Almost the entire 5′ non-translated region of hepatitis C virus is required for cap-independent translation

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Abstract To investigate which hairpin structures within the 5′ untranslated region of hepatitis C virus (HCV) are necessary for cap-independent translation, mutants were constructed that lack one or more hairpin structures. Here we demonstrate, by constructing precisely defined hairpin deletion mutants, that with the exception of the most 5′ located hairpin structure, which on deletion shows an increase on translation, each of the predicted hairpins is found to be essential for cap-independent translation. In addition, we demonstrate that HCV 5′UTR driven translation is stimulated by poliovirus 2A^{pro} co-expression.

Key words: Hepatitis-C virus; 5′ Untranslated region; Secondary structure; Translation; Internal ribosome entry site, IRES; 2A

1. Introduction

Hepatitis C virus (HCV) is the causative agent of the majority of cases of post-transfusion non-A non-B viral hepatitis [1,2]. It is related to flavi- and pestiviruses and has been classified within a separate genus of the family Flaviviridae. Members of the Flaviviridae are enveloped viruses containing an RNA genome of positive polarity with a single, large open reading frame. The HCV genome is approximately 9.5 kb and serves as a template for the viral polyprotein which is co- and post-translationally processed [3,4].

The 5′ untranslated regions (5′UTR) of both HCV and pestiviruses are relatively long (340–372 nucleotides) and contain several AUG codons upstream of the authentic translation initiation codon. Comparison of the HCV and pestivirus 5′UTR sequences have revealed several domains with a high level of sequence identity [5]. Computer assisted RNA secondary structure prediction and biochemical analysis indicated that these 5′UTRs could form a complex, but overall similar, secondary structure involving multiple stem–loop structures (Fig. 1A) [6].

Initial studies in the mechanism of protein translation initiation of HCV indicated that translation HCV RNA was 5′-end cap-dependent [7]. Other studies contradicted these results and provided evidence for the presence of an internal ribosome entry site (IRES) which allows cap-independent translation initiation of both HCV and pestivirus RNA ([8–10]; Bredenbeek et al., in preparation). The precise sequences and RNA secondary structures which are important for proper IRES functioning have yet to be determined. In this study we report the effects of precise deletion of the predicted stem–loop structures within the HCV 5′UTR on protein translation initiation.

2. Materials and methods

2.1. Cells and medium

Hep2 (human larynx carcinoma) and BT7-H cells, an African green monkey kidney cell line expressing T7 DNA dependent RNA polymerase [11], were grown in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 10% (v/v) fetal calf serum, vancomycin 50 mg/l and gentamicyn 25 mg/l. BT7-H cells were cultured in the presence of 500 μg/ml (active compound) of Geneticine (Gibco-BRL).

2.2. Construction of pWT-CAT and pWT-CAT deletion mutants

A DNA fragment containing the entire 5′UTR (341 nucleotides) and 8 nucleotides of the capsid coding region of the HCV H-strain, was PCR amplified from plasmid p19AD (Inchauspé, personal communication). The resulting PCR product is flanked by an *Eco*RI restriction site and a T7 RNA promoter at the 5′ end, and by a *Kpn*I restriction site at the 3′ end. A CAT fragment, flanked at the 5′ end by a *Kpn*I site and at the 3′ end by a *Hin*dIII restriction site was created by PCR. The *Eco*RI and *Nco*I restriction sites and the authentic translation initiation codon of the CAT gene were destroyed by site directed mutagenesis [12]. The *Eco*RI–*Kpn*I digested T7 promoter-UTR fragment and the *Kpn*I–*Hin*dIII digested CAT fragment were cloned into an *Eco*RI–*Hin*dIII digested pUC20 vector, resulting in pWT-CAT (Fig. 1B). T7 RNA polymerase directed transcripts from this plasmid have 2 additional G residues upstream of the 5′ end of the HCV 5′UTR.

Synthetic oligonucleotides containing contiguous sequences that flank predicted hairpin structures within the HCV 5′UTR (Fig. 1A) were used as primers to amplify DNA from pWT-CAT by PCR, creating DNA fragments lacking single or multiple hairpin structures. The PCR products were digested with suitable restriction enzymes and inserted into pWT-CAT to produce a series of precise deletion mutants (Fig. 1B). An additional deletion mutant, pSmaI^{1}ΔSmaI^2-CAT (SmaI^1 and SmaI^2 indicate the *Sma*I sites at position 130 and 317, respectively) was created by deletion of the sequence between the indicated restriction sites present in pWT-CAT (Fig. 1).

To construct a plasmid for the expression of bicistronic RNA the entire HCV 5′UTR and 8 nucleotides of the HCV capsid gene were amplified by PCR and fit with a 5′ *Eco*RI and a 3′ *Kpn*I restriction site. The *Kpn*I site was used to fuse the HCV 5′UTR and the CAT gene as described above. The *Eco*RI–*Hin*dIII (5′UTR-CAT) fragment of p(ΔT7)WT-CAT was subsequently cloned into *Eco*RI–*Hin*dIII digested pBluescriptKS(+) (Stratagene) to create pBL-WT-CAT. A *Bam*HI fragment containing the β-galactosidase gene was inserted into the *Bam*HI site upstream of the HCV 5′UTR in pBL-WT-CAT, creating

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Abbreviations: EMCV, encephalomyocarditis virus; RSV, Rous sarcoma virus; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase.

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pβ-WT-CAT from which a bicistronic mRNA with β-galactosidase as the first cistron and CAT as the second cistron can be transcribed by T7 RNA polymerase (Fig. 3C). Deletions in the HCV 5'UTR within pβ-WT-CAT were created by either replacement of appropriate fragments from monocistronic deletion mutants (ΔI, 5'ΔSmaI[2]) or by newly generated PCR fragments (ΔA, ΔB, Δ(ABC)). This resulted in mutant bicistronic plasmids pβ-ΔA-CAT, pβ-ΔB-CAT, pβ-Δ(ABC)-CAT, pβ-ΔI-CAT, pβ-5'ΔSmaI[2]-CAT. All PCR generated fragments were sequenced prior to translation studies.

2.3. In vitro transcription and translation

Plasmid DNAs were linearized with *Hin*dIII and transcribed in vitro using T7 RNA polymerase. Uncapped monocistronic RNAs were phenol/chloroform extracted and translated in reticulocyte lysate (20 ng/μl; Promega) as described by the supplier. [^{35}S]Methionine labelled translation products were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. DNA transfection of BT7-H cells and preparation of cell lysates

BT7-H cells ($1 \cdot 10^6$) were transfected using 200 μl of OPTIMEM (Gibco-BRL) containing 5 μg of CsCl-gradient purified plasmid DNA and 15 μl of lipofectin (Gibco-BRL). At 14–18 h post-transfection the cells were washed twice with PBS and collected with a rubber policeman in 1 ml of TEN buffer (40 mM Tris-HCl (pH7.5), 1 mM EDTA, 15 mM NaCl). The cells were pelleted for 2 min at room temperature and resuspended in 200 μl of 0.25 M Tris-HCl, pH 8.0, and lysed by 3 cycles of freeze–thawing. Cell debris was removed by centrifugation and endogenous acetylase activity was inactivated by heating the extract at 60°C for 10 min.

2.5. Determination of CAT expression

Cell lysates (0.5–50 μl) were incubated with 200 μg/ml *n*-butyryl Coenzyme A, 0.25M Tris-HCl (pH 8.0) and 2 μCi/ml D-threo-[dichloroacetyl-1-^{14}C] chloramphenicol (56 mCi/mmol) for 2 h at 37°C. CAT activity was determined by liquid scintillation counting (LSC) following xylene phase-extraction [13]. CAT values were adjusted for differences in protein concentration of the lysates; these were determined with a Coomassie blue based protein assay (Bio-Rad). CAT expression of bicistronic RNAs was determined using a CAT Elisa (Boehringer-Mannheim)

2.6. Polio 2A protease co-transfection

BT7-H cells were transfected with pWT-CAT, pEMCV-CAT [11] or pRSV-CAT [14] and co-transfected with either pEP2A or pEP2A(H20N) [15] which express an active or inactive poliovirus 2A protease, respectively. 5 μg of each plasmid, 30 μl of lipofectin and 200 μl of OPTIMEM was used for each transfection. At 48 h post-transfection cell lysates were prepared and assayed for CAT activity.

2.7. Expression of bicistronic RNAs

Near confluent monolayers of Hep2 cells were infected at a m.o.i. of 10 with vTF7-3, a vaccinia virus recombinant that expresses the T7 DNA dependent RNA polymerase [16]. After the virus was left to absorb for 45 min at room temperature, the cells were transfected with equimolar amounts of plasmid DNA. 6 h post-transfection the cells were lysed and assayed for CAT activity.

3. Results and discussion

To determine the capability of the HCV 5'UTR used in this study to direct cap-independent translation, the HCV 5'UTR driven translation in uncapped monocistronic transcripts was tested in vivo in the presence of a picornaviral induced translational host shut-off. The pWT-CAT plasmid was co-transfected with a plasmid expressing either a functional (pEP2A) or inactivated (pEP2A(H20N) [15] poliovirus 2A protease into BT7-H cells. The poliovirus 2A protease induces cleavage of the p220 component of the eukaryotic translation initiation factor eIF4-F [17]. Integrity of this initiation factor is essential for cap-dependent translation [18]. As a control, EMCV-CAT RNA [11], which is known to contain a functional EMCV IRES and should therefore be resistant to the effects induced by 2A co-expression, was used in parallel transfections. To verify the action of poliovirus 2A protease, we also tested the effect of 2A co-expression on the translation of RSV-CAT RNA. Transcription of this RNA is driven by the RSV promoter and results in capped RNA. The data shown in Fig. 2 demonstrate that translation of WT-CAT RNA is resistant to poliovirus 2A co-expression and therefore cap-independent (Fig. 2). Compared to co-transfection of pWT-CAT and pEP2A(H20N) the CAT activity is increased in the presence of an active 2A protease. A similar effect was observed for the transfection involving pEMCV-CAT. It has been suggested that this effect of 2A on EMCV directed translation initiation is due to a reduced

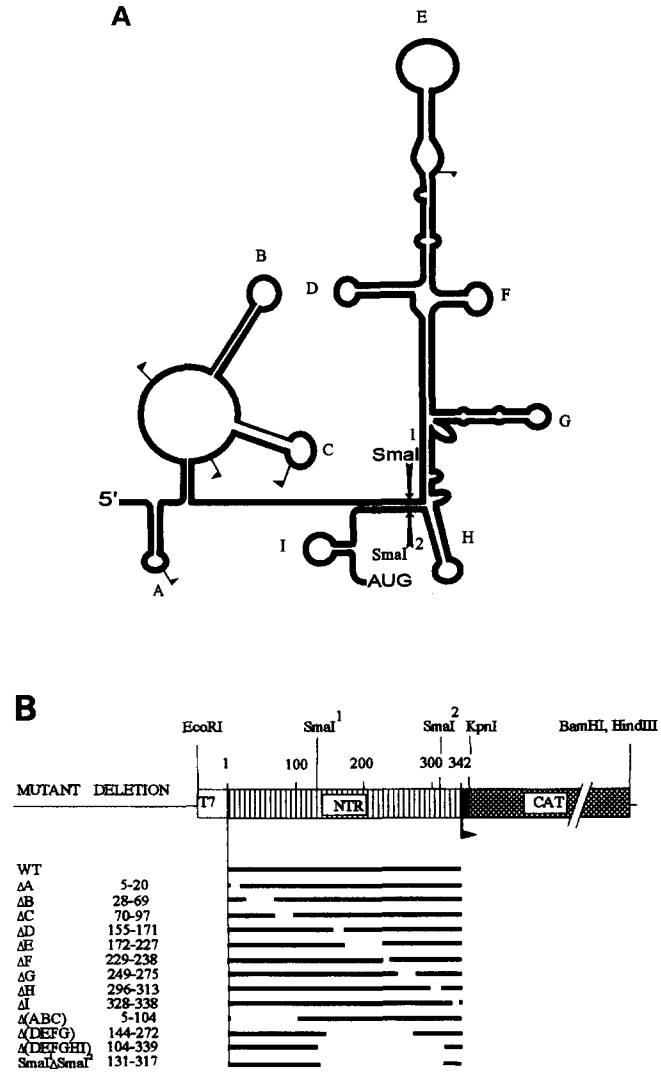

Fig. 1. (A) Proposed secondary structure model based on computer assisted folding of the HCV 5'UTR (H-strain). Individual stem–loop structures are labelled A to I. The position of the translation initiation site of the HCV polyprotein is indicated by AUG. Flags indicate the position of the upstream AUG codons in the 5'UTR sequence of this HCV isolate. (B) Diagram showing pWT-CAT and an overview of the 5' UTR deletion mutants. The actual nucleotides deleted for each mutant are indicated. T7, T7 RNA polymerase promoter. The flag indicates the position of the authentic HCV initiation site. Translation initiation at this AUG codon will result in synthesis of a HCV capsid/CAT fusion protein. Hatched box, sequences of the HCV 5'UTR; solid box, capsid coding region; cross hatched box, CAT coding region.

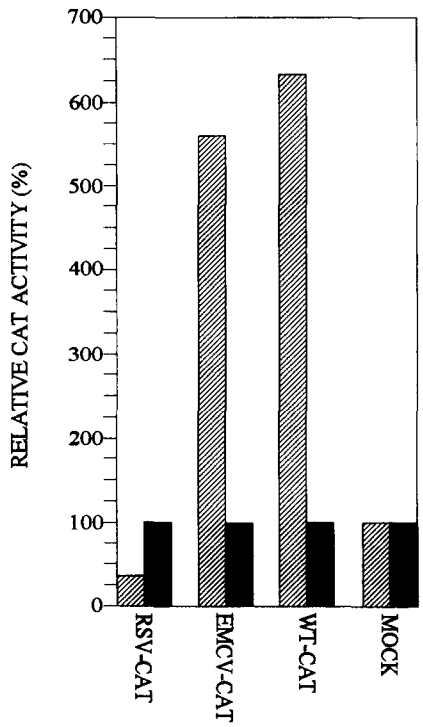

Fig. 2. Relative CAT activity of HCV 5'UTR-CAT RNAs after co-transfection with poliovirus 2A protease expressing plasmids. BT7-H cells were transfected with either pRSV-CAT, pEMCV-CAT or pWT-CAT and either pEP2A (hatched bars) or pEP2A(H20N) (black bars). Translation of constructs cotransfected with pEP2A(H20N) was set at 100%.

competition with cellular mRNAs for translation initiation factors [11]. This could also explain the observed effect of 2A on the HCV 5'UTR driven translation. The significance of this finding is not yet understood as there is no clear homology of any HCV protein with the poliovirus 2A protease. HCV therefore resembles the situation present in EMCV, which itself is resistant to a poliovirus host shut-off and lacks the equivalent of the 2A protease. As expected RSV-CAT translation was decreased in the presence of the active 2A protease as its translation is cap-dependent.

To analyze the role of hairpin structures in the HCV 5'UTR on translation a secondary structure model was used to direct mutagenesis. This model (Fig. 1A) is based on conservation and co-variance within the 5'UTR of HCV and pestiviruses and is very similar to the structure proposed by Brown et al. [6], although the predicted folding of hairpins G and H is different. A PCR based strategy was used to create 5'UTR mutants that lack the sequences involved in the formation of the hairpin structures (Fig. 1A and B).

The effect of these deletions on translation was analyzed by in vitro translation of uncapped RNAs. Translation of RNA derived from the mutant lacking hairpin A results in a significant increase in the production of CAT protein when compared to the WT-CAT RNA (Fig. 3A). In contrast, translation of RNA derived from the other deletion mutants results in a strong reduction in CAT protein production when compared to the WT-CAT RNA.

To determine whether the hairpin deletions within the HCV 5'UTR cause a similar effect on cap-independent translation in vivo, BT7-H cells were transfected with plasmid DNA of pWT-CAT or the deletion mutants and assayed for CAT activity. Deletion of hairpin A resulted in an almost 2-fold increase in translation efficiency compared to WT (Fig. 3B). Deletion of any other hairpin or combination of hairpins resulted in background levels of CAT activity. Identical results were obtained using the recombinant vaccinia vTF7-3 infected Hep2 cells in expression studies involving this set of HCV 5'UTR deletion mutants (data not shown).

A possible explanation for the translation enhancement of the monocistronic ΔA construct is that the presence of hairpin A could directly inhibit IRES activity. Alternatively, enhanced accessibility of the 5' end to ribosomes could possibly result in a more efficient scanning of ΔA-CAT RNA, as suggested by Yoo et al. [7]. To examine whether the observed translation stimulation in pΔA-CAT was due to a more efficient scanning mechanism and to verify the results obtained with some of the other mutants, the effect of these deletions was tested in a bicistronic context (Fig. 3C). The following plasmids for the expression of bicistronic mRNAs were constructed: pβ-ΔA-CAT, pβ-ΔB-CAT, pβ-Δ(ABC)-CAT, pβ-ΔI-CAT, pβ-5'ΔSmaI[2]-CAT. Plasmids encoding bicistronic RNAs were transfected into vTF7-3 infected Hep2 cells and CAT activity was determined (Fig. 3D). Translation of CAT from β-ΔA-CAT was over 2 times greater than translation from the pβ-WT-CAT construct. This result therefore demonstrates that the observed enhancement of CAT production after deleting hairpin A does not result from a more efficient scanning of this mutant HCV 5'UTR and indicates that the effect is caused by a direct effect of hairpin A on the IRES. Translation activity of the bicistronic RNAs containing the other deletion mutants (pβ-ΔB-CAT, pβ-ΔI-CAT, pβ-Δ(ABC)-CAT and pβ-5'ΔSmaI[2]-CAT) was at background levels, confirming the results obtained with the monocistronic RNAs.

The data presented in this study show that hairpin structures B through I are essential for HCV IRES function in vitro and in vivo. Northern blot analysis did not reveal significant differences in the amount of RNA transcribed from the transfected plasmids (data not shown). This demonstrates that the effect of these deletions is at the level of translation and not RNA transcription or stability.

The 5' border of the HCV IRES has been mapped between nt 38–84 of HCV UTR [9] or 110–156 [8], respectively (positions according to the numbering used in this paper). If these findings are superimposed on the proposed RNA folding for the HCV 5'UTR, then the result of Wang et al. agrees with our finding that deletion of hairpin B severely impairs HCV IRES functioning. In contrast, the results reported by Tsukiyama-Kohara et al. [8] imply that the 5' border of the IRES is located downstream of hairpin C. We found that deletion of either hairpin B or C was lethal for HCV IRES activity. We have no explanation for this discrepancy at present. Attempts to map the 3' border of the HCV IRES revealed that a deletion of 9 nucleotides upstream of the initiator AUG is lethal for IRES activity [9]. Such a deletion would include stem–loop I and is consistent with our finding that the deletion of this predicted hairpin structure abolishes HCV IRES activity. However, hairpin B and I are not the only essential elements required for proper function of the HCV IRES. Our data are the first to demonstrate that each of the predicted stem–loops B through I are essential elements of the HCV IRES.

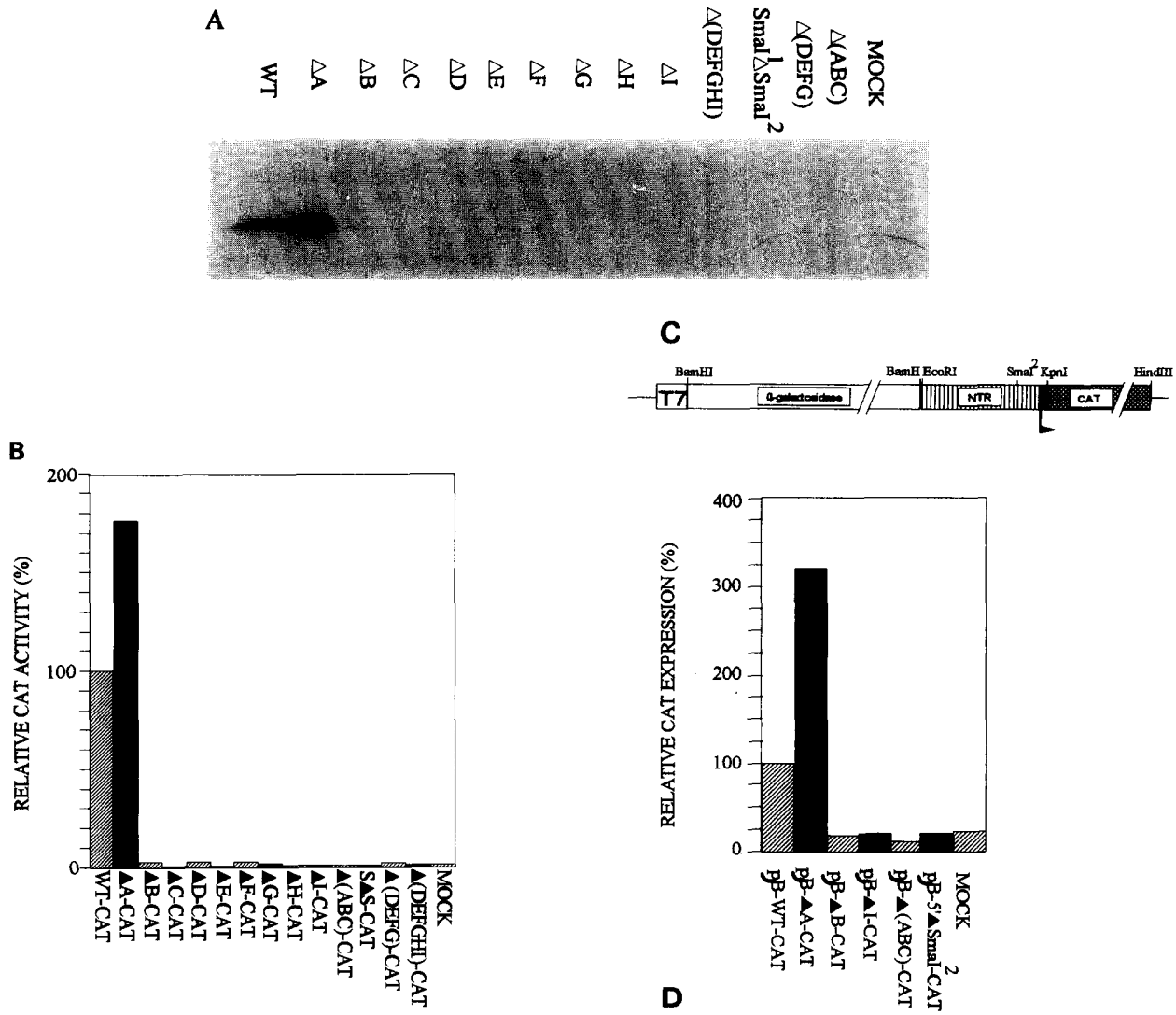

Fig. 3. (A) In vitro translation of WT-CAT and mutant HCV 5'UTR- CAT RNAs in reticulocyte lysate. ^{35}S-Labelled translation products were analyzed by SDS-PAGE. (B) The relative CAT activity obtained following DNA transfection of 5'UTR-CAT plasmids DNA into BT7- H cells. CAT activity was determined using the LSC method. The CAT activity/mg protein for pWT-CAT was set at 100%. (C) Diagram of a plasmid used in the expression of bicistronic RNAs (shown is pβ-WT- CAT). T7, T7 RNA promoter; *Eco*RI, *Bam*HI, *Hin*dIII, *Kpn*I and *Sma*I, restriction sites used. The flag indicates the position of the authentic HCV initiation site. Translation initiation at this AUG codon will result in synthesis of the HCV capsid/CAT fusion protein. Hatched box, sequences of the HCV 5'UTR; solid box, capsid coding region; cross hatched box, CAT coding region; open box, β-galactosidase gene. (D) Relative CAT expression 6 h post-transfection in vTF7-3 infected Hep2 cells transfected with the indicated plasmids. The CAT value obtained with pβ-WT-CAT was set at 100%.

The report of Yoo et al. [7] in which no IRES function of the HCV 5'UTR could be detected, shows that hairpin A is a 'potent translation inhibitor' for 5'-end dependent translation initiation. This inhibitory effect of hairpin A has not been previously observed in the context of a HCV IRES [8,9]. Our data confirm the inhibitory effect of hairpin A but in contrast to the data published by Yoo et al. we are able to show that hairpin A decreases the IRES activity for the HCV 5'UTR.

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