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SCIENCE DIRECT

Virology 339 (2005) 200 – 212

VIROLOGY

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Role of RNA structures present at the 3′UTR of dengue virus on translation, RNA synthesis, and viral replication

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Received 31 March 2005; returned to author for revision 19 April 2005; accepted 2 June 2005
Available online 5 July 2005

Abstract

We have developed a dengue virus replicon system that can be used to discriminate between translation and RNA replication. Using this system, we analyzed the functional role of well-defined RNA elements present at the 3′UTR of dengue virus in mammalian and mosquito cells. Our results show that deletion of individual domains of the 3′UTR did not significantly affect translation of the input RNA but seriously compromised or abolished RNA synthesis. We demonstrated that complementarity between sequences present at the 5′ and 3′ ends of the genome is essential for dengue virus RNA synthesis, while deletion of domains A2 or A3 within the 3′UTR resulted in replicons with decreased RNA amplification. We also characterized the vaccine candidate rDEN2Δ30 in the replicon system and found that viral attenuation is caused by inefficient RNA synthesis. Furthermore, using both the replicon system and recombinant viruses, we identified an RNA region of the 3′UTR that enhances dengue virus replication in BHK cells while is dispensable in mosquito cells.

Keywords: Dengue virus; RNA replication; Translation; 3′untranslated region; Flavivirus; Viral attenuation; Subgenomic replicon

Dengue virus belongs to the *Flaviviridae* family together with other important human pathogens such as yellow fever virus, West Nile virus, and Japanese encephalitis virus. Dengue fever is the most prevalent mosquito-borne viral disease of humans (Gubler, 1998). It is estimated that more than 50 million infections occur annually and 2.5 billion people are at risk of dengue virus infection worldwide (WHO, 2004). Despite the wide morbidity and mortality associated with dengue infections, the molecular biology of this virus is not well understood and at present neither specific antiviral therapies nor a licensed vaccine exist.

Dengue is an enveloped virus with a positive single stranded RNA genome of about 11 kb. The viral RNA encodes one large open reading frame flanked by 5′ and 3′ untranslated regions (UTRs) that are required for viral replication. The 5′ UTR is relatively short (around 100 nucleotides) and has a cap structure at the 5′ end, while the 3′ UTR is longer (around 450 nucleotides), lacks a poly(A) tail, but contains a number of conserved RNA structures (Markoff, 2003). The genomic RNA is directly used as mRNA for protein synthesis. The large viral polyprotein is co- and posttranslationally processed by viral and cellular proteases into three structural proteins, capsid (C), premembrane (prM), and envelope (E); and seven nonstructural proteins (NS) that are primarily involved in replication of the viral RNA (Rice, 2001). The mechanism by which the viral replicase initiates RNA synthesis specifically at the viral 3′UTR is not clearly understood. The RNA replication complex assembles on cellular membranes and involves the viral RNA dependent RNA polymerase-methyl transferase NS5, the helicase-protease NS3, the glycoprotein NS1, the hydrophobic proteins NS2A and NS4A, and presumably host factors (Mackenzie et al., 1998; Westaway et al., 1997, 1999). The nucleotide sequence at the 3′ end of the genome and

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doi:10.1016/j.virol.2005.06.009

the presence of specific RNA structures appear to be essential for dengue and other flavivirus RNA replication (Elghonemy et al., 2005; Khromykh et al., 2003; Tilgner and Shi, 2004; Tilgner et al., 2005; Yu and Markoff, 2005; Zeng et al., 1998).

The 3′ end of the flavivirus genomes folds into a highly conserved stem–loop (3′SL). Detailed analysis of the structure–function of the 3′SL in West Nile virus, Kunjin virus, dengue virus, and yellow fever virus revealed an absolute requirement of this RNA element for viral replication (Brinton et al., 1986; Men et al., 1996; Proutski et al., 1997; Rauscher et al., 1997; Zeng et al., 1998). Upstream of the 3′SL there is another essential RNA element for viral replication, the conserved sequence CS1 (Men et al., 1996). This element contains the cyclization sequence CS that is complementary to a sequence present at the 5′ end of the genome (Hahn et al., 1987). 5′–3′ long-range RNA–RNA interactions have been proposed to be necessary for RNA replication in West Nile virus, Kunjin virus, and dengue virus (Alvarez et al., 2005; Khromykh et al., 2001; Lo et al., 2003). In addition, using recombinant dengue virus NS5 polymerase, it has been demonstrated that in vitro RNA synthesis requires sequences present at the 5′ and 3′ends of the genome (You and Padmanabhan, 1999; You et al., 2001). While the 3′SL has been extensively studied, the function of the other RNA structures and conserved motifs present within dengue virus 3′UTR remain elusive. Folding algorithms predict two almost identical structures designed A2 and A3 preceding the 3′SL. These structures contain the highly conserved sequence CS2 and the repeated CS2 (RCS2), within A3 and A2, respectively (Shurtleff et al., 2001). CS2 and RCS2 sequences are found in Japanese encephalitis, West Nile, Murray Valley encephalitis, and dengue virus types 1 to 4 (for review, Markoff, 2003). Furthermore, between the stop codon of the viral polyprotein and domain A2 resides a variable region (VR), which displays large heterogeneity in length and nucleotide sequence among different dengue virus isolates (Shurtleff et al., 2001).

Even though previous studies reported that deletions within the 3′UTR yielded seriously impaired dengue viruses (Men et al., 1996), the role of each of these RNA elements during translation and RNA synthesis has not been analyzed due to the lack of an amenable genetic system. We have developed a dengue virus replicon that allows discrimination between viral translation and RNA synthesis. Using this replicon, we performed a systematic deletion analysis of each RNA structural element of the 3′UTR on the viral processes. We found that deletion of RNA elements at the 3′UTR greatly decreased viral RNA synthesis without compromising translation initiation. In addition, using the replicon system and recombinant dengue viruses, we identified an RNA element of the 3′UTR that differentially modulates viral replication in mosquito and mammalian cells.

Results

Construction and characterization of a subgenomic dengue virus replicon

A dengue virus replicon system has been previously described (Pang et al., 2001). This replicon allows detection of viral proteins by immunofluorescence and RNA replication in transfected cells. However, this system lacks a sensitive reporter amenable to discriminate translation of input RNA and RNA replication. In an attempt to overcome this limitation, we developed a new replicon system carrying a sensitive reporter. In the context of dengue virus 2 16681 cDNA clone (Kinney et al., 1997), we introduced the firefly luciferase (Luc) coding sequence replacing the structural proteins (Fig. 1A). The *trans* membrane domain (TM) corresponding to the C-terminal 24 amino acids of E was retained in order to maintain the topology of the viral protein NS1 inside of the ER compartment. The Luc was fused in-frame to the first 102 nucleotides of the capsid protein (C), which contain the *cis*-acting element of 11 nucleotides complementary to the 3′CS sequence (Alvarez et al., 2005; Hahn et al., 1987; Khromykh et al., 2001; You et al., 2001). A similar replicon system has been recently developed for West Nile virus (Lo et al., 2003). To ensure proper release of the Luc from the viral polyprotein, we designed three alternative constructs carrying different protease cleavage sites between the C-terminus of luciferase and the beginning of the TM domain of E (Fig. 1A). Two of these constructs contain recognition sites for NS3 protease, corresponding to the C-prM and the NS4B-NS5 junctions (DVRepCprM and DVRep4B-5, respectively). The third construct contains the *cis*-acting FMDV 2A protease (DVRep) (Ryan and Drew, 1994).

Transfection into BHK cells of the replicon RNAs carrying the NS3 recognition sites yielded low levels of Luc activity and no amplification of the RNA was detected, likely due to the slow processing by the NS3 protein (data not shown). In contrast, the processing by FMDV 2A was fast and Luc activity was readily detected few hours after transfection. To examine whether the DVRep was capable to autonomously replicate in cells, we transfected the RNA into BHK cells and assayed for Luc activity as a function of time. The levels of Luc activity peaked between 8 and 10 h after transfection. Around 20 h, the Luc signal dropped but rebounded exponentially after 30 h (Fig. 1B). To confirm that the observed time increase in Luc signal was the result of replicon RNA amplification by the viral replicase activity, a replication defective RNA was designed. We replaced the essential GDD motif of the RNA dependent RNA polymerase NS5 by AAA (DVRepNS5Mut). Similar mutations of the GDD motif have been shown to have a lethal effect on flavivirus replication (Khromykh et al., 1998). We transfected BHK cells with equal amounts of RNA of DVRep WT and NS5Mut and monitored Luc activity. During the first 20 h, the Luc signal obtained from cells transfected