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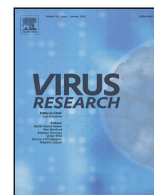

# K312 and E446 are involved in HCV RNA translation modulation by NS5A domains II and III

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ABSTRACT

HCV NS5A plays a critical role in the HCV life cycle. We previously demonstrated that NS5A down-regulates viral translation through a mechanism requiring the polyU/UC region of the viral 3′UTR and that each of the three domains is capable of carrying out this function individually. In this study, we mapped the regions and amino acid residues within domains II and III involved in the modulation of viral translation. Using a series of deletion and amino acid substitution mutants, we found that K312 and E446 play important roles in the modulation of viral translation by NS5A domains II and III, respectively. In the context of full-length NS5A, mutations of K312 and E446 alone or in combination again abrogate translation down-regulation. In a transient replication assay using HCV subgenomic replicons, the K312A mutation alone does not affect HCV replication throughout a 96-h time course. While the E446A mutation can increase HCV replication at early time points (4–24 h), the K312A and E446A double mutation can enhance viral replication at 24–96 h, suggesting both residues are involved. Our results shed more light on the functions of NS5A.

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## 1. Introduction

Hepatitis C viral (HCV) RNA contains a single open reading frame that is flanked by two highly conserved untranslated regions (UTRs) (Niepmann, 2013). The viral polyprotein is cleaved co- and post-translationally by both cellular and viral proteases into at least ten viral proteins (Hoffman and Liu, 2011). This includes three structural proteins core, E1, E2 and seven non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Hoffman and Liu, 2011). The 5′UTR contains an internal ribosomal entry site (IRES) which can directly recruit the 40S ribosomal subunit to initiate translation of the viral polyprotein (Hoffman and Liu, 2011). The 3′UTR, which serves as the initiation site for negative stand synthesis during viral replication, is composed of three regions: a variable region, a polyU/UC tract and an X-tail region (Hoffman and Liu, 2011). The 3′UTR also functions to stimulate viral translation through unknown mechanisms but may involve long range RNA–RNA interactions with the 5′UTR, binding of cellular proteins and/or the retention and recycling of ribosomes during subsequent rounds of translation (Bai et al., 2013; Hoffman and Liu, 2011).

Numerous cellular and viral proteins have been implicated in the modulation of viral translation but the mechanism remains unclear (Hoffman and Liu, 2011; Niepmann, 2013). Particularly unclear is the role that the essential viral protein NS5A may play in this process (Hoffman and Liu, 2011). NS5A is made up of an N-terminal amphipathic helix followed by three domains separated by regions consisting of low complexity sequences (LCSs) (Tellinghuisen et al., 2004). The amphipathic helix is essential for viral replication and mediates NS5A localization to the replication complex at ER derived membranes (Elazar et al., 2003; Tellinghuisen et al., 2005). The three domains of NS5A have been implicated in numerous functions in the viral life cycle and each possesses RNA binding ability toward the polyU/UC tract within the viral 3′UTR, although with differing affinities (Foster et al., 2010; Huang et al., 2005). Domain III binds to this region weakly when compared to domains I and II (Foster et al., 2010). NS5A has also been observed to bind to sites within the IRES in the 5′UTR (Huang et al., 2005). As NS5A interacts with both ends of the genome, this suggests that it may play a role in modulating viral translation and in particular the switch from translation to replication which must occur during the life cycle of a positive sense RNA virus.

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Domain I is highly structured, possesses an essential zinc-binding motif and is proposed to mediate NS5A dimerization within infected cells (Tellinghuisen et al., 2005). A correlation between NS5A dimerization, RNA binding and viral replication has been observed, supporting the biological relevance of domain I dimerization (Lim et al., 2012). Much less is known about NS5A domains II and III. Domain II (aa. 254–346), like domain I, is essential for viral replication (Appel et al., 2008; Ross-Thriepland et al., 2013; Tellinghuisen et al., 2008). However, large portions of this domain are dispensable for replication as various mutagenesis studies have found that only between 12 and 23 amino acids within the C-terminal portion of domain II are essential for viral replication (Appel et al., 2008; Ross-Thriepland et al., 2013; Tellinghuisen et al., 2008). Domain III appears to be dispensable for viral replication as deletion of this region is tolerated in viral replicons (Hughes et al., 2009). However, this region may have a role in establishing early replication efficacy as deletion of this domain leads to delayed replication kinetics in the JFH-1 cell culture system (Hughes et al., 2009). On the other hand, this region plays a critical role in viral assembly (Masaki et al., 2008).

We have previously found that NS5A down-regulates viral translation through a mechanism requiring the polyU/UC region of the 3′UTR (Hoffman et al., 2015). Furthermore, we demonstrated that each of the three NS5A domains is capable of carrying out this modulation independently (Hoffman et al., 2015). In this study, we determined the regions and residues of domains II and III of NS5A that are involved in the modulation of viral translation. We found that K312 and E446 play important roles in the modulation of viral translation by NS5A domains II and III, respectively.

## 2. Materials and methods

### 2.1. Plasmid construction and RNA synthesis

The monocistronic HCV RNA translation reporter T7 HCV 5′UTR-Core$^{aa.1-16}$-rLuc-NS5B$^{aa.5}$-3′UTR and plasmids expressing full-length NS5A, NS5A domain II with or without LCS II, NS5A domain III, and EGFP were described previously (Hoffman et al., 2015). NS5A domain II and domain III truncations and amino acid substitution mutants were constructed by a PCR-based approach. The K312A and E446A mutations were also cloned onto the full-length NS5A. NS5A and a few deletion mutants were cloned 3′ to the EGFP-coding sequence to express EGFP-NS5A fusion proteins. For all truncation constructs, a myc-tag was added to the C-termini. The amino acid substitution mutations were also cloned into an HCV subgenomic replicon with a firefly luciferase (fLuc) reporter (Lohmann, 2009). All plasmids were confirmed by DNA sequencing. HCV RNA translation reporter and replicon RNAs were generated from linearized plasmids by *in vitro* transcription using the MEGAscript T7 *In Vitro* Transcription kit (Ambion).

### 2.2. Cell lines, transfections, HCV transient replication and luciferase assay

HuH-7 and HuH-7.5 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) and supplemented with 10% fetal bovine serum and 1% gentamicin and cultured at 37 °C and 5% $CO_2$. Transfection, HCV replication and luciferase assays were performed as previously described (Hoffman et al., 2015).

### 2.3. SDS–PAGE and Western blotting

SDS–PAGE and Western blotting were performed as previously described (Hoffman et al., 2015; Jackel-Cram et al., 2010). A monoclonal anti-NS5A antibody was purchased from Virogen and a polyclonal NS5A antibody was kindly provided by Dr. Cameron (Huang et al., 2004). The myc-tag and β-actin antibodies were purchased from Cell Signaling Technology. Secondary antibodies IRDye 800CW goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG were purchased from Li-Cor Biosciences.

### 2.4. Statistical analysis

All the experiments were performed in triplicates. The experimental data were analyzed by Student's *t* test. A $p$ value of $\leq 0.05$ was considered statistically significant.

## 3. Results

### 3.1. NS5A domain II region aa. 299–346 is sufficient for the down-regulation of HCV translation

We previously showed that NS5A domain II can down-regulate HCV translation (Hoffman et al., 2015). To map the region of NS5A domain II (Fig. 1A) required for the translation modulation, we constructed a set of domain II deletion mutants and assessed their effects on viral translation. We found that the LCS II region did not play a role in modulation of translation and that the aa. 284–346 fragment lacking the first 30 residues of domain II was still capable of modulating viral translation (Fig. 1B). However, upon deletion of 60 residues from the N-terminus of domain II, the aa. 314–346 fragment could no longer down-regulate viral translation (Fig. 1B). We then deleted a further 15 amino acids from the N-terminus of the aa. 284–346 fragment to produce the aa. 299–346 fragment. We found that the aa. 299–346 fragment was still able to down-regulate viral translation (Fig. 1B). Expression of these mutants was confirmed by Western blotting (Fig. 1C). The aa. 314–346 protein with a molecular weight of 8.3 kDa was not detectable by Western blotting despite numerous attempts (data not shown). This might be due to poor transfer of the very small protein from the SDS–PAGE gel to the blot membrane. As such, we fused the aa. 314–346 sequence with EGFP and the expression of the fusion protein could be detected in Western blotting (Fig. 1E). Fusion with EGFP had no effect on translation modulation by aa. 314–346 (Fig. 1D). These results demonstrate that the region spanning aa. 299–346 of domain II is sufficient for down-regulating viral translation.

### 3.2. Residue K312 is involved in the down-regulation of viral translation by NS5A domain II aa. 299–346 region and full-length domain II

Our previous research demonstrated that translation down-regulation by domain II of NS5A requires the polyU/UC sequence, where NS5A binds (Foster et al., 2010; Hoffman et al., 2015; Hwang et al., 2010). Positively charged amino acids are favored in protein–RNA interactions as they can interact with the negatively charged phosphate backbone of RNA. Therefore, to identify residues within the aa. 299–346 region of domain II involved in the translation modulation, the four positively charged amino acids within this fragment were mutated to alanines individually and their effects on viral translation investigated. The domain II aa. 299–346 fragments that contained R308A, K309A, or K311A maintained, whereas the K312A mutation abolished the ability of this domain II region to modulate viral translation (Fig. 2A). Furthermore, the fragment which contained all four mutations behaved similarly to the K312A mutant (Fig. 2A). The expression of these domain II aa. 299–346 mutants was demonstrated by Western blotting (Fig. 2B). These results point to an essential role of K312 in the ability of aa. 299–346 to modulate viral translation.

To confirm the importance of K312 in the modulation of viral translation by NS5A domain II, the K312A mutation was introduced

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