Downregulation of viral RNA translation by hepatitis C virus non-structural protein NS5A requires the poly(U/UC) sequence in the 3’ UTR

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Hepatitis C virus (HCV) non-structural protein 5A (NS5A) is essential for viral replication; however, its effect on HCV RNA translation remains controversial partially due to the use of reporters lacking the 3’ UTR, where NS5A binds to the poly(U/UC) sequence. We investigated the role of NS5A in HCV translation using a monocistronic RNA containing a Renilla luciferase gene flanked by the HCV UTRs. We found that NS5A downregulated viral RNA translation in a dose-dependent manner. This downregulation required both the 5’ and 3’ UTRs of HCV because substitution of either sequence with the 5’ and 3’ UTRs of enterovirus 71 or a cap structure at the 5’ end eliminated the effects of NS5A on translation. Translation of the HCV genomic RNA was also downregulated by NS5A. The inhibition of HCV translation by NS5A required the poly(U/UC) sequence in the 3’ UTR as NS5A did not affect translation when it was deleted. In addition, we showed that, whilst the amphipathic α-helix of NS5A has no effect on viral translation, the three domains of NS5A can inhibit translation independently, also dependent on the presence of the poly(U/UC) sequence in the 3’ UTR. These results suggested that NS5A downregulated HCV RNA translation through a mechanism involving the poly(U/UC) sequence in the 3’ UTR.

INTRODUCTION

Hepatitis C virus (HCV) is a small, enveloped, positive-sense ssRNA virus and belongs to the family Flaviviridae as the only member in the genus Hepacivirus (Dustin & Rice, 2007). The viral genome is 9.6 kb and contains a single ORF, which encodes a polyprotein of ~3000 aa (Suzuki et al., 2007). The viral polyprotein is cleaved co- and post-translationally by both host and viral proteases into at least 10 viral proteins: three structural proteins (core, E1 and E2) and seven non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Dustin & Rice, 2007). The single ORF is flanked by highly conserved 5’ and 3’ UTRs which are involved in the control of viral translation and replication.

HCV NS5A is a multifunctional protein that plays key roles in the HCV life cycle (Macdonald & Harris, 2004; Ross-Thriepland & Harris, 2015). It is essential for viral replication and has been found to be involved in numerous processes, such as viral assembly, modulation of cellular signalling pathways and inhibition of IFN responses (Hughes et al., 2009; Kohler et al., 2014; Macdonald & Harris, 2004; Ross-Thriepland & Harris, 2015). A role for NS5A in the modulation of viral translation has, however, remained unclear as numerous contradictory studies have been published (Gonzalez et al., 2009; He et al., 2003; Imbert et al., 2003; Kalliampakou et al., 2005; Lourenço et al., 2008). Translation of the viral polyprotein is mediated by an internal ribosomal entry site (IRES) located within the viral 5’ UTR through direct recruitment of the 40S ribosomal subunit (Hoffman & Liu, 2011). The viral 3’ UTR functions to stimulate virus translation, through an unknown mechanism, in addition to serving as the initiation site for negative-strand synthesis (Bradrick et al., 2006; Bung et al., 2010; Song et al., 2006). This stimulatory effect is only observed when monocistronic RNA reporters with precise 3’ ends or complete RNA genomes are utilized (Song et al., 2006). The 3’ UTR of HCV is composed of three distinct regions: the variable region, the poly(U/UC) tract and the X-tail (Song et al., 2006). The X-tail forms three stem–loop structures (i.e. SL1, SL2 and SL3) (Bung et al., 2010). It has been found that the variable region, the poly(U/UC) tract and SL1 (the 3’-most terminal stem–loop) of the X-region contribute significantly to the translation enhancement provided by the 3’ UTR, whilst SL2 and SL3 play only minor roles (Song et al., 2006). However, the mechanism of 3’ UTR enhancement
of translation remains to be clearly defined. Possible mechanisms of this stimulatory effect include RNA–RNA interactions with the HCV IRES, recruitment of cellular factors to the 3' UTR that interact with components of the translational machinery present on the IRES and the recycling of ribosomes (Bai et al., 2013).

NS5A has been found to bind directly to the poly(U/UC) region of the viral 3' UTR with high affinity (Foster et al., 2010; Huang et al., 2005). Other potential binding sites for NS5A identified include sites within the HCV IRES (Huang et al., 2005). The fact that NS5A binds to regions within both 5' and 3' UTRs strongly suggests that NS5A may function in the modulation of HCV translation, and could perhaps be involved in regulating the switch from translation to replication (Foster et al., 2010; Huang et al., 2005; Toroney et al., 2010). As such, we sought to investigate the modulation of translation by NS5A and the potential role of the poly(U/UC) region.

RESULTS AND DISCUSSION

NS5A downregulates HCV RNA translation in a dose-dependent manner in the presence of the poly(U/UC) region of the viral 3' UTR

We generated monocistronic HCV RNA translation reporters with WT 3' UTR, Δpoly(U/UC) or Δ3' UTR using the HCV-1b N sequence (Ikeda et al., 2002). Upon transfecting these RNA reporters into Huh-7 cells, we studied the role of the 3' UTR in HCV translation. Deletion of the poly(U/UC) region decreased viral translation by ~50%, whereas when the entire 3' UTR was removed viral translation was decreased by ~90% compared with the WT.

![Graphs and images showing results of experiments](http://vir.sgmjournals.org/2115)

Fig. 1. NS5A downregulates HCV RNA translation in the presence of the poly(U/UC) region in the 3' UTR. (a) Huh-7 cells were transfected with a monocistronic HCV RNA translation Renilla luciferase (rLuc) reporter RNA containing either WT or the indicated deletions. Cells were harvested 4 h after transfection and luciferase assay performed. The rLuc value of the WT 3' UTR RNA was set to 1. Statistical differences were analysed by Student's t-test: *P<0.05, **P<0.01. (b) Huh-7 cells were transfected with plasmid expressing HCV NS5A or plasmid vector. After 24 h, cells were transfected with a monocistronic HCV RNA translation rLuc reporter RNA containing either WT or Δpoly(U/UC) 3' UTR. Luciferase assay was performed 4 h after RNA transfection. The rLuc value after vector transfection was set to 1. Statistical differences were analysed by Student's t-test: *P<0.05. (c) The levels of rLuc RNA in cells in (b) were determined by real-time-PCR. The RNA levels of a housekeeping gene GUSB (β-glucuronidase) were used for normalization. (d) Huh-7 cells were transfected sequentially with HCV NS5A-expressing plasmid or plasmid vector and viral RNA translation reporter RNAs as indicated. Luciferase assay was performed 4 h after RNA transfection. (e) MTT assay for cell viability was performed 24 h after transfection of Huh-7 cells with plasmid vector or HCV NS5A expression plasmid. DMSO treatment was used as a control. (f) Expression of NS5A was demonstrated by Western blotting using an NS5A-specific antibody. The levels of β-actin were also determined using a β-actin-specific antibody.
3' UTR (Fig. 1a). These results showed the importance of the 3' UTR in the modulation of viral translation and were similar to those obtained by others (Bai et al., 2013; Bung et al., 2010; Song et al., 2006). The mechanism behind the stimulatory effect of the individual 3' UTR regions on viral translation remains to be determined. However, it has been observed that differences in viral translation efficiency are not the result of differing RNA stability upon deletion of 3' UTR or its individual regions (Bung et al., 2010; Song et al., 2006). Therefore, we used the monocistronic RNA reporters in the following experiments.

To investigate the effect of NS5A on HCV translation, Huh-7 cells were transfected with HCV NS5A expression plasmid and the monocistronic HCV translation reporter RNA. As shown in Fig. 1(b), expression of NS5A resulted in a significant decrease in luciferase activity in comparison with vector control. These results suggested an inhibitory effect of NS5A on HCV translation.

NS5A has been shown to preferentially bind to the poly(U/UC) region in the 3' UTR (Foster et al., 2010; Huang et al., 2005); however, the function of this RNA binding is not clear. To test whether the poly(U/UC) region plays a role in translation modulation by NS5A, we utilized a RNA reporter without the poly(U/UC) in the 3' UTR. Fig. 1(b) shows that NS5A had no effect on the Renilla luciferase (rLuc) level in the absence of the poly(U/UC) region, suggesting a role of the poly(U/UC) region. NS5A did not affect the RNA levels of these HCV RNA translation reporters (Fig. 1c). The expression of NS5A was demonstrated by Western blotting (Fig. 1f). These data suggested that the poly(U/UC) region of the 3' UTR was necessary for the downregulation of viral translation by NS5A.

To investigate whether the effect of NS5A on translation was HCV-specific, we measured the RNA translation driven by the enterovirus 71 (EV71) 5' UTR. As shown in Fig. 1(d), HCV NS5A did not affect EV71 RNA translation. Furthermore, no effects of NS5A were observed on the translation of RNAs with the EV71 5' UTR and HCV 3' UTR, or with the 5' cap and HCV 3' UTR, or with the 5' cap and polyU sequence (Fig. 1d). These results suggested that NS5A only inhibited translation when both HCV 5' and 3' UTRs were present. NS5A expression had no effect on cell viability as measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Fig. 1e).

GFP did not affect the translation of any of the RNA translation reporters used (Fig. 2a), suggesting that the effects of NS5A on translation were not due to the expression of a protein. GFP expression was demonstrated by Western blotting (Fig. 2b).

To further confirm the effect of NS5A on HCV RNA translation, we performed a dose experiment by transfecting

![Graph](image.png)

**Fig. 2.** GFP has no effect on viral RNA translation. (a) Huh-7 cells were co-transfected with plasmid expressing Flag–GFP or plasmid vector, together with viral RNA translation rLuc reporter RNAs as indicated. Luciferase assay was performed 24 h after transfection. The rLuc value after vector transfection was set to 1. (b) Expression of Flag–GFP was demonstrated by Western blotting using a Flag-specific antibody. The levels of β-actin were also determined using a β-actin-specific antibody.
increasing amounts of NS5A expression plasmid. We found that NS5A downregulated viral translation in a dose-dependent manner (Fig. 3a). This effect was again dependent upon the presence of the poly(U/UC) region in the 3' UTR (Fig. 3a). The downregulation of HCV translation by NS5A was again specific to viral protein translation and increasing levels of NS5A expression did not have an effect on cap translation (Fig. 3b). Increasing levels of NS5A did not induce measurable changes in cellular viability (Fig. 3c). The increasing expression of NS5A protein was demonstrated by Western blotting (Fig. 3d). These results suggested that NS5A dose-dependently downregulated HCV RNA translation in the presence of the poly(U/UC) region.

NS5A consists of an N-terminal amphipathic α-helix (AH), and domains I, II and III with two inter-domain low complexity sequences (LCSs) (Fig. 4a). To map the domain(s) of NS5A responsible for this modulatory effect, plasmids encoding NS5A with an AH deletion or the individual domains of NS5A were utilized along with the monocistronic RNA reporters with or without the poly(U/UC) region. Our results demonstrated that the AH did not play a significant role in modulating viral translation as the AH deletion construct was capable of downregulating viral translation similarly to WT NS5A (Fig. 4b). Furthermore, each domain of NS5A was capable of downregulating HCV translation independently, albeit to a lower degree than the full-length NS5A, particularly for domain

Fig. 3. NS5A downregulates HCV RNA translation in a dose-dependent manner in the presence of the poly(U/UC) region in the 3’ UTR. (a) Huh-7 cells were transfected with HCV NS5A expression plasmid at 0, 0.25, 0.5, 1 or 2 μg for 24 h. Total amounts of DNA transfected were kept consistent by adjusting the amounts of vector. Cells were then transfected with an HCV monocistronic translation rLuc reporter RNA, containing either the WT or Δpoly(U/UC) 3’ UTR. Luciferase assay was performed 4 h after RNA transfection. (b) Huh-7 cells were transfected with increasing amounts of HCV NS5A plasmids as in (a), followed by a capped mRNA expressing rLuc. Luciferase assay was performed 4 h after RNA transfection. The rLuc value after vector plasmid or control mRNA transfection was set to 1. Statistical differences were analysed by Student’s t-test: *P≤0.05, **P≤0.01. (c) Cell viability was determined by MTT assay 24 h after transfection with increasing amounts of HCV NS5A-expressing plasmids. DMSO treatment was included as a control. (d) The levels of NS5A and β-actin were determined by Western blotting.
II (Fig. 4b). These inhibitory effects again were dependent upon the poly(U/UC) sequence (Fig. 4b). None of the NS5A domain proteins had an effect on cap translation and cell viability (Fig. 4c, d). The expression levels of NS5A and mutant proteins were demonstrated in Western blotting (Fig. 4e). The fact that each individual domain was capable of downregulating viral translation may not be completely surprising as it has been shown that each domain is capable of binding to the 3′ UTR independently (Foster et al., 2010). However, as domains I and II can bind to 3′ UTR RNA to a stronger degree than domain III (Foster et al., 2010), this raises the question as to whether 3′ UTR RNA binding is the only potential mechanism for the observed modulatory effect.

To substantiate the results obtained using the monocistronic RNA translation reporters, we generated a replication-deficient HCV genomic construct with the rLuc gene between p7 and NS2 (Fig. 5a). The reason for using a replication-deficient HCV was to avoid HCV RNA replication, which could render result interpretation difficult. When the full-length HCV genomic RNA containing the WT 3′ UTR was utilized, NS5A expression led to decreases in viral translation compared with control at 2, 4 and 8 h post-electroporation (Fig. 5b). However, when the poly(U/UC) was deleted from the 3′ UTR, NS5A expression did not affect viral translation (Fig. 5c). Western blotting showed NS5A expression (Fig. 5d). These results suggested that NS5A expression in trans could downregulate viral translation from HCV genomic RNA dependent upon the presence of the poly(U/UC) sequence in the 3′ UTR.

To confirm that the observed effect on viral translation was indeed due to NS5A, we determined HCV genomic RNA translation after knocking down NS5A expression. As shown in Fig. 5(e), knocking down NS5A by a small hairpin RNA (shRNA) significantly eliminated the down-regulation of HCV translation by NS5A. Furthermore, this elimination was negated when NS5A was expressed by a shRNA-resistant mRNA in the presence of the
shRNA (Fig. 5e). Reduced NS5A expression after knock-down by the shRNA was demonstrated in Western blotting (Fig. 5f). These results reaffirmed the downregulation of HCV translation by NS5A.

The mechanisms by which NS5A downregulates HCV RNA translation are not clear. The requirement of the poly(U/UC) region in the 3' UTR for the downregulation suggests that NS5A may affect the functions exerted by the poly(U/UC) region, most likely as a consequence of binding to this region. It has been shown that long-range interactions between the 5' and 3' UTRs may act as molecular switches for different steps in the HCV life cycle (Isken et al., 2007; Shetty et al., 2013; Tuplin et al., 2012). We showed that NS5A specifically downregulates RNA translation when both HCV 5' and 3' UTRs are present, suggesting that NS5A may interrupt this long-range interaction. It is also possible that the binding of NS5A to the poly(U/UC) region may disrupt the binding of cellular factors that function to enhance viral translation and/or the recycling of ribosomes between the 3' UTR and IRES (Bai et al., 2013; Niepmann, 2013). Whilst the exact mechanisms remain to be elucidated, data presented here as well as those already published collectively suggest that NS5A may play a role in the switch between viral translation and viral replication.

In conclusion, we have shown that NS5A downregulates viral translation through a mechanism that requires the poly(U/UC) region in the viral 3' UTR. Our results shed more light on the functions of NS5A in the HCV life cycle.

**Fig. 5.** NS5A downregulates translation of full-length HCV genomic RNA in the presence of the poly(U/UC) region in the 3' UTR. (a) Structure of the HCV p7-Luc2A ΔGDD genomic RNA. (b–e) Huh-7.5 cells were electroporated with HCV p7-rLuc2A ΔGDD full-length viral RNAs containing either the WT 3' UTR (b) or Δpoly(U/UC) 3' UTR (c), together with mRNAs expressing NS5A or firefly luciferase (fLuc). Cells were harvested at 2, 4 and 8 h after electroporation for luciferase assay.

In (e), Tet-On shRNA Huh-7.5 cells were electroporated with HCV p7-rLuc2A ΔGDD RNA and mRNAs expressing NS5A-PTEN 3' UTR WT (NS5A-WT), NS5A-PTEN 3' UTR Mut (NS5A-Mut) or fLuc. Cells were harvested at 4 h after electroporation for luciferase assay. The rLuc values were normalized to the total cell number in each sample by quantifying total protein concentration. The normalized value of control mRNA at each time point was set to 1. Statistical differences were analysed by Student’s t-test: *P<0.05, **P<0.01, NS, not significant. (d, f) Expression of NS5A was demonstrated by Western blotting using an NS5A antibody. The levels of β-actin were determined by a β-actin antibody. In (f), the ratios of the band intensities of NS5A to β-actin are given underneath the blot.
METHODS

RNA reporters and expression plasmids. All plasmids were constructed as per standard methods and confirmed by DNA sequencing. The HCV monocistronic RNA translation reporter construct contained the HCV 5' UTR, sequence encoding the first 16 aa of the core protein, an internal rLuc gene, sequence encoding the last 5 aa of the NS5B and the 3' UTR of HCV-1b N (Ikeda et al., 2002). This reporter was used to develop additional reporters with 3' UTR, the poly(U)/UC region or the entire 3' UTR deleted. A monocistronic EV71 RNA translation reporter was generated. This reporter construct contained the rLuc gene flanked by the 5' UTR and 3' UTR sequences of EV71 Anhui1 (Chang et al., 2010). In another construct, the EV71 3' UTR was replaced by the HCV 3' UTR. A restriction enzyme recognition sequence (XbaI for HCV and XmaI for EV71) was engineered around the very 3' end of the 3' UTR sequences. Plasmid DNAs digested with XbaI or XmaI were treated with mung bean nuclelease (New England Biolabs) to allow generation of RNA transcripts with authentic 3' ends (Lohmann, 2009). The HCV p7-rLuc2A construct was a full-length viral genome which contained an insertion of rLuc and the foot-and-mouth disease virus 2A peptide located between p7 and NS2 of the HCV genome following a previously described strategy (Jones et al., 2007). The GDD sequence within the NS5B viral polymerase was deleted, rendering this viral reporter replication deficient. We also generated an HCV p7-RLuc2A GDD construct without the poly(U)/UC sequence. Translation reporter RNAs and HCV genomic RNAs were generated by in vitro transcription (MEGAscript T7 In Vitro Transcription kit; Ambion). These RNA molecules contained viral 5' UTR sequences at the 5' ends (no cap) and viral 3' UTR sequences at the 3' ends.

The coding sequences for HCV NS5A, AH deletion mutant and individual domains were amplified by PCR using HCV-1b N Neo C-5b (Ikeda et al., 2002) as template, respectively, and cloned into pEF/cyto/myc vector (Invitrogen) with a stop codon or in-frame with the Myc tag sequence. In a separate construct, the 3' UTR sequence of PTEN of 1 kb length was cloned just 3' to the NS5A-coding sequence, generating plasmid NS5A-PTEN 3' UTR WT. The PTEN 3' UTR was amplified by PCR using the genomic DNA of Huh-7 cells as the template and primers PTEN-FD (5'-AAATTCGAGATTTTTTTTTTTTTATCAAGGAGGATAAACC-3') and PTEN-rev (5'-AAATCTGGGGCCGCACACTGTAATAAATGGTAGTTGTGTT-3'). The seed sequence (5'-GAGACAGACTGATGTGTAAT-3') of a shRNA in the PTEN 3' UTR was removed by mutagenesis to generate NS5A-PTEN 3' UTR Mut. The coding sequence of NS5A was also cloned into pcDNA3 vector and used as template to generate capped mRNA by in vitro transcription after linearization (mMessage mMACHINE T7 In Vitro transcription kit; Ambion). A similar plasmid encoding firefly luciferase (fLuc) was used to generate capped fLuc mRNA. These mRNAs had a cap structure at the 5' ends and a polyU sequence at the 3' ends. The cloning of GFP with a Flag-tag at its N terminus was cloned into the pEF/cyto/myc vector (Invitrogen).

Cell lines, transfections and luciferase assay. Human hepatoma cell lines Huh-7 and Huh-7.5 were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% FBS and 1% gentamicin, and cultured at 37 °C and 5% CO2. A Tet-On Huh-7.5 cell line expressing a shRNA targeting the 5'-GAGACAGACTGATGTGTAAT-3' sequence in the PTEN 3' UTR was generated (Q. Wu and Q. Liu, unpublished data). For RNA transfections, cells were transfected with reporter RNAs using DMRIE-C (Invitrogen) and incubated for 4 h. For DNA and RNA co-transfections, cells were transfected with expression plasmid DNA and translation reporter RNA using the Jet-PEI transfection reagent (Polyplus-Transfection), and incubated for 24 h. For sequential transfections, cells were transfected with plasmid DNA using the TransIT-LT1 transfection reagent (Mirus Bio) for 24 h followed by translation reporter RNA transfection using DMRIE-C (Invitrogen) and incubated for 4 h. In HCV genomic RNA experiments, Huh-7.5 cells in PBS were electroporated (270 V, 950 μF, 4 mm cuvette) with HCV RNA and NS5A or rLuc mRNA using an electroporator (Bio-Rad) before they were harvested at the indicated time points. In NS5A knockdown experiments, Tet-On shRNA Huh-7.5 cells were incubated with 1 μg doxycycline (Sigma-Aldrich) ml⁻¹ for 48 h to induce the expression of shRNA. Cells were then electroporated with HCV RNA and NS5A-PTEN 3' UTR WT, NS5A-PTEN 3' UTR Mut or rLuc mRNA and harvested at 4 h after electroporation. For luciferase assay, the cells were lysed with Passive Lysis Buffer and rLuc activity was determined according to the manufacturer's instructions (Promega). Luciferase data in the electroporation experiments were normalized to protein concentration, which was determined by the Bradford assay (BCA Protein Assay kit, Thermo Scientific).

Western blotting. Western blots were performed as described previously (Jackel-Cram et al., 2010). Briefly, cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then blocked in 5% skimmed milk in PBS for 1 h at room temperature before incubation with primary antibody overnight at 4 °C. Blots were then washed with PBS and incubated with the appropriate secondary antibodies (LI-COR) for 1 h at room temperature. Blots were then washed with PBS and incubated with the appropriate secondary antibodies (LI-COR) for 1 h at room temperature. Blots were then washed with PBS. Blots were scanned using a LI-COR Odyssey scanner at the appropriate wavelengths (LI-COR). In the NS5A knockdown experiments, protein band intensities were determined by Quantity One software (Bio-Rad). An anti-NS5A mAb (Virogen) and a polyclonal NS5A antibody (Huang et al., 2004) were used. Anti-β-actin and anti-Myc antibodies were purchased from Cell Signaling Technology. Anti-Flag antibody was from Sigma-Aldrich.

MTT assays. Cell viability was investigated using an MTT assay. MTT reagent (20 μl, 5 mg ml⁻¹; Sigma-Aldrich) was added to cells in a 96-well plate and the plate was placed on a shaker for 5 min at room temperature. The cells were then incubated for 4 h to allow the MTT to be metabolized. The medium was removed from the cells and 200 μl DMSO was added to each well. After 5 min at room temperature on a shaker, the OD₅₇₀ was obtained and the background (OD₅₇₀ of shRNA) was subtracted using a Spectra Max 340PC plate reader (Molecular Devices).

Reverse transcription and real-time PCR. RNA was extracted from Huh-7 cells with TRIzol (Invitrogen) and reverse transcribed into cDNA by Superscript II (Invitrogen) as previously described (Qiao et al., 2013). Real-time PCR experiments were performed with primers rLuc-FD (5'-TTGTGAGGGTGCCAAGAAG-3') and rLuc-rev (5'-TGAGAACTGCTCAAGACG-3') using a SYBR Green-based detection system. The transcript levels of β-glucuronidase (GUS) determined in parallel with primers GUS-FD (5'-GGTGCTGAGGATGCGAG-3') and GUS-rev (5'-GGCTCCTGCGCAAGACG-3') were used for normalization.

Statistical analysis. All the experiments were performed in triplicate. The experimental data were analysed by Student's t-test. P≤0.05 was considered statistically significant.

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