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Composition of the sequence downstream of the dengue virus 5′ cyclization sequence (dCS) affects viral RNA replication

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ABSTRACT

RNA replication of dengue virus (DENV) requires an RNA–RNA mediated circularization of the viral genome, which includes at least three sets of complementary RNA sequences on both ends of the genome. The 5′ and the 3′ untranslated regions form several additional RNA elements that are involved in regulation of translation and required for RNA replication. Communication between the genomic termini results in a structural reorganization of the RNA elements, forming a functional RNA panhandle structure. Here we report that the sequence composition downstream of the 5′ CS element in the capsid gene, designated as downstream CS (dCS) sequence – but not the capsid protein – also influences the ability of the viral genome to circularize and hence replicate by modulating the topology of the 5′ end. These results provide insights for the design of reporter sub-genomic and genomic mosquito-borne flavivirus constructs and contribute to the understanding of viral RNA replication.

Introduction

Dengue, the most common mosquito-borne viral disease in humans, is caused by the four serotypes of dengue virus (DENV 1–4), a member of the *Flaviviridae* family (Lindenbach et al., 2007). The small enveloped virus contains a plus-strand RNA genome that encodes the viral polyprotein and serves as a template for genome replication. RNA translation and replication are regulated by RNA elements located in the viral 5′ and 3′ untranslated regions (UTRs) and within the capsid-coding region immediately adjacent to the 5′ UTR (Alvarez et al., 2005a,b, 2006, 2008; Chiu et al., 2005; Clyde and Harris, 2006; Clyde et al., 2008; Filomatori et al., 2006, 2011; Harris et al., 2006; Holden and Harris, 2004; Holden et al., 2006; Lodeiro et al., 2009; Polacek et al., 2009b; Villordo and Gamarnik, 2009; Wei et al., 2009).

Several RNA elements located within the 3′UTR are believed to regulate 5′-cap-dependent translation (Alvarez et al., 2005a; Holden and Harris, 2004; Holden et al., 2006; Manzano et al., 2011; Polacek et al., 2009b; Wei et al., 2009). Besides modulating translation, RNA elements at the viral genomic 3′ end are also essential for RNA replication as well as cytopathogenicity and pathogenicity (Funk et al., 2010; Pijlman et al., 2008; Silva et al., 2010). For initiation of minus-strand synthesis, the 3′SL, the HP-3′SL and three RNA sequences – the 3′ cyclization sequence (CS), the 3′ upstream of AUG region (UAR) element, and the 3′ downstream of AUG region (DAR) – are absolutely necessary (Alvarez et al., 2005b, 2008; Friebe and Harris, 2010; Friebe et al., 2011; Villordo and Gamarnik, 2009). Several studies have shown that these three sequences can interact with their complementary 5′ elements – the 5′ CS, the 5′ UAR, and the 5′ DAR elements – resulting in an RNA–RNA-mediated circularization of the genome and a reorganization of the 3′ RNA structure, a prerequisite for initiation of minus-strand RNA synthesis (Filomatori et al., 2011; Friebe and Harris, 2010; Villordo and Gamarnik, 2009).

The formation of the 5′–3′ panhandle structure brings the 5′ stem loop A (SL-A), which has been demonstrated to bind the viral RNA-dependent RNA polymerase (RdRp) NS5, in close proximity to the structurally-reorganized 3′ end, allowing initiation of minus-strand synthesis (Filomatori et al., 2006, 2011; Friebe and Harris, 2010). While SL-A and the 5′ UAR element are located within the 5′ UTR, the 5′ DAR and 5′ CS elements reside within the capsid-coding region. An additional element within the viral coding region, the capsid-coding region hairpin (cHP), is involved in RNA replication and also directs start codon selection (Clyde and Harris, 2006; Clyde et al., 2008). As the function of the cHP is position-dependent but sequence-independent, it is believed that its role in translation is to stall the scanning initiation complex over the first AUG, favoring its recognition. The exact involvement of cHP in RNA replication is less well understood, but recent work indicates a role in the formation of a functional 5′–3′ panhandle structure, a prerequisite for RNA replication. Although the cHP is not required to initiate basepairing between the viral termini, it must be part of the panhandle structure, where it forms a functional unit with the 5′ UAR, 5′ DAR and 5′ CS elements (Friebe and Harris, 2010). In contrast to SL-A, which is 5′-end-

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dependent, the position of the remaining RNA elements are not absolutely position-dependent, as long as the elements form a single unit in close proximity to the viral 5′ terminus (Friebe and Harris, 2010).

The viability of sub-genomic DENV reporter replicons, containing the 5′ UTR and the first 72 nucleotides (nt) of the capsid-coding region but lacking the remaining open reading frame (ORF) of the structural proteins, demonstrates that all RNA elements essential for RNA replication are located within this region, including the 5′ DAR, cHP and 5′ CS elements (Alvarez et al., 2005a; Clyde et al., 2008). The ability to produce reporter viral particles (RVPs) by packaging the replicon RNA into infectious viral particles further demonstrates that any essential packaging signals must be located in this region, outside of the missing coding region for capsid, prM/M and E (Ansarah-Sobrinho et al., 2008).

For hepatitis C virus (HCV), a closely related virus within the *Flaviviridae* family, additional regulatory RNA elements have been identified within the core-coding region, the protein homologous to DENV capsid protein. An RNA–RNA interaction between nucleotides in the 5′ UTR and the core-coding region was shown to affect viral translation (Kim et al., 2003). Furthermore, two RNA stem-loop structures within the core-coding region contribute to HCV genome translation and replication (Vassilaki et al., 2008). Besides non-essential RNA elements, an impact of the core protein on HCV RNA translation has been reported (Lourenco et al., 2008; Shimoike et al., 2006). Furthermore, the capsid protein of other RNA viruses has also been implicated in modulation of RNA replication (Tzeng et al., 2006). In the case of flaviviruses, one report suggested a role for the capsid protein in either unpackaging and/or early RNA synthesis (Schrauf et al., 2009). However, a study using DENV showed no difference in RNA replication in the presence or absence of wild-type (WT) capsid protein, but expression of a mutated capsid protein with an altered subcellular localization pattern had a negative effect on RNA accumulation (Samsa et al., 2009).

We therefore analyzed the impact of the capsid-coding region – focusing on the RNA sequence and role of the capsid protein – on viral translation and RNA replication, using DENV as a model and West Nile Virus (WNV) to confirm the results with another flavivirus. Our data show that the RNA sequence composition between nucleotide positions 170–200 of the viral RNA, rather than the capsid protein, contributes to RNA replication but has no effect on RNA translation. Furthermore, enhancement of viral RNA replication is not caused by a new RNA element located downstream of the 5′ CS element, designated the dCS sequence, but rather by its sequence composition and its effect on the ability of the 5′ UAR, 5′ DAR and 5′ CS element to interact with their 3′ counterparts. This then modulates the affinity between the viral termini, affecting the ability of the viral genome to circularize, a prerequisite for RNA replication.

Results

The downstream of 5′ CS (dCS) sequence affects RNA replication

To evaluate whether non-essential regulatory RNA elements are embedded within the capsid-coding region, we included as many silent point mutations as possible in the region downstream of the DENV 5′ UTR and the first 72 nts of the capsid gene. This region, spanning nucleotide positions 171–438, was designated as the downstream CS (dCS) sequence, and the mutated sequence was designated as “SYN”. An alignment of “SYN” with the WT sequence is shown in Supplementary Fig. 1. Care was taken to avoid introducing rarely-used codons. The “SYN” sequence was then introduced into a replicon with a similar design as 5′UTR-Cap-tr-SPACER (Friebe and Harris, 2010) but with the “SPACER” sequence replaced by the capsid-coding region. The new replicon backbone was designated biRep-5′UTR-CapFL (Fig. 1A), harboring either the WT or the “SYN” capsid-coding region (resulting in biRep-5′UTR-CapFL-WT or biRep-5′UTR-CapFL-SYN, respectively).

The replication phenotype of the new replicons was tested in BHK and Huh7 cells and compared to pDRrep by transfecting the same amount of RNA of each construct into either BHK or Huh7 cells. Luciferase activity was monitored over a timecourse of 4 h (hours), 24 h, 48 h, 72 h and 96 h. The 4 h value, reflecting translation of the input RNA, served as an indicator of transfection efficiency and was set to 100%, and all later values were normalized to the 4 h value to account for transfection efficiency. Luciferase activity (relative luciferase units, RLU) was comparable among all three replicon RNAs 4 h post-transfection (p.t.) (Supplementary Fig. 2A and data not shown), revealing no difference in EMCV IRES-mediated translation efficiency between biRep-5′UTR-CapFL-WT and -SYN. As a negative control, a replicon with an inactivating mutation (GDD to GVD) in the catalytic site of the NS5 RdRp was used (Friebe and Harris, 2010). Comparison of the replication pattern in BHK cells showed that replicon biRep-5′UTR-CapFL-WT showed ~1.5 logs higher RNA accumulation levels than the mutant biRep-5′UTR-CapFL-SYN as early as 24 h p.t. (Fig. 1B), and the same difference was observed in Huh7 cells at 48 h p.t. (Fig. 1C). In BHK cells, differences at later time points (72 h and 96 h p.t.) disappear, as replication of the WT replicon is limited by the cytopathic effect of viral RNA replication on the host cell, which results in a loss of cells that support replication and hence reduced luciferase activity (data not shown).

To further validate the negative effect on RNA replication of the silent point mutations harbored by biRep-5′UTR-CapFL-SYN, we constructed a full-length reporter virus in which the complete viral open reading frame is under the authentic DENV 5′ cap-dependent translational control (Fig. 1B, IC-5′UTR-Cap228), with a similar design to mDV-R (Samsa et al., 2009). Note that the 5′ end of IC-5′ UTR-Cap228 consists of the 5′ UTR and the first 228 nt of the DENV ORF, which are fused to the *Renilla* luciferase reporter that is cleaved from the C-terminal DENV polyprotein by an engineered FMDV2A cleavage site. The results using the more authentic full-length reporter viruses confirm the observations made with reporter replicons; namely, IC-5′UTR-Cap228-SYN shows delayed RNA accumulation kinetics as compared to IC-5′UTR-Cap228-WT in BHK as well as in Huh7 cells (Figs. 1B and C). For both RNAs, RLUs 4 h p.t. were similar (Supplementary Fig. 2B and data not shown), indicating that the mutations present in the capsid-coding region do not affect overall translation efficiency. Furthermore, both viral RNAs harbor the WT sequence of the capsid-coding region downstream of the *Renilla* luciferase gene, indicating that effects of mutations in IC-5′UTR-Cap228-SYN cannot be compensated by internal positioning of the WT capsid-coding region and arguing for position dependency. It is worth mentioning that both RNAs retained their ability to produce infectious viral particles (data not shown). To simplify the presentation of results, replication kinetics will hereafter be shown by comparison of the luciferase activity 48 h p.t. in Huh7 and 24 h p.t. in BHK cells, normalized to the value measured 4 h p.t., although the full kinetic timecourse was performed in both cell lines for all experiments.

An additional predicted 5′–3′ RNA–RNA interaction and stem-loop structure in the dCS sequence do not moduate RNA replication

Using MPGAfold, a recent publication indicated a potential interaction between nucleotides 193–213 at the DENV-2 5′ end with a complementary sequence within the 3′ UTR (nucleotide positions 10419 to 10438 of the DENV-2 strain 16681 full-length sequence), although the interaction was only predicted as a suboptimal RNA folding structure (Manzano et al., 2011). Additional complementary sequences between the viral termini could assist the 5′–3′ UAR-, DAR- and CS-mediated circularization of the genome and therefore enhance RNA replication while not being absolutely required. The predicted interaction is depicted in Fig. 2A, revealing complementarity between 17 nucleotides in the viral 5′ and 3′ ends. The “SYN” silent