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Mutational analysis of the apical region of domain II of the HCV IRES

Katerina I. Kalliampakou, Loukia Psaridi-Linardaki[1], Penelope Mavromara*

Molecular Virology Laboratory, Hellenic Pasteur Institute, 127 Vassilisis Sofias Avenue, Athens 11521, Greece

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Abstract The hepatitis C virus internal ribosome entry site (IRES) binds directly to the 40S ribosomal subunit via domains III/IV while domain II induces conformational changes on the ribosome which have been implicated in the decoding process. Here, we performed an extensive mutational study within the apical portion of domain II in order to address the functional role of this region on translation. Our results showed that the conservation of most nucleotides in this region was only partially related to the IRES function. Notwithstanding, however, selected single point mutations within the apical loop had a deleterious effect on IRES activity. © **2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

Key words: Hepatitis C virus; Internal ribosome entry site; Translation; Domain II; Apical loop; Mutational analysis

1. Introduction

The hepatitis C virus (HCV) is the main causative agent of post-transfusion non-A, non-B hepatitis [1]. In most infected individuals, the virus establishes persistent infection, which often leads to the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma [2]. HCV is an enveloped, single-stranded, positive sense RNA virus classified within the Flaviviridae family. The viral genome, about 9600 nucleotides in length, encodes a polyprotein precursor which is processed by viral and cellular proteases to yield mature viral proteins. The open reading frame is flanked at the 5′ and 3′ ends by highly structured non-translated regions (NTRs) [3]. Initiation of translation of the HCV genome is controlled by an internal ribosome entry site (IRES) located mainly within the 5′NTR of the viral RNA [4–6]. The minimal sequence required for IRES activity extends approximately from nucleotides 42 through 341 or 356, the 3′ border being controversial [7–9]. This RNA segment is folded into a complex secondary structure consisting of several stem-loops, known as domains II, III and IV, a pseudoknot and a helical structure that links domain II with domains III and IV [10–13]. Both the highly ordered structure and the nucleotide sequence of selected regions of the IRES are critical in determining the IRES function [7,14–26]. Recent biochemical and biophysical studies have shown that these elements form a unique tertiary structure that allows the direct binding of the 40S ribosomal subunit at the site of the initiator AUG and the eIF3 through multiple and specific intermolecular contacts [15,18,22,27–33]. The RNA regions involved in these contacts have been mapped mainly on domains III to IV, and the importance of most interaction sites has been supported by mutational analysis. On the other hand, domain II folds away from domains III and IV and is not required for binding to the ribosome or to the eIF3 nor does it contribute to the affinity of these interactions [11,28,30]. Recent studies, however, have shown that domain II induces a conformational change on the 40S ribosomal subunit that has been implicated with the RNA decoding process. A current model suggests that the apical loop of domain II contacts ribosome at or near the E site and may be involved in holding the coding RNA into the decoding center until the translational machinery has assembled [33]. Furthermore, sequence analysis studies have shown that the apical unpaired regions of domain II contain conserved nucleotide motifs which are also present in the corresponding IRES sequences from GBV-B and pestiviruses. These include the motifs 81-ARCCA-85 (apical loop) and 71-GAA-73 and 92-UAGUA-96 (adjacent bulge) [9]. As the overall sequence similarity within the 5′NTRs of those viruses is low, the strong conservation of these motifs has suggested a key role in translation initiation. Nevertheless, despite the significance of those findings, the molecular details defining the role of domain II in IRES function remain elusive, since the majority of the studies have focused on the structural requirements of this region in the IRES-dependent translation [9,23,34–36].

To elucidate the importance of the primary structure of the apical region of domain II in the IRES function, we performed an extensive mutational analysis and tested the effects of those mutations on the HCV IRES activity. Our results confirmed the significance of the apical region of domain II on translation and demonstrated that single-point mutations at selected positions of the apical loop had a severe effect on IRES function. On the other hand, however, the integrity of the conserved motifs was only partially related to the IRES activity.

2. Materials and methods

2.1. Plasmids and site-directed mutagenesis

The dicistronic constructs pHPI933, pHPI1046 (Fig. 1E) and pHPI892 were previously described [24,26]. Plasmid pHPI1121 was

*Corresponding author. Fax: (30)-1-6478877.
E-mail address: penelopm@hol.gr (P. Mavromara).

[1] Present address: Department of Biochemistry, Hellenic Pasteur Institute, Athens, Greece.

Abbreviations: LUC, firefly luciferase; CAT, chloramphenicol acetyl transferase; IRES, internal ribosome entry site; HCV, hepatitis C virus; NTR, non-translated region

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constructed by replacing the wild type *Hin*dIII–*Bst*EII fragment of pHPI1046 with the *Hin*dIII–*Bst*EII fragment of pHPI892. Site-directed mutagenesis was performed either by the M13-based MUTAGENE (Bio-Rad) or the Quikchange™ (Stratagene) kit using the oligonucleotides shown in Table 1. All mutations were confirmed by sequence analysis. M13-based mutagenesis was performed using the plasmid pHPI803 as template [26]. Mutations were cloned into pHPI933 by replacing the wild type 271 bp *Bam*HI–*Nru*I fragment with the corresponding fragment of the mutated template. For the in vivo studies, mutations were cloned into pHPI1046 by replacing the wild type 1796 bp *Hin*dIII–*Bst*EII fragment with the corresponding fragment of the mutated pHPI933. Quikchange™ mutagenesis was performed using the plasmid pHPI933 as template. All mutations were recloned into pHPI933 by replacing the wild type 249 bp *Bam*HI–*Nhe*I fragment with the corresponding fragment of the mutated template to assure that the vector had only the desirable mutation. For the in vivo studies, mutations were cloned into the pHPI1046 by replacing the wild type 1229 bp *Hin*dIII–*Xba*I fragment with the corresponding fragment of the mutated pHPI933.

2.2. In vitro transcription and translation

Wild type plasmids pHPI933 and pHPI892 (negative control) and the mutated constructs (derived from pHPI933) were linearized with *Xho*I. 2.5 μg DNA from each plasmid was transcribed in vitro with SP6 RNA polymerase. 400–500 ng of each uncapped RNA was used for in vitro translation with Flexi Rabbit reticulocyte lysates (Promega). Transcription and translation reactions were performed as previously described [24,26].

2.3. Cells and DNA transfection experiments

HepG2 cells were maintained in minimum essential Eagle's medium (MEM; by Gibco BRL) supplemented with 10% fetal bovine serum (MEM/FBS). Cells seeded in 6-well plates (40% confluence) were transfected with 1 μg plasmid DNA using the lipofectamine plus reagent (Boehringer Mannheim) according to the manufacturer's protocol. The medium was replaced with new MEM/FBS 24 h post-transfection. At 48 h post-transfection the cells were washed twice with phosphate-buffered saline and lysed in 260 μl Luciferase Lysis Buffer 1X (Promega). Quantitation of firefly luciferase (LUC) was performed by mixing 20 μl of cell extracts and 100 μl luciferase assay reagent (Promega) and the luminescence was directly measured by a Turner TD-20/20 luminometer. Quantitation of chloramphenicol acetyl transferase (CAT) was performed with the CAT–ELISA kit (Boehringer Mannheim) according to the manufacturer's instructions. The ratio of LUC to CAT activities was estimated for each mutation and compared with that of the wild type under the constrain that the variability of the CAT amounts was lower or equal to 40%.

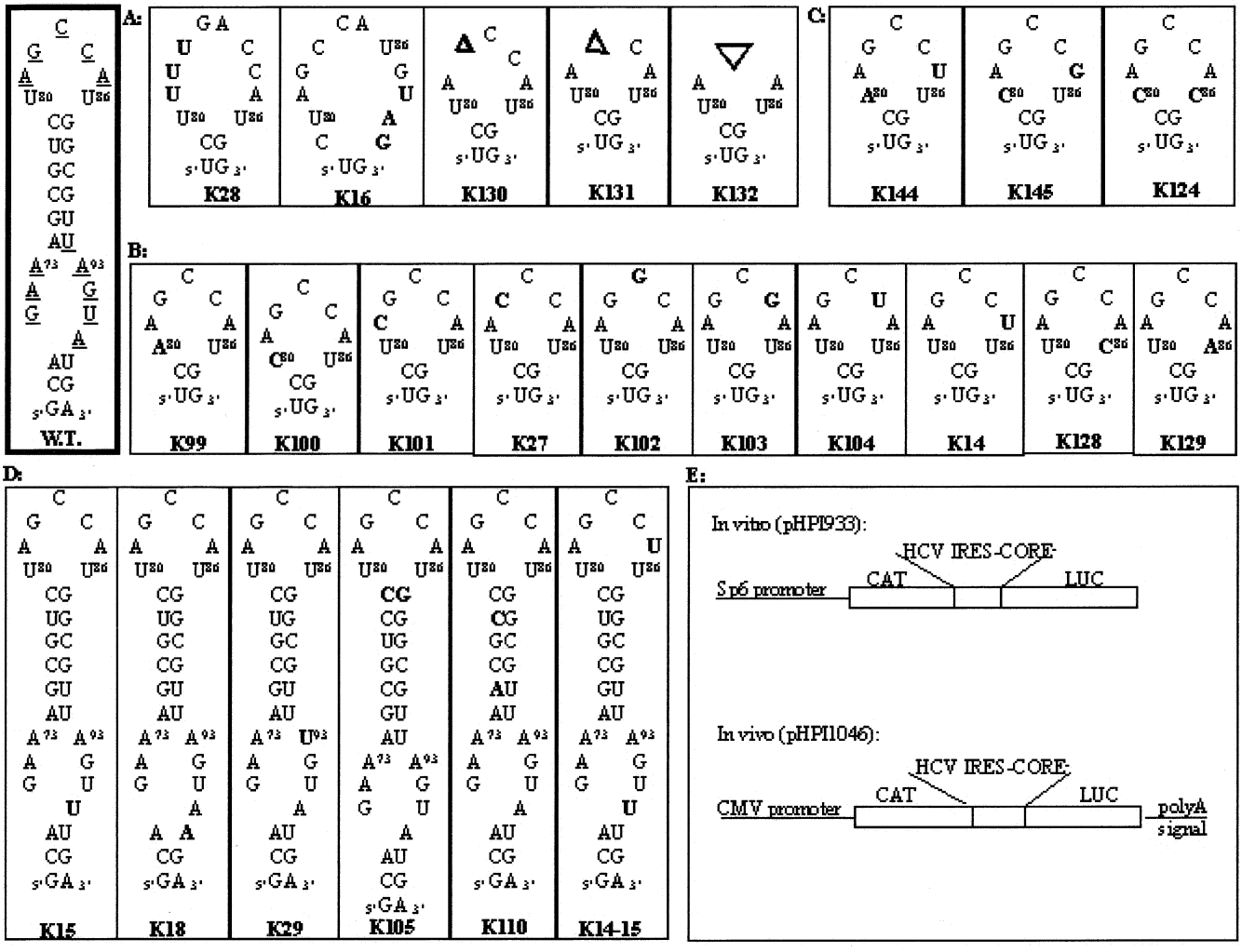


Fig. 1. Predicted secondary structure of the upper segment of domain II of HCV IRES. Conserved bases are underlined (W.T., wild type). A: Insertion and deletion mutations. B,C: Substitution mutations in the apical loop. D: Mutations affecting the apical bulge and stem. Substitution and insertion mutations are shown in bold. Deletion mutations are represented by triangles. E: Schematic representation of CAT–LUC dicistronic constructs. The HCV-1a 5′NTR and 66 bases of the coding sequence are fused in frame with the LUC gene. In vitro and in vivo expression is mediated by SP6 and CMV promoter, respectively.

3. Results

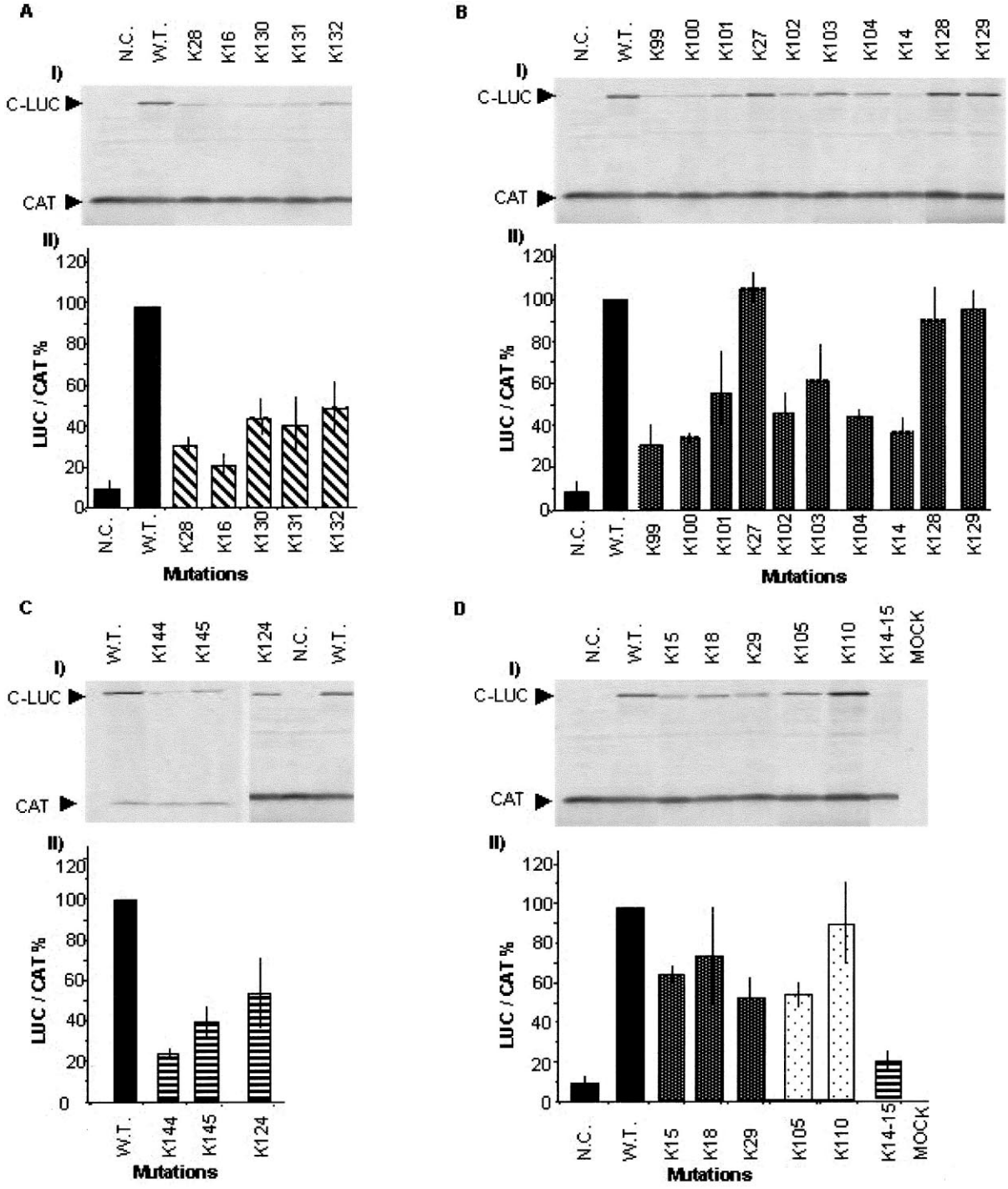
To investigate the functional role of the upper segment of domain II in the IRES translation activity, we performed extensive site-directed mutational analysis in this region. The sequence changes included insertion, deletion, and point mutations (Fig. 1A–D). The effect of each mutation, on IRES driven translation, was assessed both in vitro and in vivo with a dicistronic expression vector, which carries the CAT and the LUC genes as the first and second cistron, respectively (Fig. 1E). Expression of LUC gene was directly related to the functional properties of the HCV IRES, while the CAT activity served as an internal control to correct possible differences in transfection efficiencies in vivo or potential variations in the transcript abundance in vitro. Fig. 2A–D (I) shows representative results of in vitro translation reactions. Fig. 2A–D (II) shows the level of LUC expression relative to CAT expression in transfected HepG2 cells. The results from the in vitro and in vivo experiments (Fig. 2) were in close agreement and can be summarized as follows:

1. Insertion and deletion mutations were introduced in the apical loop of domain II to assess the importance of the structure of this region to IRES function. Mutants K16 and K28 contain a 3-nucleotide insertion within the apical loop next to nucleotides U-80 and U-86, respectively (Fig. 1A). Mutants K130, K131 and K132 have deletions of the G-82, 82-GC-83 and 82-GCC-84 nucleotide sequences, respectively (Fig. 1A). As shown in Fig. 2A, insertion mutations caused a severe effect on the expression of the LUC gene, which was about 25% of that of the wild type. Additionally, all deletion mutations inhibited the IRES activity. However, the LUC expression never reached the low levels obtained from the insertion mutations, suggesting a differential sensitivity of the apical segment to the structural constrains introduced by the different type of mutations. Notably, the K132 mutation had the least effect on translation. Our results were in agreement with previous studies and indicated that the apical loop of domain II is essential for IRES activity.
2. To assess the role of the nucleotide sequence of the apical heptanucleotide loop on IRES function, a series of single nucleotide substitutions were systematically introduced into each one of the bases (Fig. 1B). As shown in Fig. 2B, these mutations affected variably the IRES activity. The most severe effect resulted from substitutions in U-80 and A-85 positions. Transversion (K99) and transition (K100) mutations of U-80 as well as a transversion mutation of A-85 (K14) almost completely abrogated IRES-driven translation (Fig. 2B), indicating that the identity of these nucleotides is particularly important for HCV IRES activity. In contrast, changes in G-82 (K27) or U-86 (K128, K129) had no detectable effect on translation (Fig. 2B), indicating that the nucleotide sequence of these positions had no influence on IRES activity. Notably, deletion of G-82 (K130) resulted in a significant inhibition of IRES function. On the other hand, changes of the highly conserved nucleotides A-81, C-83 and C-84 (mutants K101,

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

Mutation	Oligonucleotides	In vitro plasmids	In vivo plasmids
W.T.		pHPI933	pHPI1046
N.C.		pHPI892	pHPI1121
Single substitution mutations			
K99	5′-^{71}GAAAGCGTC**A**AGCCATGGCGTTAGTA-3′	pHPI1105	pHPI1122
K100	5′-^{71}GAAAGCGTC**C**AGCCATGGCGTTAGTA-3′	pHPI1106	pHPI1123
K101	5′-^{71}GAAAGCGTCT**C**GCCATGGCGTTAGTA-3′	pHPI1107	pHPI1124
K27	5′-^{72}AAAGCGTCTA**C**CCATGGCGT-3′	pHPI1004	pHPI1064
K102	5′-^{71}GAAAGCGTCTAG**G**CATGGCGTTAGTA-3′	pHPI1108	pHPI1125
K103	5′-^{71}GAAAGCGTCTAGC**G**ATGGCGTTAGTA-3′	pHPI1109	pHPI1126
K104	5′-^{71}GAAAGCGTCTAGC**T**ATGGCGTTAGTA-3′	pHPI1110	pHPI1127
K14	5′-^{77}GTCTAGCC**T**TGGCGTTAGTATG-3′	pHPI972	pHPI1072
K128	5′-^{71}GAAAGCGTCTAGCCA**C**GGCGTTAGTA-3′	pHPI1114	pHPI1131
K129	5′-^{71}GAAAGCGTCTAGCCA**A**GGCGTTAGTA-3′	pHPI1115	pHPI1132
K29	5′-^{83}CCATGGCGTT**T**GTATGAGTG-3′	pHPI1005	pHPI1077
K15	5′-^{88}GCGTTAGT**T**TGAGTGTCGTG-3′	pHPI973	pHPI1062
K18	5′-^{89}CGTTAGTA**A**GAGTGTCGTG-3′	pHPI974	pHPI1063
Double substitution mutations			
K124	5′-^{71}GAAAGCGTC**C**AGCCA**C**GGCGTTAGTA-3′	pHPI1113	pHPI1130
K110	5′-^{65}CACGCAGAAA**A**CG**C**CTAGCCAT-3′	pHPI1112	pHPI1129
K14–15	5′-^{77}GTCTAGCC**T**TGGCGTTAGTATG-3′ (first) 5′-^{88}GCGTTAGT**T**TGAGTGTCGTG-3′ (second)	pHPI1117	pHPI1073
K144	5′-^{71}GAAAGCGTC**A**AGCC**T**TGGCGTTAGTA-3′ 5′-TACTAACGCCA**A**GGCT**T**GACGCTTTC-3′	pHPI1119	pHPI1136
K145	5′-^{71}GAAAGCGTC**C**AGCC**G**TGGCGTTAGTA-3′ 5′-TACTAACGCCA**C**GGCT**G**GACGCTTTC-3	pHPI1120	pHPI1137
Insertion mutations			
K105	5′-^{71}GAAAGCGTC**C**TGCCAT**G**GGCGTTAGTA-3′	pHPI1111	pHPI1128
K28	5′-^{71}GAAAGCGTCT**TTT**AGCCATG-3′	pHPI1034	pHPI1070
K16	5′-^{80}TAGCCATG**TAG**GCGTTAGTATG-3′	pHPI1041	pHPI1076
Deletion mutations			
K130	5′-^{71}GAAAGCGTCTA*CCATGGCGTTAGTA-3′	pHPI1116	pHPI1133
K131	5′-^{71}GAAAGCGTCTA**CATGGCGTTAGTA-3′	pHPI1117	pHPI1134
K132	5′-^{71}GAAAGCGTCTA***ATGGCGTTAGTA-3′	pHPI1118	pHPI1135

W.T.: wild type; N.C.: negative control



: insertions or deletions in the apical loop

: single substitutions in the apical loop or bulge

: mutations affecting only the upper stem

: double substitutions in the apical loop or both in the apical loop and the internal bulge.

K102 and K103, K104) resulted in a moderate effect, varying between 40 and 70% of the translation activity observed with the wild type (Fig. 2B). These data showed that the first (U-80) and sixth (A-85) bases of the apical loop of domain II are critical, while the identity of the remaining nucleotides is not essential for IRES activity. Notably, U-86 in contrast to U-80 had no effect on LUC expression although U-80 and U-86 are both located at the basis of the loop structure.

3. To investigate the importance of U-80 and A-85 in IRES function, a series of double nucleotide substitutions were introduced in the apical loop (Fig. 1C). Mutation K124 introduced a U-80-C and U-86-C double substitution in order to examine the importance of having two identical pyrimidines at the basis of the loop. Additionally, mutations K144 and K145, each introducing a double nucleotide substitution of U-80-A, A-85-U and U-80-C, A-85-G, respectively, were designed to address the existence of a putative Watson–Crick interaction between the bases U-80 and A-85. K144 and K145 mutations test the possible restoration of IRES activity from mutations K99 and K100, respectively. However, none of those mutations was able to fully restore the LUC production (Fig. 2C), suggesting that the identity of U-80 and A-85 is critical for IRES activity.
4. To further address the importance of the upper segment of domain II on IRES function, a number of mutations were introduced into the adjacent stem and bulge (Fig. 1D). In the context of the terminal bulge, mutations K29 and K15 introduce an A-to-U change of the highly conserved A-93 and A-96, respectively, while mutation K18 carries a U-to-A change of U-97. Mutations K15 and K18 did not significantly affect the IRES activity, while mutation K29 had a moderate effect on IRES function (Fig. 2D). These data suggest that similar to the case of the apical loop, the identity of most bases of the upper bulge of domain II is not absolutely essential for IRES function. Interestingly, the double substitution mutation K14–15 that introduces A-to-U changes at positions 96 and 85 of the apical part of domain II (Fig. 1D), resulted in a complete inhibition of the IRES activity (Fig. 2D). This emphasizes the importance of selected bases in the apical unpaired regions of domain II on IRES function. In the context of the upper stem, mutation K105 (Fig. 1D), which introduced an extra GC base pair at the top of the stem, caused a moderate effect on IRES activity (Fig. 2D). In contrast, mutation K110 (Fig. 1D), which introduced a double nucleotide substitution of G-75-A and U-78-C and was designed to create a more stable base-pairing interaction within the upper stem, did not alter IRES function (Fig. 2D). Thus, optimizing the base pairing in the upper stem of domain II did not appear to confer any translational advantage to the IRES.

4. Discussion

In this study, we have employed an extensive mutational analysis within the apical part of domain II in order to address the functional role of this region on IRES-dependent translation.

Our studies confirmed the structural importance of the apical stem-loop segment on the IRES function as insertion and deletion mutations affecting the secondary structure of this region severely inhibited translation. On the other hand, our data did not support an essential role on translation for the previously identified conserved nucleotide motifs of the upper segment of domain II [4,9]. Indeed, single nucleotide substitutions of the conserved A-81, C-83, C-84 (apical loop), A-93, A-96 (internal bulge) nucleotides had only moderate effect on translation efficiency. Additionally, single changes of G-82 and U-86 had absolutely no effect on IRES activity. Similarly, it was recently reported that isolated small changes in the upper single-stranded regions of domain II appeared to retain IRES function [23,25]. Notably, similar results were reached for the conserved pyrimidine-rich motifs of the apical loop of domain III [13].

On the other hand, single nucleotide substitutions of U-80 and A-85 strongly inhibited the IRES activity. Interestingly, these mutations had a more severe effect on translation than the previously described deletion mutations. Furthermore, mutation K14–15 that alters A-85 and A-96 to uridines completely abolished IRES function. Whether these nucleotides represent a sequence- or a structural-specific element is currently unknown. However, a series of double nucleotide substitutions (mutations K144, K145) designed to maintain a putative Watson–Crick interaction between positions 80 and 85 failed to restore IRES function. Additionally, mutation K124, designed to maintain two identical pyrimidines at the basis of the apical loop, restored only marginally the IRES activity. Furthermore, mutations K99 and K129 revealed opposing effects on the IRES function despite the fact that each of them is predicted to affect similarly the secondary structure of the apical loop. Thus, taken together these results suggested that, in addition to the secondary structure, selected bases of the apical loop are critical for the viral RNA translation. On the other hand, the integrity of the conserved nucleotide motifs in the apical segment of domain II was not found essential for IRES function, at least in the context of the dicistronic vector.

It is of interest to note that the most critical nucleotides for IRES activity, U-80 and A-85, are accessible to solvent upon 40S ribosomal subunit binding, in contrast to the least critical bases for IRES function, G-82 and U-86 [30]. Therefore, the strong inhibitory effect caused by mutations at positions 80 and 85 may suggest a possible contribution of those bases to the viral RNA decoding process. Because of the high degree of sequence conservation of the apical region of domain II in

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Fig. 2. I: In vitro translation activity from reticulocyte lysates programmed with equal amounts of dicistronic RNAs containing HCV IRES mutant variants. The in vitro translation products (identified on the left) were separated on SDS–PAGE and analyzed by autoradiography. II: In vivo translation activities of HCV IRES variants in the context of the dicistronic constructs. Duplicate cultures of HepG2 cells were transfected with plasmids containing the mutated IRESs and the activity of each mutation was calculated by determining the ratio of LUC to CAT activity. Bars represent the means obtained in three to five separate experiments of duplicate cultures. Error bar represents the standard deviation. Insertions, deletions (A) and single substitutions (B) within the apical loop. Double substitutions within the apical loop (C) and mutations within the internal bulge and the upper stem (D). W.T., wild type; N.C., negative control; MOCK, no RNA (in vitro) or DNA (in vivo) added.

diverse viral IRES sequences, it is tempting to speculate that this region may still play a key role on IRES function, other than its direct interaction with the basic translation machinery. One such possibility could be at the level of regulation of the IRES activity through novel RNA–RNA or RNA–protein interactions. Indeed, recent studies have shown that the HCV IRES activity is cell cycle dependent [37]. Most importantly, domain II is proposed to adopt different conformations within the IRES structure via the flexible unstructured region that joins domain II with III/IV [11,26]. Although experimental data are still lacking, we would like to propose that the apical part of domain II alone or together with the flexible unstructured region, may represent part of a regulatory switch for the IRES function. Further work is required to test this hypothesis.

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