designated sgRNA1) (Fig. 2A), but a unique domestic isolate of DV2 (D2-43) generated another two smaller additional sgRNAs (designated sgRNA2 and sgRNA3, respectively). These three species of sgRNA were also observed to occur simultaneously in D2-43 infected mouse brain tissue (Fig. 2B). Additionally, in both cases, the level of sgRNA1 was lower than that of sgRNA2 and sgRNA3. The significance of these sgRNA species is still unknown.

It is apparent that these sgRNA molecules were derived from the DV 3′ UTR and had the same 3′-terminus, so the 5′ initiation position determines the length of these sgRNAs. To identify the origin of these sgRNAs, 5′ RACE and secondary structure prediction were performed. A combination of 5′ RACE results and sequence analysis revealed that the initiation positions of the DV1–DV4 sgRNAs were at 10316, 10295, 10275, and 10263 nt, respectively (Fig. 3). This information combined with the genome lengths of DV1–DV4 allowed us to determine the lengths of the DV1–DV4 sgRNAs, 430, 429, 410, and 390 nt, respectively, which is consistent with the results of the Northern blotting assay (Fig. 1). Secondary structure prediction suggested that a conserved stem–loop (SL) was formed at the initiation sequence of the sgRNAs (Fig. 3). These findings strongly support the critical role of the SL structure during the generation of DV sgRNAs. Pijlman et al. proposed that these SL-like structures act as barriers against cellular exoribonuclease activity against viral gRNA and result in the generation of WNV sgRNA [8].

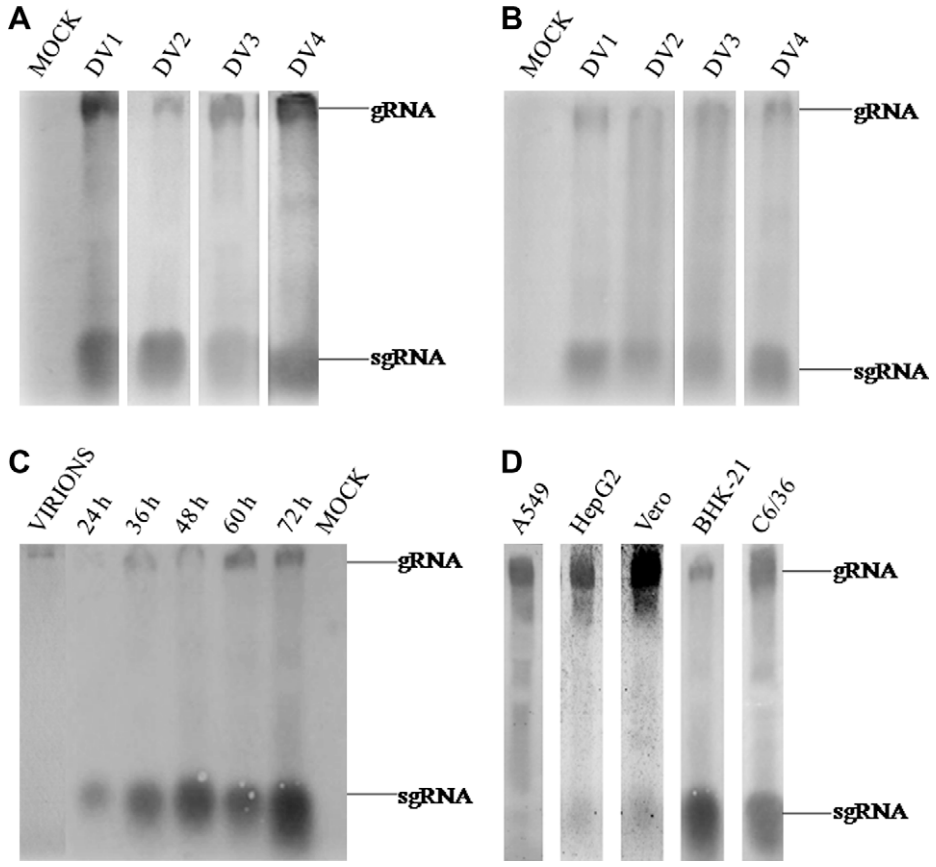

Fig. 1. Identification and characterization of sgRNAs generated by dengue 1–4 viruses *in vitro* and *in vivo*. (A) Both gRNA and sgRNAs were detected in DV1- to DV4-infected BHK-21 cells. The upper and lower blots indicate gRNAs and sgRNAs, respectively. (B) DV sgRNAs were produced in DV-infected mouse brain tissue. (C) Accumulation of gRNAs and sgRNAs in DV-infected BHK-21 cells. (D) DV sgRNAs were detected in all permissive cells.

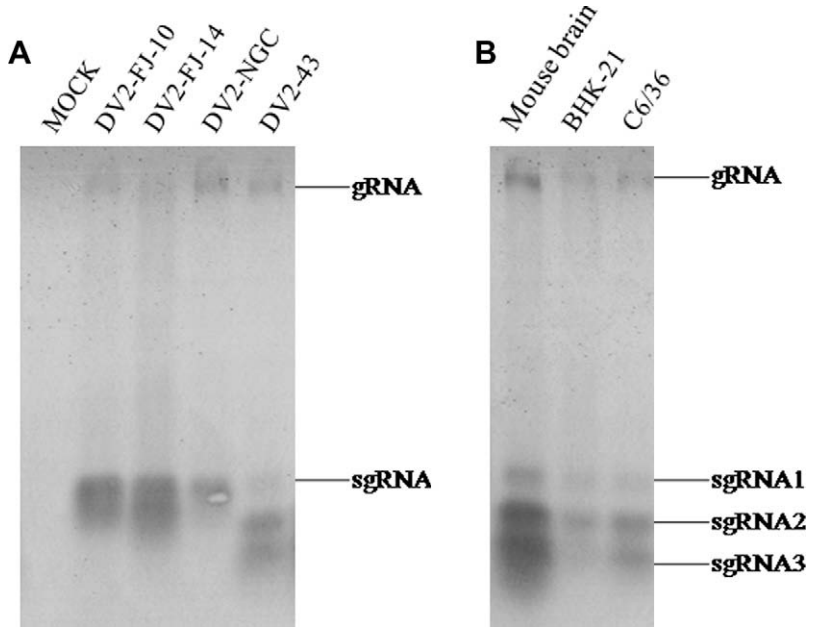

Fig. 2. Three species of sgRNA were generated by DV2-43. (A) Generation of sgRNAs by different DV2 isolates in BHK-21 cells. (B) Three species of sgRNAs were produced in D2-43-infected C6/36 cells, BHK-21 cells, and mouse brain tissue.

As for the three species of sgRNA produced by DV2-43, the results from 5′ RACE demonstrated that the initiation positions of DV2-43 sgRNA1, sgRNA2, and sgRNA3 were at 10295, 10451, and 10542 nt, respectively. The lengths of sgRNA1, sgRNA2, and sgRNA3 were about 429, 270, and 180 nt, respectively. Secondary structure prediction of the 3′ UTR of DV2-43 indicated that there

were SL structures at the initiation positions of DV2-43 sgRNA1, sgRNA2, and sgRNA3 (Fig. 4A). Compared to the other DV2 strains, the SL structure formed by the initiation sequence of DV2-43 sgRNA1 was smaller. Here, we deduced that neither the SL structure at the common initiation position nor the downstream SL could completely prevent degradation by host ribonucleases, which contributed to the rise of sgRNA2 and sgRNA3.

The discovery of DV sgRNAs has important implications. The presence of sgRNA in DV-infected cells will no doubt have an effect on both viral replication and the host response. These sgRNAs derived from the 3′ NCR consisted of several functional RNA elements [10–13], most of which have been shown to be crucial both in viral replication and RNA translation [14–16] through interactions with viral and host proteins [17–19]. A few studies have implied that flavivirus 3′ NCRs could influence either cap-dependent or -independent translation *in vitro* [20,21]. Thus, these free sgRNAs might interfere with the normal function of RNA elements within the 3′ NCRs of DV. Recent reports have also demonstrated that sgRNAs generated by some plant RNA viruses suppress viral RNA translation and progeny RNA synthesis [22,23].

A previous report showed that the production of sgRNA is essential for WNV-induced cytopathicity and pathogenicity [8]. Our study identified a unique DV2 isolate (DV2-43) that produced three species of sgRNAs that are quite different from those of other DV2 isolates. DV2-43 was isolated from a dengue fever patient from Guangxi province, China, in 1987 [10]. The biological properties of DV2-43, including cytopathic effects, neurovirulence in mice, etc., have been well characterized, while no significant difference between DV2-43 and other DV2 isolates has been recorded. To investigate the relationship of sgRNAs and DV biological characteristics, mutational experiments based on an infectious clone of DV2-43 [10] are currently underway in our laboratory.

In conclusion, in this study, we characterized sgRNAs generated by the four serotypes of DV *in vitro* and *in vivo* for the first time, and a unique domestic DV2 isolate was found to produce three species of sgRNAs. The sequence and secondary structure proper-

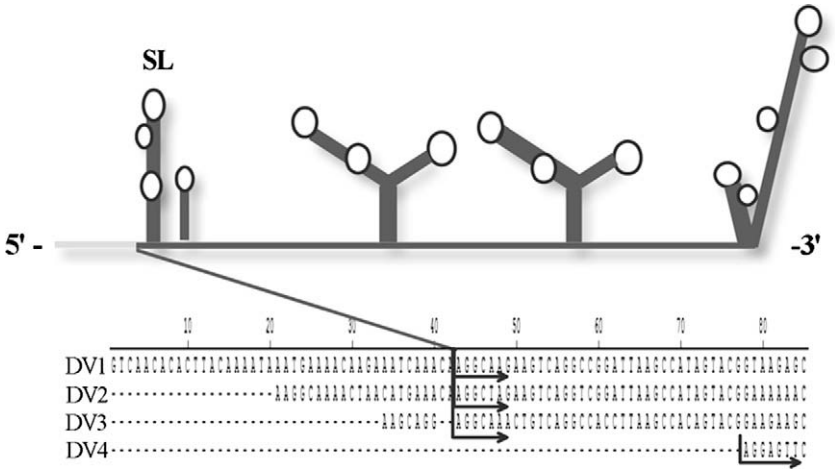

Fig. 3. The 5′ initiation sequence of DV1–DV4 sgRNAs. The arrow indicates the nucleotide position of initiation for sgRNAs generated by DV1–DV4, respectively. RNA secondary structure was predicted using the Alifold program based on the full 3′ NCR of DV1–DV4 (GenBank Accession No. EF032590, AF204178, AF317645, and AF289029). The corresponding sgRNA sequences are shown in deep color.

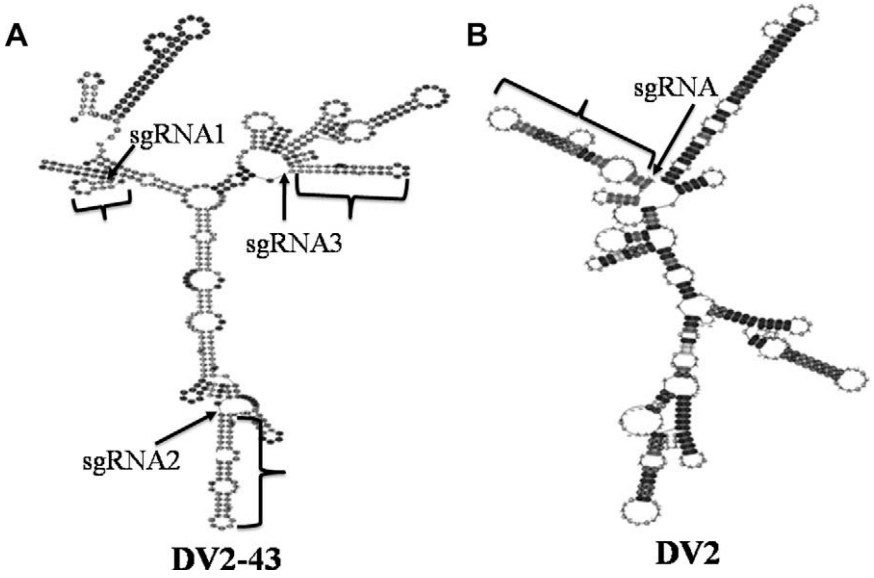

Fig. 4. Comparison of the predicted secondary structure of DV2-43 and other isolates. RNA secondary structure was predicted using the RNAfold program. (A) The arrows indicate the initiation positions of DV2-43 sgRNA1, sgRNA2, and sgRNA3, respectively. (B) The arrow indicates the initiation position of the sgRNA of other DV2 isolates. The SL structure followed by DV2-43 sgRNA1 is smaller than that of the other DV2 strains.

ties of these sgRNAs endow them with possible biological roles during the DV life cycle and pathogenesis. Currently, DV infection poses tremendous social and economic burdens on the world population, and no vaccines or antivirals against DV are commercially available. Further study on these sgRNAs will help uncover the molecular mechanisms of DV replication and contribute to vaccine and antiviral development.

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