Structure and function of RNA elements present in enteroviral genomes

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Enteroviruses are small RNA(+) viruses that encode one open reading frame flanked by two extensive noncoding regions carrying structural RNA regulatory elements that control replication and translation processes. For a long time the central, coding region was thought to remain single-stranded and its only function was supposed to be as the template for polyprotein synthesis. It turned out, however, that the protein coding region also encodes important RNA structures crucial for the viral life cycle and virus persistence in the host cells. This review considers the RNA structures in enteroviral genomes identified and characterized to date.

Key words: Enterovirus, poliovirus, coxsackievirus B3, RNA structure, structural RNA element, cis-acting element

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INTRODUCTION

The Enterovirus genus belongs to the Picornaviridae family, which includes two small non-enveloped RNA(+) viruses with an icosahedral capsid (Racaniello, 2007; Muehlenbachs et al., 2015). Based on molecular and serological analyses, 12 species of enteroviruses have been identified: Enterovirus A-J and Rhinovirus A-C.

Another enterovirus which is often chosen as a model for molecular research is the heart pathogen coxsackievirus B3 (CVB3), a member of Enterovirus B species, in part because of its similarities with PV in terms of structure and life cycle, while at the same time being much safer to work with.

The viral life cycle lasts 5 to 10 hours, depending on the serotype. Initially, the virus recognizes a specific receptor located on the surface of a host cell and is internalized. Low pH in the endosomes as well as virus' interaction with co-receptors lead to the release of the viral genetic material. In contrast to DNA viruses, RNA(+) viruses do not move into the nucleus and viral RNA can be used immediately as a template for translation machinery to begin production of the viral proteins. When virus proteins reach a high enough concentration, the genomic RNA strand is used as a template for replication. Newly synthesized RNAs can go into the next round of replication or they can be packed into virions and released by cell lysis (Garmaroudi et al., 2015).

The viral genome is the most important element in initiation of the host inflammation. This genome is a single-stranded plus RNA strand which is approximately 7400 nt in length, with about 6500 nt-long open reading frame encoding a single viral polyprotein (Fig. 1). The 5' end of the viral genome is bound by the VPg protein, whereas at the very 3' end a poly(A) tail is present (Semler, 2004; Racaniello, 2007).

Untranslated regions (UTR), flanking the open reading frame, comprise around 10% of the entire viral genome. They contain RNA structural elements which play regulatory functions in the viral life cycle. The 5' UTR contains an IRES element (internal ribosome entry site) that is responsible for initiation of cap-independent translation and a cloverleaf structure indispensable for replication. The structurally ordered 3' UTR is involved in replication initiation and viral circularization as well (Semler, 2004; Racaniello, 2007; Liu et al., 2009).

The coding region was originally thought to be single-stranded and its only function to be a template for polyprotein synthesis. As structural RNA features became better understood, it became apparent that they are present in the coding part as well. The region encoding the viral polyprotein is divided into three regions: P1, P2 and P3 (Semler, 2004). Genes in the P1 block express structural proteins, and the P2 part encodes proteins and a cre RNA structure which are both involved in the replication cycle of the virus. The most crucial protein factors, precursor 3CD, proteinase 3C\textsuperscript{pro}, RNA replicase 3D\textsuperscript{pol}, protein 3A and peptide VPg, are synthesized from the P3 region (Semler, 2004). Recently, several new RNA elements have been discovered within P3, which are also engaged in propagation of the viral cycle or virus persistence in the host cells.

In this review, we present current knowledge regarding structural elements which have been found in the enterovirus genomes (Fig. 1). Some of these motifs are common for all species (cloverleaf, IRES, cre, Y), while others are specific for particular enterovirus types (X, Z, i-RNaseL, 3D-7000, E10). These structural RNA features allow for infection and propagation in order to continue the viral cycle. Moreover, there are also motifs (E10, II',III') found in the enterovirus genomes whose function is still unknown and further research is necessary to reveal the role of these structural elements in the viral life cycle.
IRES ELEMENTS AND THEIR ROLE IN THE TRANSLATION PROCESS

With some exceptions, the translation initiation process in eukaryotes is dependent on the m'G cap at the 5' end of an mRNA. Enteroviruses are able to completely inhibit cap-dependent translation. Instead, the ribosome is recruited to the viral genomic RNA by an IRES, allowing the genomic RNA to serve as an mRNA. The IRES element is composed of several structural domains connected by single-stranded fragments of RNA present within the 5' UTR of the PV genome, which is approximately 750 nt in length. It has been demonstrated that domain II and domains IV–VI comprise an IRES element, which is involved in initiation of the translation process and allows binding of the ribosome to mRNA without a cap-structure. Interestingly, the first IRES element ever described was that of the poliovirus (Pelletier & Sonenberg, 1988).

The presence of an IRES element within viral RNA means that synthesis of host proteins can be blocked by inactivation of the complex which is responsible for cap-structure recognition, and at the same time production of viral proteins proceeds undisturbed. The stable secondary structure of the IRES makes the recruitment of the small 40S ribosome subunit possible without the involvement of the cap element. Hydrolysis of GTP allows for association of the large 60S subunit of the ribosome and translation can be initiated. The structural features of an IRES are characteristic of cis-regulatory elements which are important for viral translation due to their ability to interact with translation initiation factors and RNA-binding proteins (Filbin & Kieft, 2009; Filbin & Kieft, 2009). The sequence of an IRES is not conserved among different types, however, all IRES elements contain a tetra-loop GNRA element (where N means any nucleotide and R means a purine) with a poly-pyrimidine tract located upstream of the AUG codon (Fig. 2) (Bhattacharyya & Das, 2005; Balvay et al., 2009). It has been shown that the lack of the GNRA structure results in decrease in the translation efficiency, indicating the role of this motif in viral protein synthesis (Bhattacharyya & Das, 2005).

In the translation process of viral proteins, besides the canonical translational initiation factors (eIFs), GTP, tRNA and poly(A) tail, there are also trans-elements, initiation translation activation factors (ITAFs) and viral proteases involved (Balvay et al., 2009). In the case of PV, 2–3 hours after infection, the eIF4G factor is cleaved by the viral protease 2A, disturbing the binding of eIF3 and eIF4E to the ribosomal complex and inhibiting translation of the host proteins. Another viral protease, 3Cpro, inactivates the PABP protein (poly(A)-binding protein). Lack of functional PABP leads to complete inhibition of the cap-dependent translation of the host proteins, giving the virus a nearly complete monopoly of the host cell’s translation machinery (Balvay et al., 2009).

RNA STRUCTURES TAKING PART IN REPLICATION PROCESS OF ENTEROVIRUSES

Eukaryotes replicate their genetic material in the nucleus using a DNA polymerase and other components of the replication complex. Enteroviruses encode their own replicate, RNA-dependent RNA pol-
ymerase, and they replicate in the cytoplasm of the cell. Virus replication starts a few hours after infection and takes place in the membranous vesicles (Steil & Barton, 2009; van der Linden et al., 2015). During the first step, single-stranded RNA(–) is synthesized and then RNA(+) is produced using the minus RNA strand as a template. The newly synthetized RNA(+) strands can be incorporated into progeny virions or take part in the next round of replication or translation. There are several structural elements encoded in the RNA genome which are essential for enterovirus replication: the cloverleaf motif (also named domain I, oriL) present in the 5' UTR, its complementary structure, cloverleaf(–), located at the 3' end of the minus strand, the cre element in the coding part of 2C, the 3' UTR and the poly(A) tail (Liu et al., 2009).

Cloverleaf structure

All structural RNA elements mentioned above, particularly the cloverleaf, are involved in the first step of the synthesis of the minus strand. The cloverleaf is especially important for replication, but also has a tuning role in translation (Vogt & Andino, 2010). Presence of the cloverleaf motif is necessary for uridylylation of VPg, which plays a role as a protein starter in the replication of both, the plus and minus RNA strands (Barton et al., 2001; Sharma et al., 2009).

The cloverleaf structure is highly conserved among all enteroviruses. It is composed of four stem-loops, SL a-d (Fig. 3). Hairpin b and the cytidine-rich sequence adjacent to the cloverleaf interact with the cellular PCBP protein (poly(rC)-binding protein) which is also known as hnRNP E (Vogt & Andino, 2010). Disturbance of these interactions inhibits viral replication (Sharma et al., 2009). Stem-loop d binds the viral proteins, 3C and 3CDpro, and its interaction with 3CDpro is required for replication of the plus and minus RNA strands (Claridge et al., 2009; Sharma et al., 2009). The replication complex is formed by interactions between the cloverleaf element with PCBP and viral 3CDpro (Steil & Barton, 2009). Mutations within the cloverleaf structure cause a lower efficiency of RNA(+) synthesis. Moreover, stem-loop “a” plays a crucial role in efficient production of the plus strand RNA (Vogt & Andino, 2010).

The 3'UTR

Besides the cloverleaf element, other structural features, such as the oriR present at the 3' terminus of the
viral RNA genome, are important for the replication process. The oriR is located within the 3’ UTR and is composed of several stem-loop motifs. There are the following domains: X and Y in the Enterovirus C genomes; X, Y and Z domains in the Enterovirus B genomes (Fig. 3), and only the Y domain in the RNA of rhinoviruses. Mutations in the X and Y motifs result in a delay of PV RNA synthesis and complete inhibition of CVB3 replication (van Ooij et al., 2006a, 2006b; Zoll et al., 2009). Interaction of the X and Y domains forms a tertiary structure. This interaction was described for the first time in 1992 and was named the “kissing interaction” (Fig. 3). Recently, Zoll and coworkers (2009) described it as a “kissing-like pseudoknot”. Most of the nucleotides located in the loops of the X and Y domain are engaged in formation of this interaction. In the case of all enteroviruses, except rhinoviruses, six pairs of nucleotides are required. Site-directed mutagenesis has shown that these interactions are crucial for viral RNA synthesis, whereas they are not required for viral infection. The relationship between the structure and function of oriR is determined by the distance between the X and Y domains. The length of both helices is the same in most enteroviruses. Moreover, the spatial conformation of the domains seems to be important. Mutations in one domain can be compensated for by mutations in the other domain (Zoll et al., 2009).

The presence of a 20-150 nt-long poly(A) tail at the terminus of the 3’ UTR, results from the presence of poly(U) in the replicative intermediate (Steil & Barton, 2009). It means that it is genetically encoded, in contrast to the poly(A) tail of cellular mRNA, which is invariably added by the poly(A) polymerase. The poly(A) tail influences the efficiency of translation and the plus and minus strand synthesis via interaction with cellular PABP. The poly(A) tail is used as a template for VPg uridylylation processing by viral polymerase 3Dpol. Uridylylated VPg binds to the 3’ end of the virus RNA and acts as a
signal for initiation of replication. It is worth mentioning that there are two forms of the VPg proteins: VPg and VPgPupU₃⁰₅ (VPg covalently linked to two uridines by phosphodiester bonds between tyrosine and uridine residues). Both forms function as primers for the PV RNA replication (Paul et al., 1998).

A ribonucleoprotein (RNP) complex formed around the 5'-cloverleaf RNA structure of PV interacts with the PABP bound to the 3'-poly(A) tail, thus linking the ends of the viral RNA and circularizing it (Barton et al., 2001). Formation of this circular RNP complex is required for initiation of the synthesis of both RNA strands. Synthesis of RNA(−) is completed when the replication complex reaches the 5' end of the genomic strand. Both RNA strands are formed into heteroduplex RF (replicative form), until the next cycle of replication begins.

The cre element

A well-characterized structure, the cre element (cis-acting replication element) is located in the P2 region of the viral genome (P1 in some rhinoviruses) and it is thought to be involved in VPg uridylylation (Steil & Barton, 2009). The cre element is highly conserved among enteroviruses. It is folded into a hairpin structure with a 14-nucleotide loop rich in adenosine residues. The loop of the cre hairpin contains a sequence conserved in all enteroviruses: (RINNNNA5A6R7NNNNNNR14) (Cordy et al., 2008; Liu et al., 2009). The conserved sequence: AAAUG for CVB3, AAACA for PV, is present in the loop of the cre hairpin (Fig. 3) (Paul et al., 2000; van Ooij et al., 2006c). The first two adenosine residues are indispensable for the covalent linkage of UMP nucleotides to the VPg protein by the viral polymerase. It has been shown that disruption of the cre sequence and/or structure results in a decrease in the efficiency of the synthesis of the poliovirus plus-strand RNA. In contrast, the cre element is engaged in both steps of replication in case of the coxsackievirus B3 (van Ooij et al., 2006c).

The number of the plus RNA strands is always greater than the number of the minus strands in an infected cell. The ratio of the plus strands to the minus strands is approximately 40:1 for poliovirus (Giachetti & Semler 1991; Novak & Kirkegaard 1991). One proposed explanation is based on the observation that replication of RNA(+) via cre is much more effective than synthesis of RNA(−) due to VPg uridylylation of the poly(A) template (Paul et al., 2000). Presumably, the VPg uridylylation is responsible for differential production of the plus and minus RNA strands, which reflects the need of the virus for different numbers of each. While RNA(−) only serves as a template to generate new RNAs, the RNA(+) strand is involved in many events of the viral life cycle, such as translation, replication and virion assembly.

The 3' terminal region of CVB3 minus-strand

The second step of viral replication requires the cloverleaf structure present at the 5' UTR, the cre element and a short sequence fragment, 10 nt in length, at the 3' end of the minus strand. Additionally, viral proteins: 3CD, 3D⁰⁰⁴, 2C and hnRNP C (heterogenous nuclear ribonucleoprotein C) are involved in this process. After formation of the replication complex at the 3' terminus of RNA(−), the synthesis of new RNA(+) is initiated by the 3D⁰⁰⁴ polymerase (Steil & Barton, 2009). It has been shown for the poliovirus that two host proteins, p36 (hnRNP C) and p38, as well as two viral peptides, 2C and 2BC, interact with this 3'-terminal region of the minus strand (Banerjee et al., 1997; Banerjee & Dasgupta, 2001a, b; Rochl & Semler, 1995). Based on computer structure prediction, a cloverleaf(−) motif has been proposed to be formed at the 3' end of the minus strand of poliovirus. However, to date, detailed experimental analyses of the 3' terminal part of RNA(−) have been only performed for coxsackievirus B3. The 3' terminal region of the minus-strand of CVB3, around 450 nt in length, is composed of three domains: I', II', III' (Fig. 3) (Dutkiewicz et al., 2014). Domain I' is folded into a cloverleaf-like structure which resembles the structure present at the 5' UTR. This structural element is also comprised of four stem-loops (a', b', c' and d') which have been characterized by structural probing. Domain II', rich in the GU repeats, is composed of two hairpins. However, structural analyses revealed that the smaller hairpin is less thermodynamically stable. Domain III', 200 nt in length, is folded into three structural motifs which are characterized by the presence of stem-loop features and internal loops (Fig. 3) (Dutkiewicz et al., 2014).

What is the role of cloverleaf(−) at the 3' end of the minus RNA strand? This highly conserved structure might function during CVB3 replication via interactions with proteins which are analogous to proteins binding to the poliovirus RNA. Since there are strong structural similarities between PV and CVB3, it is possible that in the CVB3 replication the same cellular and viral proteins are engaged in an analogous way. The presence of the cloverleaf(−) structure has a positive impact on the binding of the viral protein 2C and precursor 2BC during RNA(+) synthesis (Banerjee et al., 1997; Banerjee and Dasgupta, 2001a,b). Protein 2C is an NTPase that contains two regions of RNA-binding activity. The 2C binding is dependent on the presence of the intact sequence 5'-UGUUUUU-3' of stem a' and occurs only when it is embedded in a double-stranded structure, like that present in the cloverleaf motif (Fig. 3) (Banerjee et al., 1997). The binding of 2BC requires the intact stem b' of the cloverleaf(−) motif and its special spatial orientation to stem a' (Banerjee and Dasgupta, 2001a,b). The interactions described above have been characterized for the poliovirus, however they seem to be highly probable for coxsackievirus B3 as well. The sequence of stem a' and the secondary structure of stem-loop b' of CVB3 closely resemble that found in the PV virus. Protein 2C also seems to be highly conserved among enteroviruses and may play the same role for both viruses. Motifs a' and b' are located very close to each other in a reverse-parallel orientation (Dutkiewicz et al., 2014). A possible function of such interactions for RNA(+) synthesis is anchoring of the minus viral strand to the membrane vesicles, where the replication process occurs. Additionally, due to the proposed NT-Pase activity of protein 2C, it might help to unwind the double-stranded stem a' to make the 3' end of the minus strand more accessible to other members of the replication complex. One such member might be hnRNP C protein, which binds to the oligo(U) or poly(A) fragments preferentially in single-stranded regions. Since the stability of stem a' is relatively low, it could be a potential binding site for hnRNP C, as has been shown for the poliovirus (Brunner et al., 2005; Ertel et al., 2010; Li & Nagy, 2011).

Besides the role of structures present within the 3' terminus of the replicative minus strand in protein interactions, they might act to prevent re-hybridization with complementary plus RNA strand during the synthesis of viral genomes. Presumably, they may allow for the repeated association of the replication complex with the same minus strand and synthesis of up to six nascent RNA strands from a single template, which is still partly
INHIBITION OF RNase L BY A STRUCTURAL RNA ELEMENT ENCODED IN THE 3C REGION

Ribonuclease L (RNase L) is one of the key effector enzymes involved in an interferon pathway, whose main role is to defend the host against viruses and other pathogens (Chakrabarti et al., 2011; Jackowiak et al., 2011). The antiviral activity of RNase L comes from at least two different mechanisms. One of them involves the degradation of viral RNA, and the second promotes cell apoptosis (antiproliferative activity) (Han et al., 2007). RNase L is expressed in a variety of cells as a latent enzyme in its basic, monomeric form. Activation via dimerization occurs after accumulation of viral RNA, and the second promotes cell apoptosis (antiproliferative activity) (Han et al., 2007; Chakrabarti et al., 2011). The motifs essential for RNase L inhibition are: loops 1 and 4, as well as loop E. Especially important for inhibitory activity is the G5761 residue located in the loop E motif. Nucleotide sequences of loops 1 and 4 are complementary to each other, thus enabling the “kissing interaction” which is mandatory for activity of the inhibitor (Townsend et al., 2008a; Townsend et al., 2008b). Encoded by the 3C gene of the poliovirus, as well as by several coxsackieviruses A from the Enterovirus C genus, a conserved RNA structural element has been found that competitively inhibits the activity of RNase L (Townsend et al., 2008b). This element, named i-RNaseL or RNase L ciRNA, has an extended, branched stem-loop structure and consists of hairpins, double-stranded arms, internal loops and minor mismatches (Fig. 4). The motifs essential for RNase L inhibition are: loops 1 and 4, as well as loop E. Especially important for inhibitory activity is the G5761 residue located in the loop E motif. Nucleotide sequences of loops 1 and 4 are complementary to each other, thus enabling the “kissing interaction” which is mandatory for activity of the inhibitor (Townsend et al., 2008b). How can such an extended RNA structure inhibit activity of RNase L? It binds the active site of the endoribonuclease domain of the enzyme in a competitive manner. Kinetic analysis demonstrated that it binds with much higher affinity to the active site of RNase L than RNase L’s normal substrate, phosphorylated 2’-5’ oligoadenylans (Townsend et al., 2008a). In the case of Enterovirus B, such RNA structure like i-RNaseL has not
been found. These viruses also are much more sensitive to RNase L cleavage. Research based on inhibition of the RNase L activity with RNA structural element may help in antiviral drug design against Enterovirus C.

THE SEARCH FOR NEW ORDERED RNA STRUCTURES IN THE CODING REGION OF ENTEROVIRAL GENOMES

The nucleotide sequence encoding enteroviral polyproteins contains not only information about the protein composition, but also secondary RNA structures important at different points of the viral life cycle, like cre or i-RNaseL. A putative presence of other important RNA elements has been investigated. Several algorithms have been developed that search for conserved RNA structures within coding regions of the RNA viruses. Recently, experimental methods have been also developed that enable structural scanning of large RNA fragments, or even whole viral genomes (Burrill et al., 2013).

Based on computer structural predictions and sequence comparison, Witwer et al. (2001) proposed that enteroviruses, with the exception of the rhinoviruses, contain a strongly conserved and thermodynamically determined RNA hairpin motif E10 at the 3′ terminal part of the coding sequence [http://www.ncbi.nlm.nih.gov:80/nuccore/RNA-4/]. It is encompassed within the 3D gene between nucleotide positions 7414 and 7430 of the PV genome (Fig. 4). To date, the E10 hairpin structure has not yet been experimentally confirmed. However, 11 years later another investigation of the polioviral genome lead to the identification of a larger RNA element called β that contains hairpin E10 as a smaller motif within itself (Song et al., 2012). The same publication proposed the existence of a one more RNA element: the α element, present upstream of β in the 3′-terminal 450-nucleotide segment of the 3D RNA coding sequence (nucleotide positions: 6920–7369). Both RNA structures turned out to be functionally redundant. Presence of those elements is important for efficient replication but more detailed information about their role and structure has not been experimentally verified (Song et al., 2012).

More or less at the same time, another research group performed genome-scale RNA structure characterization of PV using the SHAPE method (selective 2′-O-alkylation analyzed by primer extension) (Burrill et al., 2013). They searched for new ordered RNA structures in the coding region and found one very interesting RNA element, 3D-7000, that overlaps extensively with the α structure (Fig. 4). Moreover, the importance of this element for the virus was proven. Mutagenesis studies on this structure revealed that it is important for viral kinetics, dynamic RNA synthesis and infectivity. It was suggested that it might interact with the 3C or 3CD protein, since some compensatory mutations were found in the 3C coding region that eliminated the harmful mutations in the 3D-7000 element (Burrill et al., 2013). However, the detailed mechanism of its function has yet to be discovered.

CONCLUDING REMARKS

This review summarizes current knowledge regarding structural elements which have been found in enteroviral genomes, mainly those of poliovirus 1 and coxsackievirus B3. These elements are especially characteristic for regulatory, untranslated regions (UTRs) of the viral genomes. However, the protein coding region also encodes important RNA structures crucial for the viral life cycle and virus persistence in the host cells. It turns out that the 3′ terminal region of the minus strand is also able to fold into ordered RNA structures, but their presence in partially dissociated replicative intermediate form (RF) and function is yet to be proven. Further research is necessary to search for new ordered RNA structures which presumably still hide undiscovered in the coding region, as well as to reveal their role in the virus propagation.

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