Hepatitis C virus (HCV) NS5A protein downregulates HCV IRES-dependent translation

Katerina I. Kalliampakou, Maria Kalamvoki and Penelope Mavromara

Molecular Virology Laboratory, Hellenic Pasteur Institute, 127 Vas. Sofias Avenue, Athens 115 21, Greece

Correspondence
Penelope Mavromara
penelopm@hol.gr

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Translation of the hepatitis C virus (HCV) polyprotein is mediated by an internal ribosome entry site (IRES) that is located mainly within the 5′ non-translated region of the viral genome. In this study, the effect of the HCV non-structural 5A (NS5A) protein on the HCV IRES-dependent translation was investigated by using a transient transfection system. Three different cell lines (HepG2, WRL-68 and BHK-21) were co-transfected with a plasmid vector containing a bicistronic transcript carrying the chloramphenicol acetyltransferase (CAT) and the firefly luciferase genes separated by the HCV IRES sequences, and an expression vector producing the NS5A protein. Here, it was shown that the HCV NS5A protein inhibited HCV IRES-dependent translation in a dose-dependent manner. In contrast, NS5A had no detectable effect on cap-dependent translation of the upstream gene (CAT) nor on translation from another viral IRES. Further analysis using deleted forms of the NS5A protein revealed that a region of about 120 aa located just upstream of the nuclear localization signal of the protein is critical for this suppression. Overall, these results suggest that HCV NS5A protein negatively modulates the HCV IRES activity in a specific manner.

INTRODUCTION

Hepatitis C virus (HCV) infects about 3 % of the world's population and is a leading cause of chronic liver disease that often progresses to cirrhosis and hepatocellular carcinoma (Alter, 1997; Hoofnagle, 1997; WHO, 1999). A vaccine against HCV is not available at present and therapeutic approaches are still limited (Moradpour & Blum, 1999; Gale & Beard, 2001).

HCV is classified into the genus *Hepacivirus* of the family *Flaviviridae* (Reed & Rice, 2000). Like all the members of the family, HCV is an enveloped, single-stranded, positive-sense RNA virus. Its genome (about 9600 nt) is flanked at both termini by conserved, highly structured non-translated regions (NTRs) and encodes a polyprotein precursor (about 3000 aa), which is proteolytically processed by host and viral proteases to produce the structural (core, E1, E2 and p7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins of the virus. Recently, an additional protein has been identified. This protein is encoded by an alternative open reading frame within the core coding region and its function remains unknown (Walewski *et al.*, 2001; Xu *et al.*, 2001; Varaklioti *et al.*, 2002).

Translation initiation of the HCV genome is controlled by an IRES (Hellen & Pestova, 1999; Rijnbrand & Lemon, 2000). This mechanism first identified for the members of the family *Picornaviridae*, is also used by the members of *Hepacivirus* and *Pestivirus* genera of *Flaviviridae*. The HCV IRES is located mainly within the 5′ NTR of the viral RNA and directs the binding of ribosomes in close proximity to the start codon of the viral open reading frame (Reynolds *et al.*, 1995; Rijnbrand *et al.*, 1995; Honda *et al.*, 1996). Interestingly, the HCV IRES does not have a strict requirement for canonical translation initiation factors (eIFs) other than eIF2 and eIF3, and its activity varies with the cell cycle (Pestova *et al.*, 1998; Honda *et al.*, 2000). On the other hand, a number of transacting cellular factors have been shown to interact with the HCV IRES. These include the polypyrimidine-tract-binding protein (Ali & Siddiqui, 1995), the human La antigen (Pudi *et al.*, 2003; Izumi *et al.*, 2004), the poly(rC)-binding protein 2 (Fukushi *et al.*, 2001a), the heterogeneous nuclear ribonucleoprotein L (Hahm *et al.*, 1998) and ribosomal protein factors S9 (Fukushi *et al.*, 1999) and S5 (Fukushi *et al.*, 2001b). Furthermore, viral sequences located at distal regions from the HCV IRES (Ito *et al.*, 1998; Ito & Lai, 1999; Wang *et al.*, 2000; Murakami *et al.*, 2001; Imbert *et al.*, 2003; Kim *et al.*, 2003) as well as selected viral proteins (Shimoike *et al.*, 1999; Kato *et al.*, 2002; Zhang *et al.*, 2002; He *et al.*, 2003; Li *et al.*, 2003) appear to modulate the efficiency of the HCV IRES activity. However, regulation of IRES-mediated translation initiation is poorly understood and most of the results concerning the effects of viral proteins on IRES function remain controversial.

Among the HCV viral proteins, NS5A is a multifunctional

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serine phosphoprotein of 56–58 kDa (Tanji *et al.*, 1995; Reed *et al.*, 1997; Hirota *et al.*, 1999) that is implicated in viral pathogenesis (Bartenschlager & Lohmann, 2000; Blight *et al.*, 2000; Krieger *et al.*, 2001; Tan & Katze, 2001; Tellinghuisen & Rice, 2002). Although a functional nuclear localization signal (NLS) exists in its carboxy-terminal part, the NS5A protein is found anchored to the cytoplasmic side of the endoplasmic reticulum membranes through an amino-terminal amphipathic α-helix, and modulates virus replication by its direct association with the virus replication complex (Ide *et al.*, 1996; Elazar *et al.*, 2003). Interestingly, cell culture-adaptive mutations in the NS5A amino acid sequence significantly enhance the efficiency of HCV replicons (Krieger *et al.*, 2001; Lohmann *et al.*, 2001; Blight *et al.*, 2003). Moreover, NS5A interacts with a number of cellular proteins, thereby affecting numerous cellular pathways (Tan & Katze, 2001; Macdonald & Harris, 2004), and plays a major role in controlling host antiviral mechanisms. Notably, recent studies suggest that NS5A may be cleaved by calpains and caspases to produce stable carboxy-terminal truncated forms of the protein (Satoh *et al.*, 2000; Goh *et al.*, 2001; Kalamvoki & Mavromara, 2004).

In this study, we investigated the effect of the HCV NS5A protein on HCV IRES-dependent translation by using a transient cell-based expression system. We found that the HCV NS5A protein inhibited HCV IRES-dependent translation in a specific and dose-dependent manner. Moreover, we found that a region of about 120 aa located at the carboxy-terminal part of the protein was critical for this suppression. These findings might help elucidate the predicted temporal regulation of viral RNA translation in the context of a switch from the translation mode to the replication mode of the virus life cycle.

METHODS

Plasmids. All plasmids were constructed using standard methods. Plasmid pHPI1484 carries, under the control of the human cytomegalovirus (HCMV) immediate early promoter, the CAT-IRES^{HCV}-LUC bicistronic transcriptional unit, containing the chloramphenicol acetyltransferase (CAT) as the upstream and the firefly luciferase (LUC) as the downstream cistron, respectively, with the sequence of the HCV IRES placed in-between the two cistrons. This construct carries the HCV IRES together with the first 23 aa of the HCV core coding sequence fused in-frame to the LUC protein, and it was made by inserting the *Hin*dIII–*Sac*I blunt-ended fragment of pHPI933 that contains the above bicistronic unit (Kalliampakou *et al.*, 2002) into the *Xba*I blunt-ended site of the pA-EUA2 expression plasmid vector. This plasmid will be referred to as the CAT-IRES^{HCV}-LUC vector. Plasmid pA-EUA2 (kindly provided by A. L. Epstein, Centre de Genetique Moleculaire et Cellulaire, University Claude Bernard Lyon 1, France) has been described elsewhere (Kalamvoki & Mavromara, 2004). Briefly, this plasmid carries two expression cassettes that are transcribed in opposite directions. The first comprises the herpes simplex virus type 1 immediate early (α22/α47) promoter that controls the expression of green fluorescent protein, which permits the estimation of transfection efficiency. The second comprises the HCMV promoter that is followed by a short polylinker (restriction sites: *Nhe*I, *Xba*I, *Not*I). Plasmid pHPI1487 carries the CAT-IRES^{EMCV}-LUC bicistronic transcriptional unit in which the HCV IRES has been replaced by the encephalomyocarditis virus (EMCV) IRES and it was constructed by the following three-step cloning procedure. Firstly, the *Bam*HI–*Tth*III1 blunt-ended EMCV IRES containing fragment of the pIREShyg vector (vector that expresses the hygromycin B phosphotransferase protein under the translational control of the IRES of EMCV; Clontech) was inserted into the *Bam*HI blunt-ended site of pGEM-luc (Invitrogen). This gave rise to plasmid pHPI1485, where the sequence corresponding to the first 23 aa of hygromycin B phosphotransferase is fused in-frame to the LUC coding sequence. The junction between the two proteins was verified by dideoxy-sequence analysis. The fusion protein of this plasmid is under the translational control of the IRES of EMCV. Secondly, the *Xba*I–*Bam*HI fragment of pHPI1484 encoding the CAT protein, and the *Bgl*II–*Xba*I fragment of pHPI1485 containing the IRES^{EMCV}-LUC* sequences were inserted simultaneously into the *Xba*I site of pUC19 (New England BioLabs) to produce plasmid pHPI1486. Plasmid pHPI1486 carries the CAT-IRES^{EMCV}-LUC* sequences (coding sequence of LUC protein until the *Xba*I site). Thirdly, the *Xba*I–*Xba*I fragment of pHPI1484 containing the CAT-IRES^{HCV}-LUC* sequences was replaced by the *Xba*I–*Xba*I fragment of pHPI1486 containing the CAT-IRES^{EMCV}-LUC* sequences to yield plasmid pHPI1487 that contains the complete CAT-IRES^{EMCV}-LUC cassette. This plasmid will be referred to as the CAT-IRES^{EMCV}-LUC vector. Plasmid pA-EUA2 + *lacZ* (kindly provided by A. L. Epstein, Centre de Genetique Moleculaire et Cellulaire, University Claude Bernard Lyon 1, France) carries the coding sequence of β-galactosidase, cloned into the pA-EUA2 expression vector, under the control of the HCMV promoter. This plasmid will be referred to as the LACZ vector.

For the expression of the HCV-1a NS5A protein and its deleted forms the following plasmids were constructed. Plasmid pHPI1419 expresses the entire NS5A protein (aa 1–447) (HCV-1a) and was constructed by insertion of the *Hin*dIII blunt-ended fragment from pHPI611 (Kalamvoki *et al.*, 2002), which contains the coding sequence of NS5A, into the *Xba*I blunt-ended site of pA-EUA2. This plasmid will be referred to as the F-NS5A vector. Plasmid pHPI1433 expresses the amino-terminal half of NS5A protein (aa 1–230) and it was constructed by a two-step procedure. Firstly, the *Bam*HI–*Pvu*II blunt-ended fragment from pHPI611 (Kalamvoki *et al.*, 2002), which encodes the amino-terminal half of the NS5A protein (HCV-1a), was ligated into the *Xba*I blunt-ended site of pCI (Promega) giving rise to pHPI1405. Secondly, the *Nhe*I–*Nhe*I blunt-ended fragment of pHPI1405, which encodes the amino-terminal half of the NS5A protein, was ligated into the *Xba*I blunt-ended site of pA-EUA2. This plasmid will be referred to as the N1-NS5A vector. Plasmid pHPI1435 expresses the carboxy-terminal half of NS5A protein (aa 236–447) and it was constructed by a two-step procedure. Firstly, the nucleotide sequence encoding the carboxy-terminal half of NS5A (aa 236–447) was amplified by PCR from pHPI611 (Kalamvoki *et al.*, 2002). The primers used were: sense, 5′-CCAAGCTTGCC**ATG**GCTCCATCT-CTC-3′ and antisense, 5′-CTCGAGAAGC**TTA**GCAGCACACGA-3′ where the *Hin*dIII restriction sites are underlined and the translation initiation and stop codons, respectively, are shown in bold. The PCR conditions were as follows: 95 °C for 60 s followed by 35 cycles of 95 °C for 30 s, 60 °C for 60 s and 75 °C for 60 s and a final extension at 75 °C for 10 min. The amplified fragment was digested with *Hin*dIII, blunt-ended and inserted into the *Xba*I blunt-ended site of pCI, giving rise to pHPI1407. The coding sequence of the carboxy-terminal part of the NS5A protein was verified by dideoxy-sequence analysis. Secondly, the *Eco*RI–*Not*I blunt-ended fragment of pHPI1407, which encodes the carboxy-terminal half of the NS5A protein, was ligated into the *Xba*I blunt-ended site of pA-EUA2. This plasmid will be referred to as the C-NS5A vector. Plasmid pHPI1436 expresses the amino-terminal part of NS5A protein up to the NLS (aa 1–354), and it was constructed by a two-step procedure. Firstly, the *Hin*dIII–*Eco*RV blunt-ended PCR

product of NS5A from pHPI611 (Kalamvoki *et al.*, 2002) was ligated into the *Xba*I blunt-ended site of pCI giving rise to pHPI1409. The coding sequence of the protein was verified by dideoxy-sequence analysis. Secondly, the *Eco*RI–*Not*I blunt-ended fragment of pHPI1409 that encodes the above amino-terminal part of NS5A was ligated into the *Xba*I blunt-ended site of pA-EUA2. This plasmid will be referred to as the N2-NS5A vector. All of the above expression cassettes are shown in Fig. 1.

Plasmid pHPI1046 has been described elsewhere (Kalliampakou *et al.*, 2002). Briefly, this expression vector carries the previously described CAT-IRES^{HCV}-LUC bicistronic transcriptional unit controlled by the HCMV promoter. This plasmid will be referred to as the HCMV/CAT-IRES^{HCV}-LUC vector. Plasmid pHPI1483 results from pHPI1046 by deletion of the *Bgl*II–*Hin*dIII fragment followed by blunt-ending and religation of the vector. This procedure resulted in the deletion of the entire HCMV promoter sequence. Theoretically, this plasmid does not carry any eukaryotic promoter and it will be referred to as the HCMVdel/CAT-IRES^{HCV}-LUC vector.

Cells and transfection experiments. HepG2 cells (epithelium from human hepatocellular carcinoma) obtained from the ATCC, WRL-68 cells (human liver embryonic hepatoma) kindly provided by A. Budkowska (Institute Pasteur, Paris) and BHK-21 cells (baby hamster kidney) obtained from the ATCC were maintained in minimal essential medium (Gibco-BRL) (HepG2) and Dulbecco's modified eagle medium (Biochrom KG) (WRL-68 and BHK-21), supplemented with 10 % fetal bovine serum (Gibco-BRL), penicillin/streptomycin (5 IU ml^{-1}/50 mg ml^{-1}) and 2 mM L-glutamine. Cells, seeded in 12-well plates at confluence of about 30 % for HepG2 and 50 % for WRL-68 and BHK-21, were transfected using Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's protocol. In the transfection experiments that were performed with only one plasmid vector, 0·3 μg DNA per well was used. In the co-transfection experiments either 0·8 or 0·6 μg total plasmid DNA per well was used. At least three independent experiments were carried out for each set of transfections. The medium was replaced 24 h post-transfection. At 48 h post-transfection the cells were washed twice with ice-cold 1 × PBS and lysed in 160 μl 1 × Luciferase lysis buffer (Promega) according to the manufacturer's instructions.

Quantification of LUC and CAT. Quantification of LUC protein was performed by mixing 20 μl (out of the 160 μl) cell extracts with 100 μl Luciferase assay reagent (Promega) and the luminescence was measured directly by a Turner TD-20/20 luminometer. Quantification of CAT protein was performed with the CAT-ELISA kit (Boehringer Mannheim) according to manufacturer's instructions.

Immunoblotting. Samples (40 of 160 μl) of cell extracts were used in Western blot analysis. SDS-PAGE loading buffer was added to each sample, the samples were boiled for 3 min, analysed in 12 % denaturing polyacrylamide gels and transferred onto nitrocellulose membranes.

After blocking for 1·5 h [in 1 × PBS, 0·02 % (v/v) Tween 20, 10 % (w/v) dried milk], the membranes were incubated with the primary antibodies overnight at 4 °C. A rabbit polyclonal anti-NS5A antibody [diluted 1 : 100 in 1 × PBS, 0·04 % (v/v) Tween 20, 2 % (w/v) dried milk] was used for the detection of NS5A protein and deletion mutants of this protein (Kalamvoki *et al.*, 2002; Kalamvoki & Mavromara, 2004), whereas a mouse monoclonal anti-β-galactosidase antibody (Gibco-BRL) (diluted 1 : 500 in the same buffer as anti-NS5A antibody) was used for the detection of β-galactosidase protein. The membranes then were washed three times (10 min each) with a solution containing 1 × PBS, 0·04 % (v/v) Tween 20, 2 % (w/v) dried milk, and incubated at room temperature with the secondary antibodies diluted 1 : 1000 in the same solution. The secondary antibodies, anti-rabbit for NS5A protein (Dako) and anti-mouse for β-galactosidase protein (Dako), are conjugated with horseradish peroxidase. After washing, the membranes were soaked in enhanced chemiluminescence reagent (Pierce) and exposed to film (Kodak).

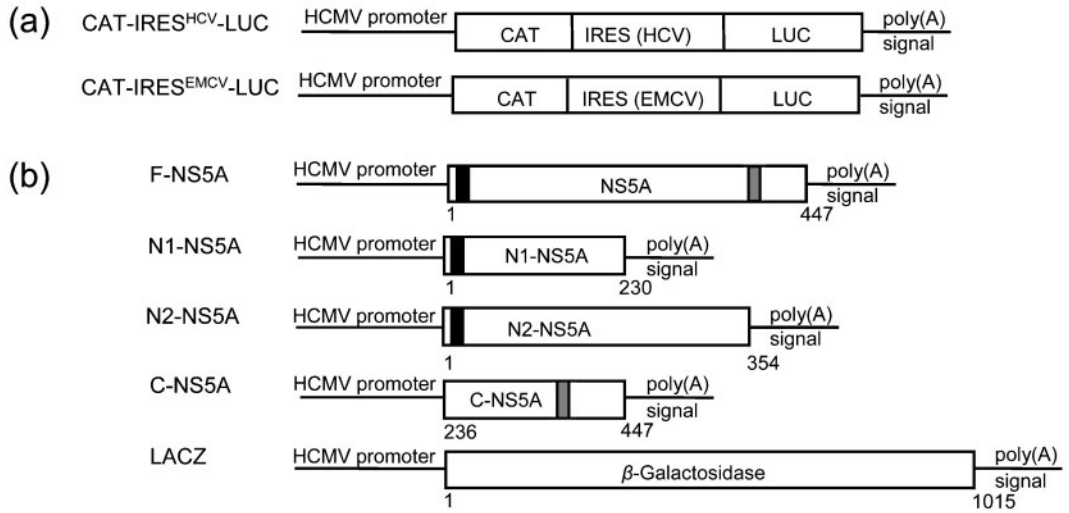

Fig. 1. Schematic representation of the bicistronic (a) and monocistronic (b) transcriptional units that were used in order to assess the effect of NS5A protein on HCV IRES. CAT and LUC correspond to chloramphenicol acetyltransferase and firefly luciferase coding sequences, respectively. In the schematic representation of the full-length NS5A and its deleted forms, the grey and black boxes correspond to the NLS and the amino-terminal amphipathic α-helix, respectively. All of the above transcriptional units are cloned into the pA-EUA2 plasmid vector and are under the control of HCMV promoter. Numbers correspond to amino acids. Names of vectors are shown on the left.

RESULTS

NS5A protein inhibits HCV IRES-mediated translation

To examine the effect of the HCV NS5A protein on HCV IRES activity we performed transient transfection experiments using a reporter plasmid. This plasmid carries a bicistronic transcriptional unit (under the control of HCMV promoter) consisting of the coding sequences of the CAT and LUC genes with the 5′ NTR of HCV genotype 1a inserted in-between (CAT-IRES^{HCV}-LUC) (Fig. 1). This construction was designed to allow analysis of IRES activity presented in the 5′ NTR of the HCV genome by LUC activity quantification, with cap-dependent translation simultaneously monitored by measurement of CAT production. HepG2, WRL-68 and BHK-21 cell lines were transiently co-transfected with the above reporter plasmid in combination with the NS5A expressing vector (F-NS5A). Cell cultures were analysed, 48 h post-transfection, for LUC activity and CAT production. As a control, we used the plasmid vector, LACZ, expressing an unrelated protein, β-galactosidase, in place of NS5A. The results in Fig. 2(a) show relative IRES activity expressed as the ratio of LUC expression levels over those of CAT. The LUC/CAT ratio in the presence of the β-galactosidase expressing vector was set at 100 %. As shown, expression of the NS5A protein strongly suppressed the LUC production compared with that observed in the β-galactosidase expressing cells suggesting an inhibitory effect of NS5A protein on the HCV IRES-driven translation. On the other hand, as shown in Fig. 2(b) the NS5A expression did not have an obvious effect on CAT production levels suggesting that cap-dependent translation is not affected by NS5A under the experimental conditions used. Furthermore, the NS5A inhibitory effect on HCV IRES-mediated translation was independent of cell type, albeit inhibition was more pronounced in cell lines of hepatic origin. Expression of NS5A and β-galactosidase proteins was confirmed by immunoblot analysis using a rabbit anti-NS5A polyclonal and a mouse anti-β-galactosidase monoclonal antibody, respectively. The results shown in Fig. 2(c) indicate that both proteins were successfully expressed in this system.

Next, we determined whether the above inhibitory effect on the HCV IRES activity was NS5A dose-dependent. For this purpose, HepG2 cells were co-transfected with the bicistronic CAT-IRES^{HCV}-LUC vector and various amounts of NS5A or β-galactosidase expressing vector. The total amount of DNA was fixed by adding the parental vector (pA-EUA2). The results are shown in Fig. 3. For each of the F-NS5A or LACZ vector concentrations used, the LUC/CAT ratio in the β-galactosidase expressing cells was set at 100 % with the LUC/CAT ratio in the presence of the same amount of F-NS5A vector expressed relative to this. As shown in Fig. 3(a), the HCV IRES activity was repressed,

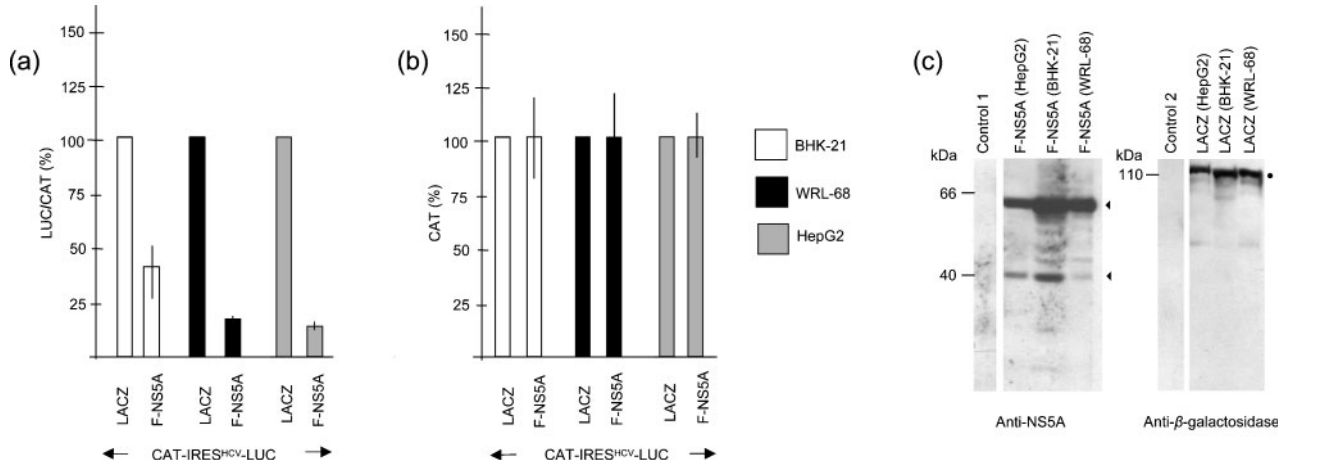

Fig. 2. Effect of HCV NS5A on the HCV IRES. Co-transfection experiments in three different cell lines (BHK-21, WRL-68 and HepG2) using the bicistronic vector (Fig. 1) expressing the CAT-IRES^{HCV}-LUC transcriptional unit, together with the monocistronic vector that expresses either NS5A (F-NS5A) or β-galactosidase protein (LACZ). In each experiment, 0·3 μg DNA per well of the bicistronic and 0·5 μg DNA per well of the monocistronic vector were used. (a) The percentage of LUC/CAT for each plasmid combination is depicted. In each cell line in the presence of the LACZ vector the LUC/CAT ratio was set at 100 %. (b) The percentage of CAT expression levels for each plasmid combination is represented. In each cell line in the presence of the LACZ vector the CAT expression level was set at 100 %. The mean values are shown with bars and the standard deviations with lines. (c) Expression of the NS5A and β-galactosidase proteins detected by Western blot analysis. Control 1, HepG2 cells co-transfected with CAT-IRES^{HCV}-LUC (0·3 μg DNA per well) and β-galactosidase (0·5 μg DNA per well) expressing vectors. Control 2, HepG2 cells co-transfected with CAT-IRES^{HCV}-LUC (0·3 μg DNA per well) and NS5A (0·5 μg DNA per well) expressing vectors. Arrowheads indicate the entire NS5A and its cleavage products (Kalamvoki & Mavromara, 2004) and the dot indicates the β-galactosidase protein. The molecular mass markers are shown on the left.

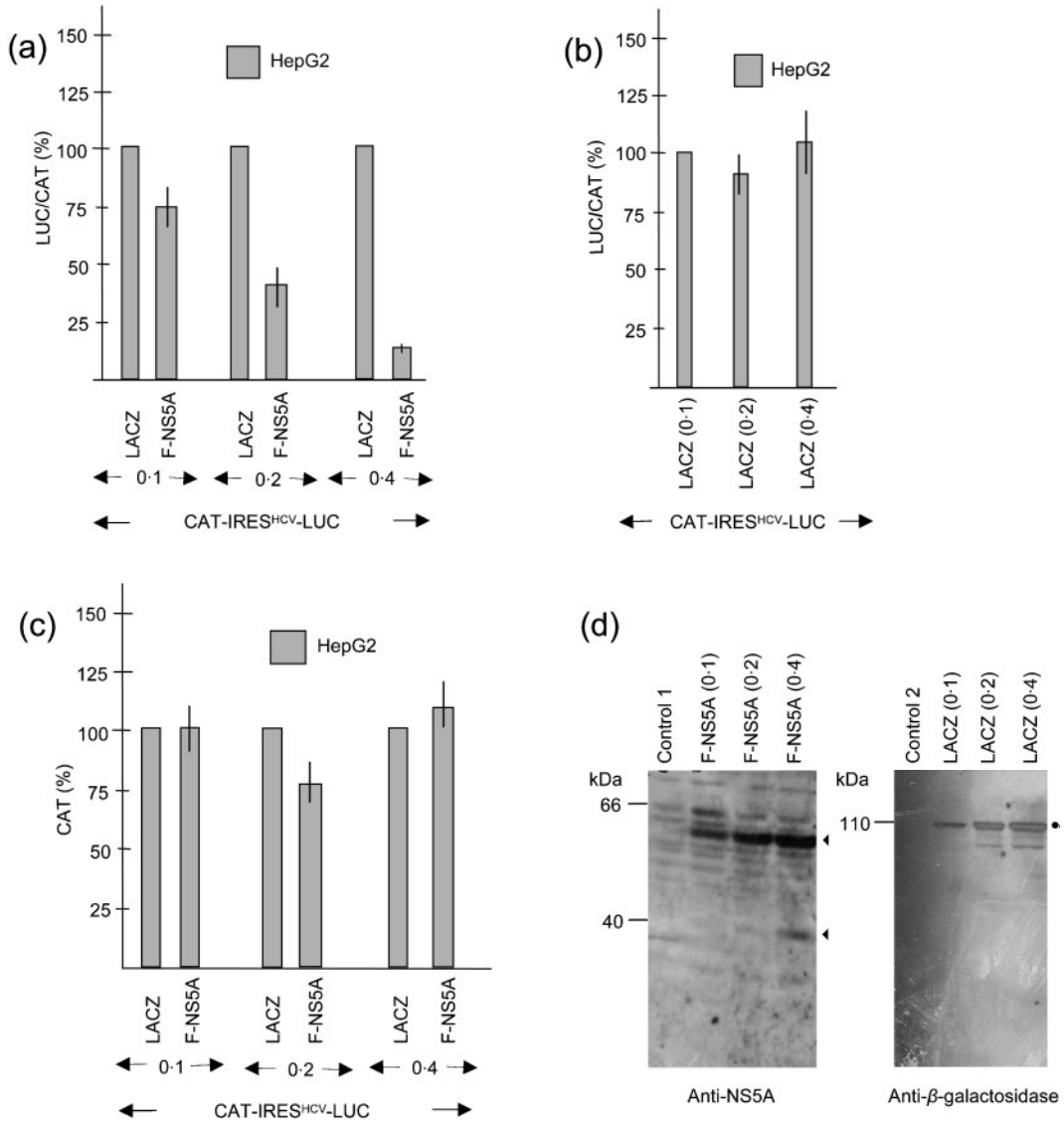

Fig. 3. Dose-dependent effect of HCV NS5A on the HCV IRES. Co-transfection experiments in the HepG2 cell line using the bicistronic vector expressing the CAT-IRES^{HCV}-LUC transcriptional unit (Fig. 1), together with various amounts of the monocistronic vector that expresses either the NS5A (F-NS5A) or the β-galactosidase protein (LACZ). In each experiment, the total amount of DNA was kept constant (0·6 μg DNA per well) by adding pA-EUA2 (parental) vector. For each experiment, 0·2 μg DNA per well of the bicistronic vector in combination with 0·1, 0·2 or 0·4 μg DNA per well of the monocistronic vector were used. (a) The percentage LUC/CAT for each vector combination is depicted. In the presence of each of the different amounts of LACZ vector the LUC/CAT ratio was set at 100 % with the corresponding LUC/CAT percentage in the presence of the NS5A protein expressed relative to this. (b) The percentage LUC/CAT for each different amount of LACZ vector is depicted. In the presence of 0·1 μg LACZ vector per well the LUC/CAT ratio was arbitrarily set at 100 %. (c) The percentage of CAT expression levels for each vector combination is depicted. In the presence of each of the different amounts of LACZ vector, the CAT expression level was set at 100 %, with the corresponding percentage in the presence of the NS5A protein expressed relative to this. The mean values are shown with bars and the standard deviations with lines. (d) Dose-dependent expression of NS5A and β-galactosidase proteins detected by Western blot analysis. Control 1, HepG2 cells co-transfected with CAT-IRES^{HCV}-LUC (0·2 μg DNA per well) and β-galactosidase (0·4 μg DNA per well) expressing vectors. Control 2, HepG2 cells co-transfected with CAT-IRES^{HCV}-LUC (0·2 μg DNA per well) and NS5A (0·4 μg DNA per well) expressing vectors. Arrowheads indicate the entire NS5A and its cleavage products, dot indicates the β-galactosidase protein. The molecular mass markers are shown on the left.

by the increase of NS5A protein production, in a dose-dependent manner, ranging from about 20 to 90 % compared with the corresponding transfection experiments where the LACZ vector was used. Increasing amounts of LACZ vector in co-transfections did not significantly affect the LUC/CAT ratio (Fig. 3b) thus excluding the possibility that the reduction of the LUC production by the NS5A protein was due to limitation of cellular translation resulting from the overexpression of a foreign protein. As expected, CAT production levels remained largely unchanged following the co-transfections with increasing amounts of F-NS5A or LACZ vectors (Fig. 3c). Immunoblot analysis confirmed that the expression levels of NS5A and β-galactosidase proteins were changed in a dose-dependent manner (Fig. 3d). The above results indicate that the inhibitory effect of NS5A on HCV IRES activity (with the amount of the bicistronic vector fixed) is directly related to the production levels of the NS5A protein.

Although these data strongly suggest an inhibitory effect of the NS5A protein on HCV IRES-mediated translation, the possibility of an artificial effect on the LUC expression due to the presence of a recently described cryptic promoter within the HCV 5′ NTR DNA corresponding sequences (Dumas *et al.*, 2003) remained a concern. Therefore, we sought to test for activity levels of such a putative eukaryotic promoter under our experimental conditions. For this purpose, a new bicistronic vector lacking the upstream HCMV promoter sequences was constructed (HCMVdel/CAT-IRES^{HCV}-LUC) and tested for CAT and LUC protein expression in HepG2, WRL-68 and BHK-21 transfected cells in parallel to the corresponding HCMV-carrying vector (HCMV/CAT-IRES^{HCV}-LUC) (Fig. 4a). As expected, transfection with the promoterless bicistronic vector resulted in the disruption of CAT expression in the three cell lines (Fig. 4b). Furthermore, as shown in Fig. 4(c), the LUC activity was severely reduced both in HepG2 and

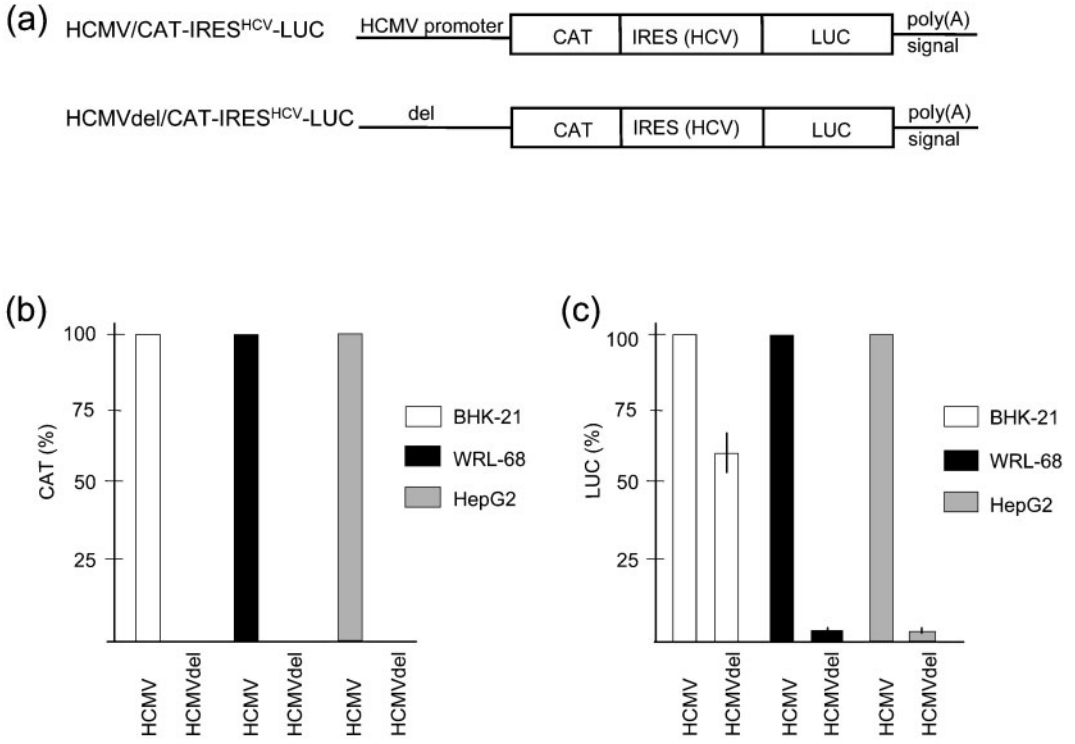

Fig. 4. Cryptic promoter activity of the HCV 5′ NTR. (a) Schematic representation of the bicistronic units used. The HCMV/CAT-IRES^{HCV}-LUC plasmid contains the CAT-IRES^{HCV}-LUC bicistronic unit under the control of the HCMV promoter. The HCMVdel/CAT-IRES^{HCV}-LUC vector contains the same CAT-IRES^{HCV}-LUC bicistronic unit but lacks the entire sequence of the HCMV promoter. (b) Results from transfection experiments in the three different cell lines using 0·3 μg DNA per well of the above plasmid vectors performed in parallel experiments. The percentage of CAT expression levels for each vector and cell line is represented. In the presence of the HCMV-carrying vector (HCMV), the CAT expression level in each cell line was set at 100 % with the CAT expression level produced by the HCMV deletion-carrying vector (HCMVdel) expressed relative to this. (c) Results from transfection experiments in the three different cell lines using 0·3 μg DNA per well of the above vectors performed in parallel experiments. The percentage of LUC expression levels for each vector and cell line is represented. In the presence of the HCMV-carrying vector (HCMV), the LUC expression level in each cell line was set at 100 % with the LUC expression level produced by the HCMV deletion-carrying vector (HCMVdel) expressed relative to this. The mean values are shown with bars and the standard deviations with lines.

WRL-68 cells compared with the LUC activity from the corresponding HCMV-containing bicistronic vector (HCMV/CAT-IRES^{HCV}-LUC), suggesting the negligible activity of a putative cryptic promoter in liver cells. These data strongly imply that the NS5A acts at a translational level, inhibiting the HCV IRES activity. On the other hand, a portion of LUC activity (about 50 %) was retained in BHK-21 cells when the HCMVdel/CAT-IRES^{HCV}-LUC vector was used, despite the lack of CAT production. This may account for the reduced inhibitory effect of NS5A on the LUC production that was observed in this cell line (Fig. 2a).

Mapping the region of HCV NS5A protein responsible for HCV IRES inhibition

To define the region of the NS5A protein responsible for repressing HCV IRES activity, three NS5A deletion constructs were used. The first carries the amino-terminal half of NS5A (N1-NS5A: 1–230 aa), the second carries the amino-terminal part of NS5A up to NLS (N2-NS5A: 1–354 aa), and the third carries the carboxy-terminal half of NS5A (C-NS5A: 236–447 aa). HepG2 cells were co-transfected with the CAT-IRES^{HCV}-LUC vector (Fig. 1) in combination with a vector that expresses either one of the NS5A constructs (full-length or deleted forms) or the β-galactosidase protein. As shown in Fig. 5(a) expression of the N1-NS5A form exhibited no significant effect on the HCV IRES activity as compared to that from the cells expressing the β-galactosidase protein. In contrast, expression of the N2-NS5A or C-NS5A forms caused a strong inhibitory effect on the HCV IRES activity to nearly the same degree as that caused by the full-length NS5A protein. The expression of each of the deleted forms of the NS5A protein was confirmed by Western blot analysis (Fig. 5b). These data suggest that the region expanded about 120 aa just upstream of the NLS of the NS5A protein plays a crucial role for the inhibitory activity on HCV IRES function.

HCV NS5A protein specifically inhibits HCV IRES-mediated translation

In order to investigate the possible inhibitory effect of NS5A on other viral IRES elements, the effect of NS5A on the EMCV IRES activity was also examined. For this purpose an analogous CAT-LUC bicistronic transcriptional unit was constructed carrying the EMCV IRES element in place of the HCV IRES (vector: CAT-IRES^{EMCV}-LUC) (Fig. 1). HepG2 cells were co-transfected separately with the above vector or the CAT-IRES^{HCV}-LUC reporter plasmid (Fig. 1) in combination with increasing amounts of F-NS5A or LACZ vector, and the relative EMCV and HCV IRES activity was determined. In addition, the effect of two deleted forms of NS5A (N1-NS5A and C-NS5A) on the function of EMCV IRES was analysed. As shown in

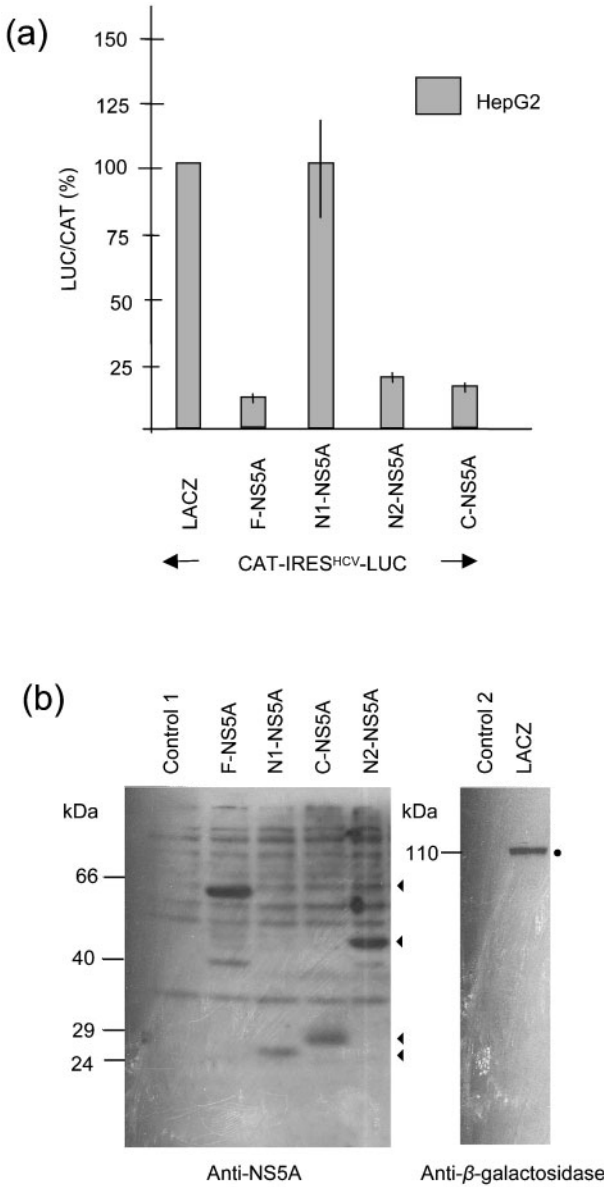

Fig. 5. Mapping the region of NS5A responsible for the inhibitory effect on HCV IRES. Results from co-transfection experiments in the HepG2 cell line using the bicistronic vector expressing the CAT-IRES^{HCV}-LUC transcriptional unit together with monocistronic vectors expressing either the NS5A or one of the deleted forms of this protein, or the β-galactosidase protein (Fig. 1). For each experiment, 0·2 μg DNA per well of the bicistronic and 0·4 μg DNA per well of the monocistronic vector were used. (a) The percentage LUC/CAT for each vector combination is depicted. In the presence of the LACZ vector, the LUC/CAT ratio was set at 100 %. The mean values are shown with bars and the standard deviations with lines. (b) Western blot analysis depicting the expression of the full-length and deleted forms of NS5A protein as well as of β-galactosidase protein. Control 1, HepG2 cells co-transfected with CAT-IRES^{HCV}-LUC (0·2 μg DNA per well) and β-galactosidase (0·4 μg DNA per well) expressing vectors. Control 2, HepG2 cells co-transfected with CAT-IRES^{HCV}-LUC (0·2 μg DNA per well) and NS5A (0·4 μg DNA per well) expressing vectors. Arrowheads indicate the main products of the entire NS5A and its deleted forms, dot indicates the β-galactosidase protein. The molecular mass markers are shown on the left.

Fig. 6 the different amounts of NS5A-expressing vector had no effect on EMCV IRES activity, whereas as previously shown, NS5A exerted a dose-dependent inhibitory effect on the HCV IRES activity. Furthermore, in contrast to the HCV IRES, the production of both deleted forms of the NS5A protein exhibited no effect on the EMCV IRES. Collectively, these data suggest that NS5A inhibits HCV IRES-mediated translation in a specific manner.

DISCUSSION

We report here that expression of the HCV NS5A protein from genotype 1a caused a strong repression of HCV IRES-mediated translation initiation. The NS5A inhibitory effect was assessed in two hepatic cell lines (HepG2 and WRL-68) as well as in a non-hepatic cell line (BHK-21) using a CAT-IRES^{HCV}-LUC bicistronic construct in transient transfection experiments. The NS5A inhibitory effect on translation was specific for the HCV IRES activity in as much as analysis of the effect of the NS5A on HCV IRES-dependent LUC production and on cap-dependent CAT production showed that NS5A inhibited only the first and had no impact on the second (Fig. 2). Furthermore, no obvious effect was observed when using the CAT-IRES^{EMCV}-LUC bicistronic construct carrying the EMCV IRES element instead of the HCV IRES element in the intercistronic space (Fig. 6). This difference is consistent with previous findings showing that these two IRESs are only distantly related and exhibit to some degree different requirements for translation initiation (Martínez-Salas *et al.*, 2001).

Notably, the inhibitory effect on HCV IRES was NS5A dose-dependent as it was clearly proportional to the protein amount, ranging from almost no inhibition (20 %) to almost total inhibition (90 %) (Fig. 3). Therefore, the inhibitory effect of the NS5A protein on the HCV IRES activity depends either on the absolute levels of the NS5A protein or on the stoichiometry of the HCV IRES and NS5A molecules. Moreover, the ability of NS5A to repress HCV IRES-mediated activity appeared to be cell-type independent, even though in BHK-21 cells LUC production was not as significantly inhibited as in cells of hepatic origin. This inhibition was not related to the recently described HCV 5′ NTR-related promoter activity (Dumas *et al.*, 2003), because the production of the LUC protein from the vector that carries the HCV IRES bicistronic cassette, but lacks the HCMV promoter sequence that controls the transcription of the bicistronic unit, was negligible in the cell lines of hepatic origin (Fig. 4).

The use of different NS5A deletion mutants suggested that the functional domain of the NS5A protein that is critical for the inhibitory effect on HCV IRES activity resides at the carboxy-terminal half of the protein and includes a sequence of about 120 aa that contains the interferon sensitivity-determining region and the proline-rich region. Clearly, the amino-terminal part of the protein, based on the results from the N1-NS5A form as well as from shorter

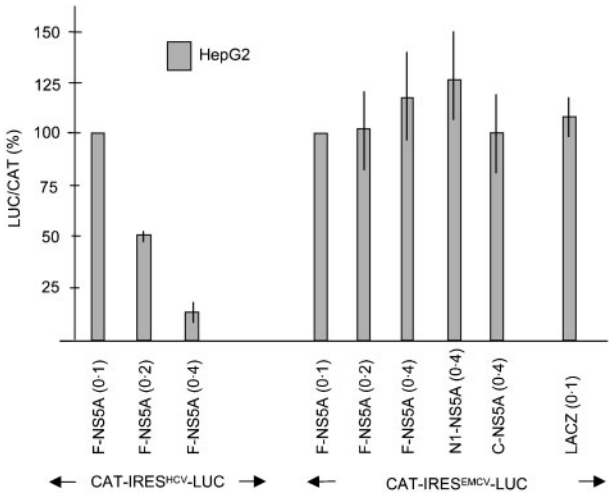

Fig. 6. Specific inhibition of HCV IRES-dependent translation. Results from co-transfection experiments in the HepG2 cell line using separately in parallel experiments the CAT-IRES^{HCV}-LUC and CAT-IRES^{EMCV}-LUC bicistronic vectors (0·2 μg DNA per well) (Fig. 1), in combination with: (i) various amounts (0·1, 0·2 or 0·4 μg DNA per well) of the monocistronic vector that expresses either the entire NS5A (F-NS5A) or the β-galactosidase protein (LACZ), and (ii) monocistronic vector (0·4 μg DNA per well) that expresses either the amino-terminal half (N1-NS5A) or the carboxy-terminal half (C-NS5A) of the NS5A protein. In each experiment, the total amount of DNA was kept constant (0·6 μg DNA per well) by adding pA-EUA2 (parental) vector. Setting the LUC/CAT ratio at 100 % in the presence of 0·1 μg F-NS5A vector per well (for each bicistronic vector), the percentage LUC/CAT for any other amount of F-NS5A expressing vector and for the deleted forms of the protein (in case of the EMCV IRES containing vector) is depicted relative to this. The percentage of LUC/CAT, in the case of the EMCV IRES containing vector in combination with 0·1 μg LACZ vector per well, is also shown on the right. The mean values are shown with bars and the standard deviations with lines.

amino-terminal forms of the protein (data not shown), had no effect on HCV IRES function (Fig. 5). Although the molecular basis of the inhibitory effect of NS5A on HCV IRES remains to be elucidated, it is of interest to note that the putative inhibitory domain of NS5A is known to represent a 'hot spot' for mutations that accumulate in the replicon system (Krieger *et al.*, 2001; Lohmann *et al.*, 2001; Blight *et al.*, 2003).

While our work was in progress, it was reported that the NS5A protein enhances the HCV IRES-mediated translation while NS3, NS4A, NS4B and NS5B do not exhibit any significant effect on the activity level of the HCV IRES (He *et al.*, 2003). Although the reason for this discrepancy is still not known, we have considered a number of possibilities to explain these data. Firstly, that the study was largely based on the use of NS5A sequences derived from the HCV-1b replicon, which is known to contain adaptive mutations within the carboxy-terminal half of the NS5A

protein (Lohmann *et al.*, 1999; Blight *et al.*, 2000; Bartenschlager & Lohmann, 2001). However, the selection of this replicon is based on the use of neomycin antibiotic and the gene that is responsible for the resistance (neomycin phosphotransferase) is placed under the translational control of the HCV IRES. Neomycin affects the translation process of the cell (Eustice & Wilhelm, 1984). Thus, it is tempting to speculate that the selection for neomycin might result in the selection of mutations in NS5A that will suppress or even reverse the negative effect of NS5A on HCV IRES-dependent translation, allowing the survival of the replicon.

Secondly, according to our data, the inhibitory effect of NS5A on the HCV IRES activity is dose-dependent. Because He *et al.* (2003) have reported results from only a single plasmid concentration, it is likely that the experimental conditions used in that study may not be appropriate to detect repression of the IRES activity. Interestingly, He *et al.* (2003) failed to detect repression of the HCV IRES activity by the HCV NS4A and NS4B proteins. Both proteins were shown to have a dose-dependent inhibitory effect on HCV IRES-driven translation (Kato *et al.*, 2002). A third explanation for this discrepancy might be the HCV genotype studied. In this study, HCV genotype 1a was used, whereas in the other study (He *et al.*, 2003) HCV 1b was examined. Although this explanation may be less likely, it is strengthened by the fact that NS5A functions are affected by the sequence variability of the protein (Gale *et al.*, 1997; Pellerin *et al.*, 2004).

To summarize our work, we showed that NS5A acts as a negative regulator for HCV IRES-mediated translation. The role of NS5A inhibitory effect on HCV RNA translation in virus life cycle remains currently unknown. However, in the case of other positive-sense RNA viruses such as poliovirus, it has been demonstrated that the replication of viral RNA begins only after translation has been inhibited, because the RNA-dependent RNA polymerase of the virus cannot replicate the viral RNA while it is being translated by ribosomes (Gamarnik & Andino, 1998; Barton *et al.*, 1999). Poliovirus inhibits the translation of its own RNA by producing a non-structural precursor protein, 3CD. 3CD protein binds to the cloverleaf just before the IRES of the virus, represses translation and facilitates negative-strand synthesis (Gamarnik & Andino, 1998). Furthermore, the existence of a negative-feedback mechanism for the regulation of the initiation of viral RNA replication has been suggested for EMCV, another member of the family *Picornaviridae* (Svitkin & Sonenberg, 2003).

Thus, it is intriguing to speculate that the NS5A protein might be part of the biological switch mechanism that is responsible for the inhibition of HCV IRES-driven translation favouring the initiation of the HCV genome replication. Furthermore, this NS5A inhibitory effect on HCV IRES occurs under conditions that do not disturb the translation of the cellular mRNAs and by this way may allow the establishment of the viral persistence.

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