Mutational analysis of a conserved tetraloop in the 5′ untranslated region of hepatitis C virus identifies a novel RNA element essential for the internal ribosome entry site function

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Abstract The 5′ untranslated region of hepatitis C virus RNA forms an extensive secondary structure including several hairpin motifs and mediates translation initiation by an internal ribosome entry site-dependent pathway. We report, here, an extensive mutagenesis analysis of a highly conserved tetraloop in the 5′ untranslated region of hepatitis C virus, namely hairpin IIIe (295′-GAUA-298′). Our results demonstrate that hairpin IIIe is essential for the internal ribosome entry site function. Moreover, they indicate the importance of the primary structure of this motif because mutations in all four nucleotides of the loop caused a severe loss of internal ribosome entry site activity. These data represent the first experimental evidence for the functional significance of tetraloops in internal ribosome entry site-driven translation of hepatitis C virus.

Key words: Hepatitis C virus; Internal ribosome entry site; 5′ untranslated region; Tetraloop

1. Introduction

Hepatitis C virus (HCV) is a positive strand RNA virus and together with the flaviviruses, pestiviruses and GB-viruses, it is a member of the Flaviviridae family [1]. Unlike flaviviruses, however, HCV, pestiviruses and GB-viruses have relatively lengthy and highly structured 5′ untranslated regions (5′UTR) harboring an internal ribosome entry site (IRES) [2–8]. For HCV, almost the entire 5′UTR, including 12–30 nucleotides from the encoding region of the HCV capsid protein, is required for an efficient IRES function, while recent reports place the ribosome binding site close to the initiator AUG [7–15]. Secondary structure modeling of the 5′UTR of HCV, GBV-B and pestiviruses revealed a remarkable folding similarity, despite the low level of overall sequence homology (lower than 50%) [16–18]. A striking common structural element is the presence of a pseudoknot structure upstream of the initiator AUG formed via interactions between a conserved six base sequence element 11 nucleotides upstream of the initiator AUG and bases from the terminal loop IIIf [5,19]. Mutational analyses have shown that this structure is essential for the HCV and pestivirus IRES function [5,19]. Moreover, several short stretches with a significant nucleotide sequence identity have been recognized in the 5′UTR of these viruses [16–18]. These regions primarily represent stem-loop structures and some of those, namely IIIa, IIIc and IIIe, contain tetraloop motifs. The high conservation of those sequences among flaviviral IRESs implies their importance in the IRES function.

Tetraloops are common elements in many RNAs and often serve as sites for tertiary interactions, recognition signals for RNA binding proteins or nucleation sites for RNA folding [20]. Certain sequences, namely GNRA, UNCG, CUUG, are found with a surprising frequency in large RNA molecules and are characterized by an exceptionally high thermodynamic stability [20–24]. These loops are very stable because of a striking base stacking and hydrogen bonding within the loop including an unusual base pair between the first and the last residue [23,25]. Interestingly, an interaction between the GNRA tetraloop and a receptor domain has been identified in the catalytic core of group I self-splicing introns and has been shown to be critical for the molecule's tertiary structure [23,26–29]. Recently, a structural motif similar to the catalytic core structure of group I introns was identified at the 3′ end of all viral IRES types [17,30]. This element includes the pseudoknot structure, two stems and in the case of flaviviral IRESs, an absolutely conserved tetraloop motif in the IIIe hairpin. In addition, a GNRA-type tetraloop has been identified at similar locations (domain III) in all known picornavirus IRESs [31]. Since invariant positions are often required for tertiary structure folding, this GNRA loop is considered to be a candidate element for determining the tertiary structure of the 5′UTR of these RNAs. Recent mutagenesis studies have shown that the maintenance of the GNRA motif in loop 3A was absolutely required in the aphthoviral IRES function [32].

Despite the extensive mutagenesis studies within the HCV 5′UTR [10–15,33–35], experimental evidence regarding the requirement for the primary structure within the HCV tetraloops is lacking. In our study, extended mutagenesis analysis was carried out on the 295′-GAUA-298′ loop (IIIe) which is the only tetraloop that is absolutely conserved in all HCV strains and is also present in the GBV-B and pestiviral IRESs [16,18]. This element is also part of the common core structure identified at the 3′ end in all viral IRESs [17,30]. We demonstrate here that the GAUA tetraloop is an essential element for the HCV IRES function and show that changes in each of the four nucleotide positions of the loop severely impair the IRES activity. Finally, our data indicate that this element does not conform with the requirements for the known GNRA tetraloop because the nucleotide of the second position is not variable and the third position cannot tolerate a purine residue.

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Abbreviations: HCV, hepatitis C virus; UTR, untranslated region; IRES, internal ribosome entry site; LUC, luciferase; CAT, chloramphenicol acetyltransferase

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Table 1
List of oligonucleotides and constructs used in the mutational analysis of loop IIIe

Mutation	Oligonucleotides used for in vitro mutagenesis	Constructs for in vitro translation	Constructs for in vivo translation
Wild-type	5'-TACTGCCT*GATA*GGGTGCTTG-3'	pHPI933	pHPI1046
mut L19	5'-TACTGCCT*GAT*<u>*G*</u>GGGTGCTTG-3'	pHPI1000	pHPI1047
mut L20	5'-TACTGCCT*GAT*<u>*T*</u>GGGTGCTTG-3'	pHPI993	pHPI1048
mut L21	5'-TACTGCCT*GA*<u>*A*</u>*A*GGGTGCTTG-3'	pHPI1039	pHPI1060
mut L22	5'-TACTGCCT*GA*<u>*C*</u>AGGGTGCTTG-3'	pHPI1031	pHPI1058
mut L23	5'-TACTGCCT*G*<u>*TT*</u>AGGGTGCTTG-3'	pHPI1032	pHPI1059
mut L24	5'-TACTGCCT*G*<u>*G*</u>*T*AGGGTGCTTG-3'	pHPI1045	pHPI1071
mut L25	5'-TACTGCCT<u>*C*</u>*ATA*GGGTGCTTG-3'	pHPI1035	pHPI1075
mut L26	5'-TACTGCCT<u>*A*</u>*ATA*GGGTGCTTG-3'	pHPI1042	pHPI1061
mut L27	5'-TACTGCCT*G***GGGTGCTTG-3'	pHPI1003	pHPI1065

Loop IIIe nucleotides are illustrated in italics.
Underlined nucleotides represent substituted nucleotides.
*, deleted nucleotides.

2. Materials and methods

2.1. Plasmid constructs and site-directed mutagenesis

Site-directed mutagenesis was performed using the M13-based MUTA-GENE kit (Bio-Rad), plasmid pHPI800 as template [34] and the oligonucleotides shown in Table 1. Plasmid pHPI800 carries the cDNA of the HCV 1a 5′UTR (nucleotides 9–341) isolated from a patient as described previously [34]. Mutations were identified by sequence analysis.

The *Sma*I/*Sma*I fragments (nucleotides 130–317) from the mutated templates were cloned into the *Sma*I cloning site of plasmid pHPI892. Plasmid pHPI892 was derived from plasmid pHPI933 [34], which carries the HCV 5′UTR together with the first 66 bases of the encoding region (nucleotides 9–407) between chloramphenicol acetyltrans-

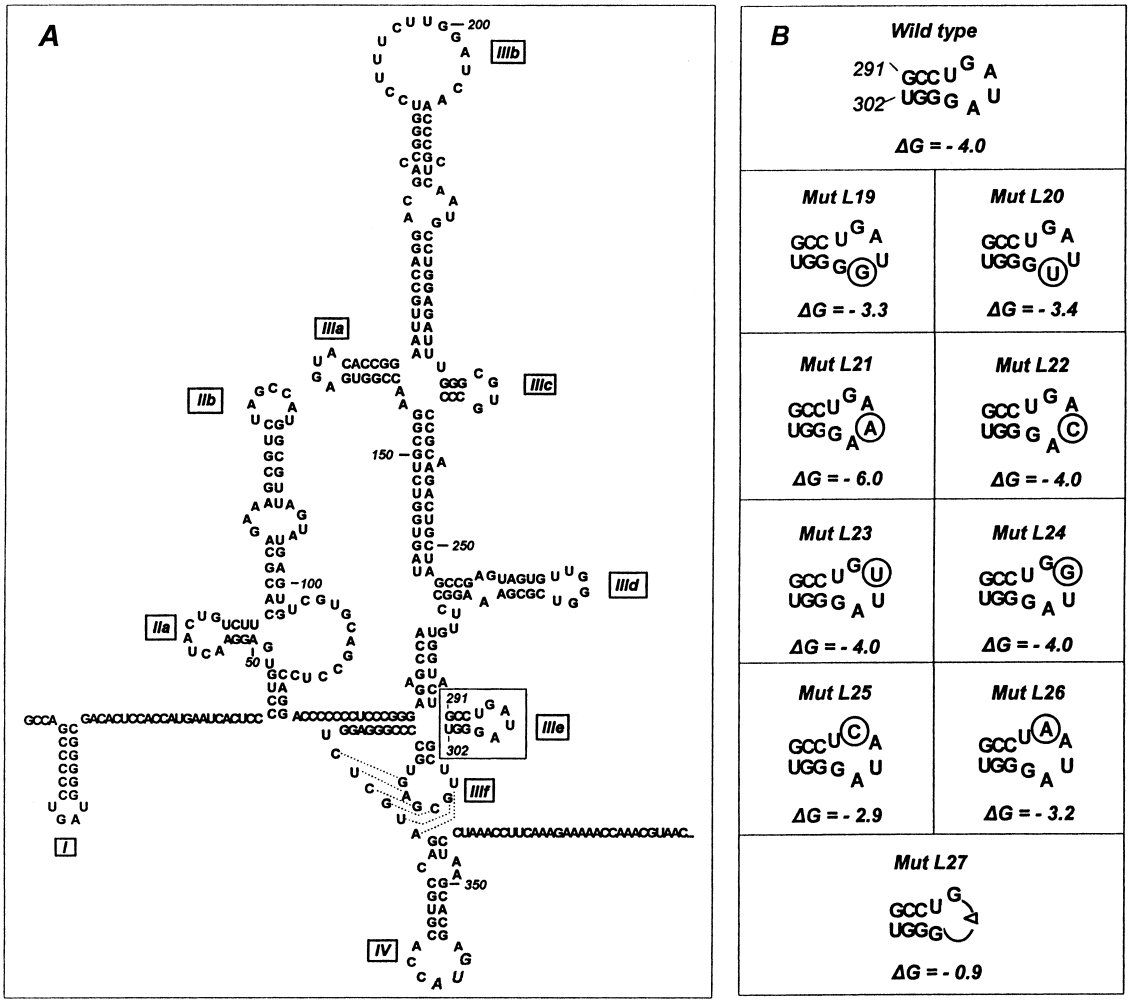

Fig. 1. (A) Secondary and tertiary structure model of the complete HCV 5′UTR [10]. Loop IIIe is boxed. (B) Transition and transversion mutations at each base of the tetraloop are shown (mut L26 and mut L25 represent transition-transversion mutations for the first residue, mut L24 and mut L23 for the second, mut L22 and mut L21 for the third and mut L19, mut L20 for the fourth; mut L27 represents a three-nucleotide deletion). The predicted ΔG is indicated.

ferase (CAT) and luciferase (LUC) reporter genes, by deletion of the *Sma*I/*Sma*I fragment (nucleotides 130–317). The derived plasmids are shown in Table 1.

For the in vivo studies, the *Hin*dIII/*Bst*EII fragment of pHPI933, carrying the CAT gene, the HCV sequence (nucleotides 9–407) and part of LUC gene were cloned into the *Hin*dIII/*Bst*EII cloning sites of plasmid pCMVNCRluc (kindly provided by Dr W.H. Caselmann [9]), producing construct pHPI1046. Thus, plasmid pHPI1046 contains the CMV promoter at the 5′ end and the bovine growth hormone polyA signal at the 3′ end of the CAT-IRES-LUC dicistronic construct. In the same way, the mutant fragments *Hin*dIII/*Bst*EII were cloned into plasmid pCMVNCRluc, producing the constructs listed in Table 1. Calculation of free energy (ΔG) in the wild-type and mutated structures was performed by using the MFOLD program (University of Wisconsin Genetics Computer Group) (Fig. 1).

2.2. In vitro transcription and translation

Wild-type plasmid pHPI933 and the corresponding mutated dicistronic constructs were linearized with *Xho*I. 3 μg DNA was transcribed in vitro with *SP6* RNA polymerase (Promega) as instructed by the manufacturer. RNA was quantitated photometrically and two different amounts (250, 500 ng) of RNA were used for in vitro translation with Flexi rabbit reticulocyte lysates (Promega). In vitro translation experiments were carried out in a total volume of 25 μl in the presence of 120 mM KCl and 0.5 mM MgAcO. Proteins were labelled with [^{35}S]Met (Amersham Life Sciences), analyzed by 12% SDS-PAGE and transferred onto nitrocellulose membranes followed by autoradiography. The exposed films were scanned and bands corresponding to CAT and LUC products were quantitated by computer analysis using the Gel-pro program. In addition, luciferase activities were measured as described below.

2.3. DNA transfection, CAT ELISA and luciferase assay

The wild-type plasmid pHPI1046 and corresponding mutated constructs were used to transfect COS-7 cells using DOTAP reagent (Boehringer Mannheim) according to the manufacturer's protocol. Cells were seeded in 100 mm dishes and after 16–18 h incubation, they were transfected with 7.5 μg of each plasmid in a DMEM 5% serum medium. After 5 h, the culture medium was replaced. Cells were harvested after 48 h and divided into two aliquots, one for CAT and one for luciferase assays. Protein was assayed in each aliquot using a Bradford assay (Bio-Rad) and luciferase and the CAT activity was estimated per μg of protein. Quantitation of CAT was performed using the CAT-ELISA kit (Boehringer Mannheim) according to the manufacturer's instructions. The cells of the second aliquot were prepared and assayed for luminescence using a Turner TD-20/20 luminometer and the Luciferase Assay kit (Promega).

3. Results

To analyze the potential significance of the 295′-GAUA-298′ tetraloop (IIIe) in HCV IRES-driven translation, we performed extensive site-directed mutational analysis including a three-nucleotide deletion and a series of transition and transversion mutations at each of the four bases of the motif. The nature of these changes is summarized in Fig. 1. The effect of these mutations on IRES-driven translation was assessed using a dicistronic expression system containing the HCV 5′UTR with the first 66 nucleotides of the encoding region between the CAT and the LUC reporter genes, both in vitro and in vivo.

In vitro synthesized wild-type and mutant dicistronic RNAs were both translated in vitro using rabbit reticulocyte lysates (RRL) in the presence of 120 mM KCl, a concentration which allows for HCV RNA translation in an IRES-dependent manner. Translation products were then analyzed by SDS-PAGE and autoradiography. The in vitro assays were performed at least three times with independent RNA preparations. The results shown in Fig. 2 can be summarized as follows. Firstly, the three nucleotide deletion within the GAUA loop (mut L27) almost completely abrogated translation indicating that this motif is essential for IRES activity (lanes 10 and 20). Secondly, all nucleotide substitutions within the tetraloop drastically reduced the amount of luciferase expression as compared to wild-type despite the relative constant quantities of the CAT product. In general, no significant differences were observed between transition and transversion mutations (lanes 3, 5, 7, 9, 13, 15, 17, 19 and 2, 4, 6, 8, 12, 14, 16, 18, respectively), with the exception of mut L19 (GAU<u>G</u>), which is a transition mutation and had a greater effect on translation initiation (lanes 3, 13) than mut L20 (GAU<u>U</u>) which is the corresponding transversion mutation (lanes 2, 12). Interest-

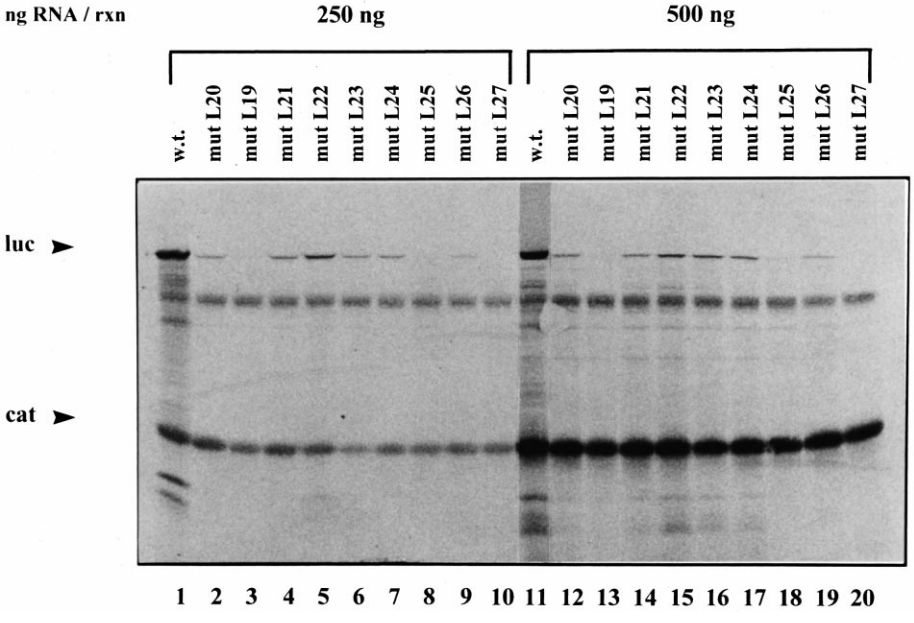

Fig. 2. The effect of mutations within loop IIIe on in vitro translation. 250 or 500 ng of each dicistronic RNA was used for in vitro translation with Flexi RRL (as described in Section 2). Translation products were resolved by 12% SDS-PAGE and the resulting autoradiograph is shown. The positions of CAT and LUC genes are indicated.

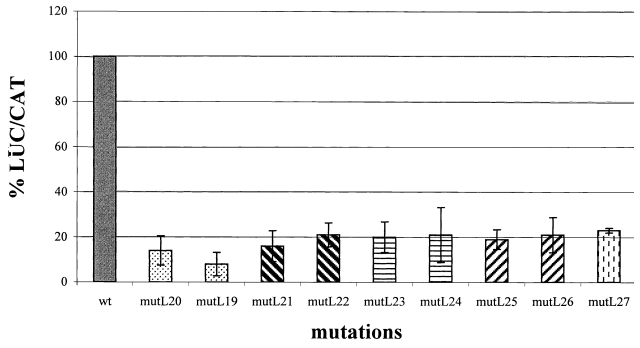

Fig. 3. The effect of mutations in the loop IIIe on in vivo experiments. 7.5 μg of each DNA was used for transfection in COS-7 cells (as described in Section 2). For clarity, bars corresponding to substitutions at the same residue have been similarly shaded. Luciferase activity (RLU/μg protein) was normalized to the CAT quantity per μg of protein present in the same extracts. Relative efficiency refers to the activity produced by the mutant IRES divided by that of wild-type. Error bars correspond to the S.E.M.

ingly, mut L19 generated an AUG in the tetraloop. Thirdly, the relative efficiency of translation for the mutated IRES varied between 0–30%. Mutations of the first (mut L25, mut L26) or fourth positions of the tetraloop (mut L19, mut L20) reproducibly had the most severe effect on the translation efficiency (lanes 8, 9, 18, 19 and 2, 3, 12, 13, respectively), similar to mut L27 which represents the three-nucleotide deletion (lanes 10, 20). Mutations of the second (lanes 4, 5, 14, 15) and third position (lanes 6, 7, 16, 17) severely reduced the IRES activity but never abolished translation (allowing for some IRES activity as high as 30% of wild-type).

It should be noted that mutations at the first or fourth base of the loop as well as the deletion of three nucleotides increase the ΔG of the structure (Fig. 1). However, mutations at the second or third base of the tetraloop do not affect the stability of the structure, with the exception of mut L21 which generates the most stable GNRA-type tetraloop.

Since all mutations had a severe effect on the luciferase expression, we wanted to exclude the possibility of having introduced accidentally a mutation on the expression vector pHPI892, other than the designed changes in the HCV 5′UTR. For this purpose, we chose plasmid pHPI1039 and rescued the corresponding mutation mut L21 with the wild-type sequences. The resulted plasmid expressed luciferase levels equal to that of wild-type pHPI933 (data not shown).

In another series of experiments, we studied the same mutations in vivo by transfections in COS-7 cells. The in vivo transfection assays were performed at least three times and the results are summarized in Fig. 3. These experiments are in good agreement with the in vitro assays because all mutations including both transition and transversion mutations severely impaired the IRES function. The decrease in the translation efficiency was 60–90% with no significant variation between mutations at different positions of the tetraloop.

Overall, these results reveal that the GAUA tetraloop is an essential element for the IRES function and demonstrate the critical importance of the primary structure of the motif.

4. Discussion

While the molecular mechanisms that control the HCV IRES function are not clearly understood, a general model [18] for internal initiation of translation predicts that the helical structures of an IRES element function primarily as structural elements to direct the proper folding of the RNA molecule. This directs the unpaired nucleotides of loops and bulges at the correct three-dimensional position to allow the recognition and binding of the ribosomal subunit. According to this model, loop structures contain the critical nucleotide sequences for protein and/or RNA interactions, a hypothesis which is further supported by the conservation of the primary structure of most the loops and bulges. However, since the experimental evidence to support this hypothesis is rather limited, it is unclear whether all conserved loops and bulges in the HCV IRES element are essential for the IRES function.

This report provides the first experimental evidence on the functional importance of tetraloops in the HCV IRES. We studied the IIIe tetraloop (295′-GAUA-298′), which is a highly conserved tetraloop in the HCV IRES and resembles the well-characterized GNRA loop found in picornavirus IRESs with the exception of the third residue which is a pyrimidine instead of purine.

Our data show that the GAUA motif is essential for the IRES function since deletion and point mutations that altered or deleted the loop are detrimental to the IRES activity. Most importantly, all single nucleotide substitutions in each of the four positions of the loop resulted in a strong reduction of the luciferase reporter activity and no significant difference was observed between transition and transversion mutations. Thus, no other nucleotide was acceptable at any of the four positions of the motif without any significant effect on translation, suggesting that the GAUA tetraloop has unique properties and does not conform to the structural requirements of the most common tetraloops (GNRA, UNCG).

It should be noted that a common feature to all known tetraloops is the interaction between the first and last residue of the loop [23,25,36]. This extra base pair effectively leaves a small two-base loop and contributes to the stability of the structure. In fact, mutations in the first and last nucleotides of the GAUA loop led to an increase in free energy and resulted in the most dramatic effect on the IRES function in vitro. In contrast, mutations at the second and third positions severely impaired translation without affecting the stability of the tetraloop. Interestingly, mut L21 (GAUA to GAAA), which converts the GAUA to a more stable GNRA-type motif, had a deleterious effect on translation. Overall, these results suggest that the thermodynamic stability of loop IIIe alone is not important for the IRES function. Rather, the nucleotides of this loop are potentially involved in protein or/and RNA interactions. However, further studies are required to define its exact function. Moreover, mut L21 provides additional evidence indicating that the GAUA motif has different structural and functional properties from the GNRA tetraloop, since the two loop motifs are not interchangeable. It is of interest that tetraloop sequences sometimes act as units and cases have been reported where the UNCG-type of loop can be changed to a GNRA or vice versa [23].

Tetraloops are believed to provide nucleation sites for proper folding of the secondary and tertiary structure in large RNA molecules or alternatively may serve as recognition sites for protein binding [20]. It has been reported that the GNRA motif can interact with helical segments at distant locations within the same RNA molecule [23]. A conserved 11 nucleotide RNA motif has been proposed to be the receptor for this

loop [27,37] and comprises an asymmetric internal loop with three adenosines stacked in a cross strand or a zipper-like fashion close to a G-C base pair. These A residues create a platform (A-platform), when the loop is bound. Although such a motif is not exactly present in the HCV IRES, there is an analogous segment consisting of an asymmetric internal loop with three A residues close to a G-C base pair (181′-GACG-184′ and 213′-CAAUGC-218′). It is of additional interest that this region has been reported as the binding site of initiator factor eIF-3 [38,39].

Our data are in agreement with a previous report, which addressed the functional importance of all hairpin structures in the HCV 5′UTR. In this study, Rijnbrand et al. [15], using precise deletion analysis, demonstrated that, with the exception of the most 5′-located hairpin, each of the predicted hairpins in the 5′UTR of HCV is essential for a proper IRES function. It should be noted, however, that the corresponding deletion designed to test the effect of the hairpin IIIe was extended to loop IIIf and removed bases important for the formation of the pseudoknot. Consequently, this mutation had a dual effect on the IRES structure and was not directly addressed to the role of hairpin IIIe.

Finally, recent mutagenesis studies with the BVDV IRES element indicate that the corresponding hairpin IIIe together with the terminal loop of domain III are only partially required for the IRES function in vivo [2]. Thus, despite the overall structural similarities of the IRES of pestiviruses and HCV, the details on the functional role of hairpin IIIe may be different.

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