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The structural and phylogenetic profile of the 3′ terminus of coxsackievirus B3 negative strand

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ABSTRACT

In the replication process of RNA(+) viruses both the positive-strand template and the newly synthesized negative strand appear in a double-stranded form, RF. It has been shown for poliovirus that prior to the initiation of positive-strand synthesis, the 5′-terminus of the positive strand must adopt a cloverleaf structure. When that happens, the 3′-terminal region of the negative strand is released from the RF form and is able to form into its own defined structure. In order to determine the secondary structure of this region, a comprehensive approach consisting of experimental mapping methods, phylogenetic analysis and computer predictions was applied. Here we propose the first structural model of the 3′-terminal region of the coxsackievirus B3 (CV-B3) negative strand, approximately 450 nucleotides in length. The region folds into three highly defined structural domains, I′–III′. The most 3′-terminal part of this region is domain I′, which folds into a cloverleaf structure similar to that found in the viral RNA strand of positive-polarity. Remarkably, this motif is conserved among all analyzed viral isolates of CV-B3 despite the observed sequence diversity. Several other conserved structural motifs within the 3′-terminal region of the viral negative strand were also identified. The structure of this region may be crucial for the replication complex assembly.

1. Introduction

Coxsackievirus B3 (CV-B3) is a heart pathogen against which no specific treatment has been approved to date. As a member of *Picornaviridae*, genus *Enterovirus*, the genome of CV-B3 is a single-stranded RNA molecule of positive-polarity that serves as a messenger RNA after the entrance to the host cell cytoplasm. It encodes one large open reading frame for viral polyprotein flanked by untranslated regions (UTRs) (Oberste, 2008; Racaniello, 2007) (Fig. 1). Both UTRs contain RNA motifs conserved in sequence and structure, the so called "*ori*" – elements (*origins of replication*), which are indispensable for the replication process (Liu et al., 2009; Ogram and Flanegan, 2011; Steil and Barton, 2009).

Replication occurs in membranous vesicles created during infection in the cytoplasm. The two major steps of replication are the synthesis of a complementary, negative RNA strand and, then, the synthesis of several genomic positive RNA strands, simultaneously on each negative template. The negative RNA strand can be found in host cytoplasm in two forms: RF, a replicative form and RI, a replicative intermediate. The RF is believed to be a fully double-stranded hybrid of positive and negative strands, whereas in RI the negative strand is partially single-stranded and partially hybridized with several (one to six) positive strands being newly synthesized (Sean and Semler, 2008; Steil and Barton, 2009).

It has been indicated for another enterovirus, poliovirus type 1 (PV-1) that prior to the initiation of synthesis of the new genomes, the cloverleaf structure at the 5′ end of the positive strand in the RF form must be formed (Vogt and Andino, 2010). Likely, a very similar situation occurs for coxsackievirus B3 since the importance of its cloverleaf subdomain "d" structure in positive-strand synthesis initiation has been shown (Sharma et al., 2009). Thus, the 3′-terminal region of the negative strand is released from the heteroduplex and can fold into its own higher order structure. Since the synthesis of the positive strand is supposed to be initiated at the 3′ end of the negative strand, possible higher order structures present near this end are expected to play an important role in this process. Due to the GC-rich sequence, this region is very likely to fold into defined structural elements. For poliovirus type 1, a cloverleaf structure has been proposed to be present at the 3′ end of the negative strand (Andino et al., 1990; Banerjee and Dasgupta,

Abbreviations: CV-B3, coxsackievirus B3; UTR, untranslated region; DMS, dimethyl sulfate; PV-1, poliovirus type 1; HCV, hepatitis C virus.

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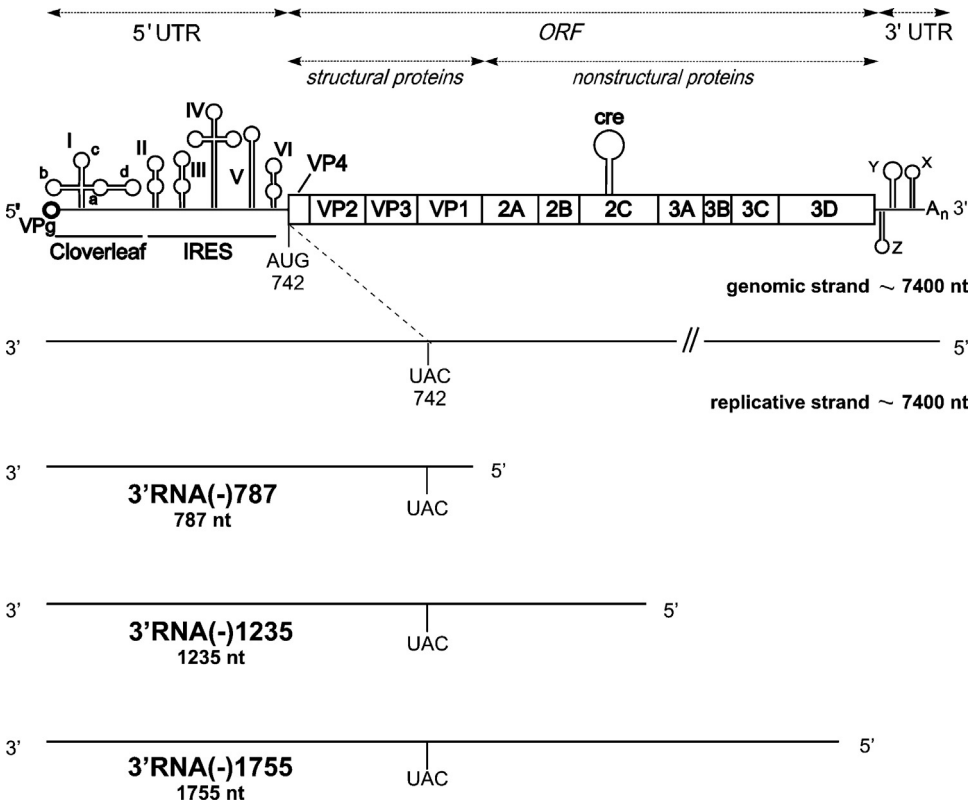

Fig. 1. Schematic organization of CV-B3 genome with functional RNA motifs indicated. The negative strand and three RNA sub-fragments investigated in this study are shown. UTR – untranslated region; IRES – internal ribosome entry site; AUG–translation start codon; VP4, VP2, VP1, 2A, 2B, 2C, 3A, 3B, 3C, 3D – regions encoding particular viral proteins; cre – cis-acting replication element; VPg – covalently linked peptide; An – poly(A) tail.

2001a,b; Pilipenko et al., 1992; Roehl and Semler, 1995). Two host proteins, p36 and p38, as well as two viral peptides 2C and 2BC have been shown to interact with this 3′-terminal region of the negative strand (Banerjee et al., 1997; Banerjee and Dasgupta, 2001a,b; Roehl et al., 1997; Roehl and Semler, 1995). The p36 has been identified as the hnRNP C and it could play multiple roles in the positive-strand synthesis (Brunner et al., 2005; Ertel et al., 2010; Li and Nagy, 2011).

For another RNA plus virus, the hepatitis C virus (HCV), a secondary structure model of the 3′-terminal region of negative strand has been proposed, approximately 340-nucleotide-long (Dutkiewicz et al., 2008; Schuster et al., 2002; Smith et al., 2002). Binding sites for helicase and viral polymerase have been mapped within that region. Interestingly, the strongest binding site for polymerase was quite remote from the very 3′ end of the RNA molecule (Astier-Gin et al., 2005; Mahias et al., 2010).

We decided to elucidate the structure of the 3′-terminal region of CV-B3 negative strand which, to the best of our knowledge, has not been investigated yet. A complex analysis of the RNA secondary structure was performed using experimental mapping methods with dimethyl sulfate (DMS) and Pb^{2+} ions, phylogenetic analysis and computer structure prediction methods. We found RNA structural elements which, as it was shown for related viruses, may be essential for the replication process.

2. Materials and methods

2.1. Materials

The materials used in this study were from the following sources: (γ-^{32}P)ATP (4600 Ci/mmol) from Hartmann Analitic (Braunschweig, Germany) and all the chemicals from Sigma–Aldrich (St. Louis, Missouri, USA) or Serva Electrophoresis (Heidelberg, Germany). Enzymes: Taq polymerase, T4 polynucleotide kinase, RevertAid^{TM} M-MuLV reverse transcriptase, RNase H, T4 RNA ligase and T1 ribonuclease were purchased from Fermentas UAB (Vilnius, Lithuania).

2.2. DNA templates and RNA synthesis

All oligodeoxyribonucleotides used in the construction of DNA templates (Supplementary Table 1) were deprotected after synthesis and purified on 8% polyacrylamide gels.

In order to obtain the dsDNA templates (including nucleotides 1–1735, 1–1255 and 1–787 under T7 promoter), single-stranded DNA complementary to the 5′ portion of CV-B3 genome was synthesized by RT-PCR. To this end the MMLV-reverse transcriptase and primers Rev1786 or Rev1735 were used. The total RNA from HeLa cells infected with the “Nancy” strain of CV-B3 (ATCC VR-30) served as a template. Virus titer: 1.3×10^8 PFU/ml was used. RNA extraction was performed using the TRI-reagent, 5 h post-infection, and approx. 100 μg of total RNA was harvested. The cDNAs were kindly provided by Prof. Heinz Zeichhardt (CHARITE, Berlin).

During the next steps cDNAs were amplified by PCR involving specific primers. Synthesis of dsDNA templates for 3′RNA(−)786 and 3′RNA(−)1255 were carried out in two steps. In the first step, dsDNAs encoding the desired RNA sequence were obtained, to which the T7 promoter was attached in the next step. The synthesis of the dsDNA template for 3′RNA(−)1735 was carried out in one step. As a result, dsDNA1735, dsDNA1255 and dsDNA787 were generated.

The reverse transcription and PCR reactions were performed according to standard protocols. The reaction products were purified by phenol/chloroform (1:1) extraction, precipitated with ethanol, and the obtained dsDNA templates were dissolved in TE buffer.