INVESTIGATING THE MODULATION OF VIRAL TRANSLATION BY THE HEPATITIS C VIRUS NONSTRUCTURAL PROTEIN 5A

A Thesis submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Vaccinology and Immunotherapeutics, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

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ABSTRACT

Hepatitis C virus NS5A is a multi-functional viral protein essential for viral replication and assembly, although the exact role the protein plays in the viral lifecycle remains unclear. A vast array of functions have been attributed to NS5A in recent years, despite the lack of enzymatic activity. NS5A has been found to interact with over 130 host proteins including many which are central to cellular signaling pathways. NS5A is composed of three domains separated by regions of low complexity. All three domains perform important functions in the viral lifecycle. Domains I and II are essential for viral replication whereas domain III is required for viral assembly. However, the role that NS5A and its individual domains may play in modulating viral translation remains controversial. Previous studies have utilized translation reporter systems that do not accurately reflect the role of the viral 3´-UTR in modulating viral translation. We and others have shown that NS5A binds to the poly-U/UC region of the 3´-UTR. In addition to serving as the initiation site for negative strand synthesis the 3´-UTR functions to significantly enhance viral translation. The mechanism of translation enhancement remains unclear but may involve long range RNA-RNA interaction with the IRES, the binding of cellular proteins which stimulate translation and/or the recycling of ribosomes. Therefore, the protein-RNA interaction between NS5A and the poly-U/UC region has the potential to modulate viral translation. Therefore we set out to determine the role of NS5A and its individual domains in modulating viral translation and the role of the NS5A-poly-U/UC region interaction in this modulation.

Utilizing monocistronic RNA reporters which contain the viral 5´- and 3´-UTRs and an internal Renilla luciferase reporter gene, we determined that NS5A specifically down-regulates viral translation in a dose-dependent manner through a mechanism dependent upon the presence of the poly-U/UC region in the viral 3´-UTR. Furthermore, we have re-tested the effect using full-length HCV genomic RNA reporters. These results suggest that NS5A is able to interfere with the stimulation of viral translation exerted by the 3´-UTR. This down-regulatory function of NS5A may function in mediating a switch from translation to replication, a step required in the lifecycle of a positive sensed RNA virus. Having established a role for NS5A in modulating viral translation, we then aimed to determine which region of NS5A was responsible for this effect. We found that each of NS5A domains was capable of this modulatory effect on viral translation independently. Although surprising, this finding is not entirely unexpected as each domain has been shown to retain the ability to bind to the poly-U/UC region.

Within NS5A domain I we identified a 61 aa. region sufficient for translation down-regulation. Furthermore, we have identified a number of positively charged residues within this region involved in

the modulation of viral translation, in particular arginine 112 (R112). This residue has previously been found to be at the domain I dimer contact interface and to form an intermolecular hydrogen bond with glutamic acid 148 (E148). We found that mutations R112A and E148A individually negate the ability of domain I to modulate viral translation and these mutations impede the formation of domain I dimers. Additionally, the R112A mutation appears to affect the ability of domain I to interact with the poly-U/UC region of the viral 3´-UTR alluding to the possible mechanism of translation modulation. Finally this mutation was lethal in an HCV subgenomic replication, confirming the link between NS5A dimerization, RNA binding and viral replication. These results collectively point to a crucial role for the NS5A arginine 112 residue in the modulation of HCV lifecycle by NS5A.

Within NS5A domain II, we identified a 47 aa. region sufficient for translation modulation. Through the mutation of positively charged amino acids within this region, we found that lysine 312 (K312) was essential for this effect. The ability of this domain to modulate viral translation was completely lost when K312 was mutated within a full domain II protein fragment. The mechanism behind this modulation remains unclear but the 47 aa. region identified has been previously found to contain a region proposed to make contact with poly-U RNA and the K312 residue was suspected to interact directly with such RNA. Furthermore, this region interacts with the host protein cyclophilin A, an interaction that enhances the RNA binding ability of domain II. These findings strongly suggest that domain II modulates viral translation by binding within the poly-U/UC region.

While investigating the modulation of viral translation by NS5A domain III we determined that the C-terminal 31 aa. are sufficient for the effect of this domain on viral translation. Through alanine scanning mutagenesis we identified glutamic acid 446 (E446) as playing a key role in the modulatory function of this region. Within a domain III protein fragment mutation of this E446 residue abolishes the modulatory function of this domain towards HCV translation. The mechanism behind this modulation and the role of E446 in this effect remains to be determined.

These findings suggest that in addition to being essential for viral replication and assembly, NS5A has an important role in modulating viral translation through a mechanism requiring the poly-U/UC region of the viral 3´-UTR. Furthermore, each domain of NS5A appears to contribute to this effect. These results support the description of NS5A as a multi-functional protein and the further characterization of its functions may aid in the development of novel antivirals targeting the numerous functions of this complex, and at times puzzling, viral protein.

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Finally, I would like to thank my amazing family, especially my parents, Gary and Carol, for their constant support and for teaching me that I could accomplish anything. Most importantly I would like to thank my wife Jessica, who was there for me every single day of graduate school. Each day I consider myself lucky for having met you.

DEDICATION

This thesis is dedicated to my brilliant and beautiful wife,

Jessica,

Without her constant love, support and unwavering belief in me I would not have been able to accomplish this work.

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LIST OF ABBREVIATIONS

aa.	-	amino acid
AH	-	amphipathic helix
ARF	-	alternative reading frame
ARFPs	-	ARF proteins
CKI-α	-	casein kinase I alpha
CKII	-	casein kinase II
CLDN1	-	claudin-1
CypA	-	cyclophilin A
CypB	-	cyclophilin B
DAA	-	direct acting antiviral
DCV	-	Daclatasvir
DGAT1	-	diacylglycerol acyltransferase 1
DMVs	-	double membrane vesicles
eIF	-	eukaryotic initiation factor
ER	-	endoplasmic reticulum
FAPP2	-	four-phosphate adaptor protein 2
FBP	-	FUSE binding protein
FKBP8	-	FK506 binding protein 8
FUSE	-	far upstream element
HCC	-	hepatocellular carcinoma
HCV	-	Hepatitis C virus

HCVcc - HCV cell culture

HIV - human immunodeficiency virus

hnRNP - heterogeneous ribonucleoprotein

Hsp90 - heat shock protein 90

HSPGs - heparin sulfate proteoglycans

IDP - intrinsically disordered protein

IL-6 - interleukin-6

IL-8 - interleukin-8

IRES - internal ribosomal entry site

ITAFs - IRES-trans-acting factors

LCS - low complexity sequence

LDV - Ledipasvir

LDLR - low-density lipoprotein receptor

LD - lipid droplet

MCP-1 - monocyte chemoattractant protein-1

miRNA - micro-RNA

MMVs - multiple membrane vesicles

NANBH - non-A, non-B hepatitis

NLS - nuclear localization signal

NPC - Nuclear pore complex

NPC1L1 - Niemann-Pick C1-like 1

NS5A - non-structural 5A protein

OCLN - occludin

ORF - open reading frame

OSBP - oxysterol-binding protein

PABP - poly(A)-binding protein

PCH - Pombe Cdc15 homology

PCR - polymerase chain reaction

Peg-IFN - pegylated interferon-α

PH - Pleckstrin homology

PI3K - phosphatidylinositol 3-kinase

PI4KIIIα - phosphatidylinositol 4-kinase IIIα

PI4P - phosphatidylinositol 4-phosphate

PKA - protein kinase A

PKR - protein kinase R

PLA2G4A - phospholipase A2, group IVA

PSTPIP2 - proline-serine-threonine phosphatase interacting protein 2

PTB - polypyrimidine tract-binding protein

RBV - Ribavirin

RNAi - RNA interference

RRM - RNA recognition motif

SgR - Subgenomic replicon

siRNA - small interfering RNA

SL - stem-loop

SMVs - single membrane vesicles

SMYD3 - SET and MYND domain containing protein 3

SOF - Sofosbuvir

SRB1 - scavenger receptor B-1

SVR - sustained virological response

TIP47 - tail-interacting protein 47

uPA-SCID - urokinase-type plasminogen activator-severe combined immunodeficiency

UTR - untranslated region

VLDL - very low-density lipoprotein

Wt - wild-type

1.0 LITERATURE REVIEW

1.1 Hepatitis C Virus

1.1.1 Identification and Molecular Characteristics

Hepatitis C virus (HCV) was first identified in 1989 as the cause of transfusion associated Non-A, Non-B hepatitis (NANBH) (1, 2). This discovery was the result of significant advances in the field of virology as HCV was the first virus identified using exclusively modern molecular cloning techniques (3). Identification of HCV was accomplished by utilizing a bacterial cDNA clone library derived from chimpanzees infected with NANBH patient blood (3). This clone library was then screened with antibodies isolated from the serum of NANBH patients (3). One of the clones identified through this screening process was subsequently found to originate from a large extrachromosomal RNA molecule found only in NANBH infected patients therefore identifying the HCV viral genome (3).

HCV is an enveloped, positive sense RNA virus classified as member of the *Flaviviridae* family within the *Hepacivirus* genus (4) . Other members of *Flaviviridae* include yellow fever virus, dengue and West Nile virus (5). The only other members of the *Hepacivirus* genus are GB virus B, of which only a single isolate obtained from a laboratory-housed new world primate exists, and novel *Hepacivirus* recently identified in various animals including dog, horses, rodents, bats and non-human primates (4, 6, 7). HCV exhibits a very limited host species tropism (6). Humans are the only naturally infected hosts, although chimpanzees can be infected experimentally (6). The evolutionary origin of HCV remains unclear as no closely related virus capable of crossing the species barrier has been isolated (6).

HCV is characterized by a high degree of genetic heterogeneity, it is currently classified into 7 distinct genotypes (7). The genotypes differ by approximately 30-35% at the nucleotide level and are further subdivided into at least 67 subtypes that differ in their nucleotide sequence by 20-25% (8, 9). Furthermore, within an infected patient the virus exists as quasi-species, a mixture of distinct but closely related viral genomes differing in sequence by up to 10% (10). This substantial genetic diversity is the result of the high level of viral replication during infection combined with the absence of proof-reading capability by the viral NS5B polymerase which results in the accumulation of mutations within the viral genome (10).

The HCV virion is pleomorphic, ranging between 40-80 nm in diameter, and enveloped by a cellular derived lipid bi-layer acquired during viral particle secretion (11). Two viral glycoproteins, E1 and E2, which mediate viral entry are displayed on the surface of the virion (11). Within this envelope is the viral nucleocapsid which contains a single copy of the viral genome (11). Furthermore, virus particles exist as

complex heterogeneous lipoviroparticles within the infected host due to association with both low-density and very-low density lipoproteins that contain various apolipoproteins, particularly ApoE (11, 12). The precise composition and structure of these lipoviroparticles remains unclear as does the role this association plays in the viral lifecycle. Association with lipoproteins may protect the virus from neutralizing antibodies as well play a role in viral entry (13). Association with ApoE is required for infectivity in cell culture and high lipid content correlates with high infectivity (13).

The 9.6 kb viral genome contains a single large open reading frame (ORF) which is flanked by two highly conserved untranslated regions (UTRs) referred to as the 5´- and 3´-UTRs which play key roles in viral translation and replication (**Figure 1.1**) (14). Translation of this single ORF is mediated by an internal ribosomal entry site (IRES) within the 5´-UTR and results in the production of the viral polyprotein of approximately 3000 amino acids (15). The viral polyprotein is cleaved co- and post translationally by cellular and viral proteases to produce at least 10 viral proteins (16). This includes three structural proteins; Core, E1, E2, and seven non-structural proteins; p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Additional proteins such as the F protein may be produced by alternate reading frames within the core coding region but expression of such proteins has yet to be demonstrated *in vivo* (17).

The core protein forms the viral nucleocapsid though its interaction with, and packaging of, the viral genome (18). In addition to its structural role, core is thought to interact with numerous cellular proteins and modulate cellular gene expression, cellular signalling networks and lipid metabolism (19). The E1 and E2 proteins, which form a non-covalent heterodimer, are the viral glycoproteins present within the viral envelope (20). They and are required for viral assembly and entry (20). The P7 protein is a viroporin that forms an ion channel in artificial lipid membranes and is essential for viral assembly and release (20, 21). However, the exact role of p7 in the viral lifecycle is unclear but (20, 21). NS2 is a cysteine protease which cleaves the viral polyprotein the NS2/NS3 junction, a process which is strongly enhanced by the N-terminal region of NS3 (20). NS2 also appears to be involved in viral assembly independently of its protease activity (20). NS3 is a multifunctional viral protein with a serine protease domain located within its N-terminal region (22). In association with co-factor NS4A, which ensures proper folding of NS3 and membrane association, the NS3/4A complex mediates cleavage of the NS3 to NS5B region of the viral polyprotein (22). The NS3/4A protease is also involved in controlling the innate immune response by cleaving the cellular adaptor protein IPS-1 in the RIG-I ssRNA recognition pathway (23-25) The C-terminal domain of NS3 possesses NTpase/helicase functions that are essential for viral replication and assembly, though the specific function in the viral lifecycle is still uncertain (22). NS4B is proposed to be involved in both the

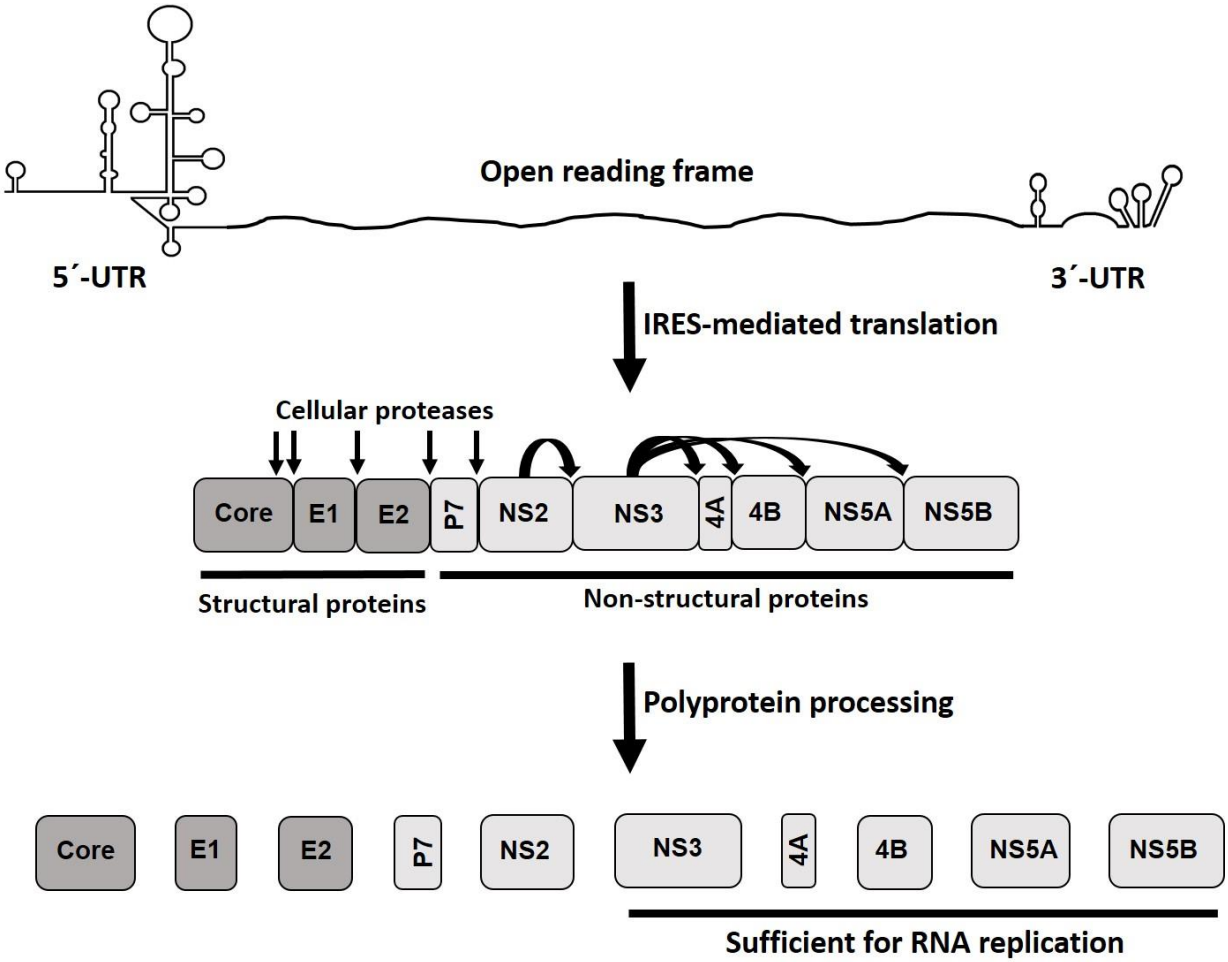

Figure 1.1 - The HCV genome and viral proteins. Diagram of the HCV genome and viral proteins. The positive-sense HCV genome is approximately 9.6 kb and flanked by two highly structured UTRs. Translation of the viral genome is mediated by the IRES within the 5´UTR and results in the production of the viral polyprotein. Cleavage of the polyprotein is accomplished through use of both cellular and the viral NS2 and NS3/4A proteases at the indicated cleavage sites. The NS3-NS5B proteins are sufficient for viral replication.

formation of an ER-associated membranous web and in the organization of the viral replication complex at this membranous structure (20). NS5A is a phosphoprotein that plays essential, but unclear, roles in viral replication and assembly (26). NS5A is also speculated to act as a signalling hub due to its interaction with a wide array of cellular proteins, suggestive of a key role in modulating the cellular environment in favour of viral survival and pathogenesis (27). NS5B is the RNA-dependent RNA polymerase which catalyzes the synthesis of new viral RNA genome via a negative strand intermediate (20).

1.1.2 Epidemiology

According to the World Health Organization, there are an estimated 130-150 million people globally infected with HCV which represents a worldwide prevalence of 2% (28). This prevalence varies considerably between different regions of the world. HCV prevalence is highest in Africa and the Middle East with rates as high as 15% in Egypt (29, 30). Conversely, in developed regions such as North America, Northern and Western Europe HCV prevalence is less than 2% (29, 31). Countries with the highest number of HCV infections; China (29.8 million), India (18.2 million), Egypt (11.8 million), Pakistan (9.4 million) and Indonesia (9.4 million) contain approximately half of worldwide HCV infections (29, 31). The incidence of HCV infection is difficult to estimate due to the challenges in identifying new infections, as acute infections are generally asymptomatic. However, up to 4 million people may become newly infected with HCV annually (31, 32). Of the seven viral genotypes, genotype 1 is the most common worldwide accounting for an estimated 46% of infections (9, 30). Genotype 3 is the second most common at 22% and is followed by Genotype 2 (13%), Genotype 4 (13%), Genotype 6 (2%) and finally Genotype 5 (1%) (30). Genotype 7 has currently only been isolated in a single case (9). The global distribution of HCV genotypes is highly varied and also characterized by regional differences affected by such factors as modes of transmission and immigration (31). Genotypes 1, 2 and 3 are fairly widespread globally while genotypes 4, 5 and 6 are relatively centralized in specific regions of the world (31). Genotype 1 is the most widespread and is most prevalent genotype in the Americas, Australia, Northern and Western Europe and Japan (30). Genotype 2 is more prevalent east Asia and west Africa (9). Genotype 3 is predominant in Asia, primarily within the Indian subcontinent (30, 33). Genotypes 4 is found in Africa and the Middle East, genotype 5 is limited to South Africa while genotype 6 is found in regions of Asia (29).

HCV is a blood-borne virus and transmission occurs primarily through parenteral routes (33, 34). However, the main routes of viral transmission differ throughout the world. In developed countries, HCV

infection is primarily spread through the sharing of contaminated needles and supplies among intravenous drug users (34, 35). In Canada, for example, up to 80% of newly acquired HCV infections are associated with injection drug use (36, 37). In developing countries, HCV is primarily spread through unsafe therapeutic injections (34, 35, 38). It is estimated that up to 39% of injections in the developing world are performed with reused medical equipment resulting in up to 2 million new HCV infections per year (35, 38). An example comes from the reuse of contaminated syringes during national schistosomiasis treatment campaigns in Egypt between 1960 and 1987 that resulted in widespread HCV transmission (39). As a result, Egypt currently has the highest prevalence of HCV in the world at 15% (39, 40). Similar mass treatment or vaccination campaigns are suspected to be responsible for HCV epidemics in a number of central African countries including Cameroon, Gabon and the Democratic Republic of Congo (34). The introduction of widespread HCV screening in the blood supply during the early 1990's virtually eliminated transfusion associated HCV in developed countries (34, 41). However, transmission of the virus though blood transfusion remains a route of infection in countries that either fail to screen for infectious diseases or lack the rigid quality control found in the blood supply system in developed countries (34, 38, 41).

Other, less frequent routes of infection include household exposure, such as the sharing of razor blades, occupational exposure, tattooing, and intranasal drug use (34, 35). Although the efficiency of sexual transmission remains controversial, as transmission between monogamous heterosexual partners is exceedingly low, it is associated with high-risk sexual activity and men who have sex with men (35, 42, 43). HCV can also be transmitted vertically from mother to child during pregnancy at rates of approximately 5% in HCV mono-infected women and up to 11% in HCV/HIV co-infected women and represents the leading cause of pediatric HCV in industrialized countries (44, 45).

1.1.3 Clinical presentation

HCV primarily infects hepatocytes, the most abundant and main functional cells of the liver (46, 47). Hepatocytes perform multiple metabolic, endocrine and secretory functions (46, 47). Of those individuals infected with HCV, an estimated 75% develop a chronic infection (29). The reasons why up to 25% of acutely infected individuals can resolve the infection remain unclear but factors include ethnicity, gender and other host genetic variations (29, 32). Acute HCV infection is asymptomatic in the majority of those infected and thus typically goes undiagnosed (32). While rare, acute infection may present with jaundice and even fulminant hepatic failure (35). Those who do develop symptoms of acute infection are

more likely to clear the virus than in asymptomatic cases (35). In the majority of patients who develop a chronic HCV infection, decades of clinically silent infection can result in severe liver diseases, such as steatosis, cirrhosis, hepatocellular carcinoma and end-stage liver disease (32). Accordingly, HCV is the leading cause of both hepatocellular carcinoma (HCC) and end-stage liver disease in developed countries (32). The chronic inflammation and associated unresolved wound-healing process associated with chronic HCV infection can lead to progressive liver fibrosis which can advance to cirrhosis (48). This cirrhosis can progress to decompensated liver disease and culminate in end-stage liver diseases (48). At this stage the only treatment option is a liver transplant, however there is universal reoccurrence of HCV infection in the liver graft and disease progression is typically accelerated (48, 49). HCV associated cirrhosis is also a significant risk factor in the development of hepatocellular carcinoma (48). Factors found to exacerbate the progression of HCV associated liver diseases include patient age at time of infection (>40), alcohol consumption and co-infection with human immunodeficiency virus (HIV) (35, 50).

HCV is also associated with a number of extrahepatic manifestations including autoimmune disorders such as cryoglobulinemia and thyroid disease in addition to glomerulonephritis, lymphoproliferative disorders, type 2 diabetes and neuropsychiatric conditions (35, 48, 51). The mechanism underlying many of these conditions remains ulcer but such conditions contribute to the lower-health quality of life of those infected with HCV (35).

1.1.4 Treatment

1.1.4.1 Sustained virological response: The goal of HCV treatment

Tremendous progress has been achieved in the treatment of chronic HCV infection in recent years. Many direct acting antivirals (DAAs) targeting various aspects of the viral lifecycle have been approved for clinical use in the last 3 years alone. These new treatment options have resulted in dramatically increased treatment efficacy. The goal and measurement of successful treatment of chronic HCV infection is a sustained virological response (SVR) (52). SVR is defined as undetectable serum HCV RNA 24 weeks after the end of treatment (53). Serum HCV RNA levels are determined by use of a sensitive polymerase chain reaction (PCR) assay with a detection limit of least than 50 IU/mL (53). Assays with lower detection limits of between 10-15 IU/mL have recently been developed and put into clinical use (53). This SVR benchmark has been proven to be indicative of a cure, as numerous follow-up studies have found SVR to be durable (52, 54). A large cohort study that followed patients for approximately 4 years after

achieving SVR found that over 99% of these patients maintained undetectable levels of serum HCV RNA throughout this period (54). Similar results have been obtained in longer term studies of up to 23 years post SVR (55, 56). Findings from studies such as these suggest the probability of HCV reoccurrence after SVR is achieved is exceedingly low. Furthermore, for the small percentage of patients in follow-up studies who were found to be HCV RNA positive after achieving SVR it is unclear whether this represents a relapse or a reinfection (53, 54). Through the use of highly sensitive molecular techniques, very low levels of HCV RNA have been occasionally detected in circulating peripheral blood mononuclear cells, such as lymphocytes and macrophages, in addition to tissues within of the liver of patients who have achieved SVR (53, 57-60). However, it remains unclear if these findings represent infectious viral RNA or have any clinical relevance.

In addition to the clearance of viral RNA to levels below detection, SVR is also associated with the abrogation of disease progression and histological improvements within the liver (53, 61). Liver fibrosis and inflammatory activity have been observed to regress in the majority of patients achieving SVR and complete resolution of these impairments is also possible (53). Furthermore, even a regression in liver cirrhosis has been observed, contrary to the previous thought that this damage was irreversible (53, 61). As such, the risk of developing hepatocellular carcinoma is significantly reduced in patients that have achieved SVR (53). Therefore, SVR leads to significant reduction in liver-related complications and mortality associated with HCV (53).

1.1.4.2 History of HCV Treatment

The standard of care for all HCV genotypes between 2001 and 2011 consisted of pegylated interferon-α (Peg-IFN) and the nucleoside analogue Ribavirin (RBV) (62). IFNα was first used as a treatment for NANBH in 1986, 3 years before HCV was even identified as the causative agent, due to its use against hepatitis B (63). IFNα does not act directly on the virus itself but rather activates a general antiviral state within the cell through induction of IFN-stimulated genes (64). However, this early treatment regime was poorly effective, with SVR achieved in only 6% to 16% of patients undergoing 24 and 48 week treatments, respectively, and the rate of relapse was high (63). In 1998 RBV was added to this treatment regimen in combination with IFNα (63). This dual therapy resulted in increased rates of SVR; approximately 34% and 42% upon completion of 24 or 48 week courses of treatment, respectively, in addition to a reduction in relapse rates (63, 64). The precise molecular mechanism behind RBV antiviral

activity against HCV is unclear (64). Multiple mechanisms have been proposed (64). The next improvement to this dual therapy occurred in 2001 when the half-life of IFNα was enhanced though the molecular modification of pegylation, a process which involves the covalent conjugation of IFN with polyethylene glycol (63, 65). Although leading to increased rates of SVR, it was found that HCV genotype significantly affected the antiviral response to this dual PEG-IFN/RBV treatment (4). While SVR could be achieved in up to 80% of patients infected with genotype 2/3 virus after 24 weeks of treatment, those infected with genotype 1/4 virus at best achieved SVR 50% of the time after a 48 week course of treatment (4). This dual therapy is also associated with a wide array of adverse side-effects (4). These include flu-like symptoms, haemolytic anaemia, autoimmune syndromes and various neuropsychiatric side effects such as severe fatigue, apathy and depression with an increased risk of suicide (66). These adverse side effects combined with the low efficacy, particularly in genotype 1 patients, contribute to poor adherence and treatment avoidance. Despite these significant issues this PEG-IFN/RBV combination therapy remained the standard of care for non-genotype 1 infected individuals until only recently (52, 67).

As the understanding of HCV has expanded substantially over the past 26 years, an increasing amount of research has gone into developing DAAs that directly target essential viral proteins. Development of DAAs was initially focused on genotype 1 virus due to both the poor response of genotype 1 to PEG-IFNα/RBV therapy and its predominance in developed countries compared to other viral genotypes (63). The first two DAAs were approved in 2011 for clinical use against this genotype (63). These two drugs, Telaprevir (Vertex) and Boceprevir (Merk) directly impede the viral lifecycle by inhibiting the activity of the viral NS3/4A serine protease (52). Addition of one of these protease inhibitors to PEG-IFN/RBV therapy results in SVR rates of up to 75% in treatment naïve genotype 1 infected individuals (4). The continued requirement for PEG-IFN/RBV in combination with protease inhibitors was due in part to the rapid selection for resistance mutations during studies of monotherapy (62). Furthermore, these protease inhibitors add additional side effects such as anemia, nausea and rash to an already harsh treatment (68). A third protease inhibitor, Simeprevir (Janssen), was approved for use in 2013 and further increased SVR up to 80% for genotype 1 patients (62). Despite the impressive advances in treatment efficacy these first generation protease inhibitors have already been replaced by more recent and more effective DAAs.

1.1.4.3 Current Standard of Care

In October of 2014, Harvoni (Gilead), the first PEG-IFN/RBV free treatment was approved for use in treatment of HCV genotype 1 infections (69). Harvoni is a once-a-day single tablet combination therapy that consists of two DAAs; Ledipasvir (LDV) and Sofosbuvir (SOF) (69). LDV is a potent inhibitor of the essential viral protein NS5A although the precise mechanism of action is unclear (70, 71). However, LDV has been found to inhibit both viral assembly and replication (72). SOF is a nucleotide analog inhibitor of the viral polymerase NS5B (73). The drug acts as a mimic of the uridine nucleotide and leads to RNA chain termination after its incorporation into the viral RNA during replication by NS5B (73). Treatment duration of Harvoni is typically 12 weeks but may be extended up to 24 weeks in treatment experienced patients who display cirrhosis or shorted to 8 weeks in treatment naïve patients without cirrhosis and low baseline HCV RNA levels (70, 74, 75). This combination treatment has been found to be highly effective, with SVR rates consistently above 90% and as high as 100% (1b subtype) in clinical trials of genotype 1 infected individuals (70, 74, 75). Additionally, this therapy is well tolerated with few side effects reported, headache and fatigue being most common (70, 74, 75). The convenience of a daily single pill, compared with weekly injection of IFN, twice daily RBV and three times daily protease inhibitor, and lack of the severe side effects associated with IFN will undoubtedly significantly improve patient adherence and treatment uptake. However, the cost of this state of the art treatment remains a significant barrier to widespread use, as a single pill is estimated to cost over $1100, which puts the price of treatment at an estimated $63,000 to $189,000 for 8 and 24 week courses, respectively. These prohibitive costs will significantly slow the global use of this treatment as the majority of HCV infected individuals reside in resource limited countries (38).

Due to the pan-genotypic activity of SOF this drug can also be used in the treatment of non-genotype 1 infections (73). Unfortunately, the use of LDV is restricted to genotype 1 virus due to genotypic differences (73). Treatment with SOF/RBV for 12 or 24 weeks is currently recommended for those infected with genotype 2 or genotype 3, respectively (76-80). With this treatment regime over 90% of genotype 2 or 85% of genotype 3 infected patients have been shown to achieve SVR in clinical trials (76-78, 80). For the remaining viral genotypes (4, 5 and 6) it was found that the addition of peg-IFNα was beneficial so the recommended therapy is 12 weeks of triple IFNα / RBV / SOF (79). As with the Harvoni treatment for genotype 1, SOF costs are in a similar range, over $1000 per pill, so again this represents a significant barrier to widespread access (81).

Despite the drastically improved efficacy of newly approved therapeutics, treatment of patients who have failed to respond to previous treatments, and those with varying levels of cirrhosis, remain particularly challenging (82). Due to unknown factors, patients with cirrhosis often display a reduced ability to clear the infection upon treatment (82). Investigations into new treatment combinations, dosages and treatment duration continue with hopes of improved efficacy in these hard to treat populations (82).

1.1.4.4 HCV antivirals in clinical trials

An impressive number of new DAAs are currently in phase II/III clinical trials (68, 82). These include new NS3/NS4 protease inhibitors, NS5A inhibitors and both nucleoside analogue and non-nucleoside inhibitors of the viral polymerase NS5B (68, 82). Some of these new antivirals offer increased potency, higher barriers to resistance and pan-genotypic activity (82). In addition to the numerous DAAs currently in development and clinical trials, a number of drugs targeting essential host factors are being investigated (83). Advantages of such therapies include pan-genotypic activity and a high-barrier to resistance (83). These treatments include Cyclophilin A inhibitors, miR-122 antagonists and viral entry inhibitors (4, 83). Cyclophilin A is a host factor and its interaction with the viral NS5A protein is required for viral replication (84). One inhibitor, Alisporivir (Novartis), is currently in phase III clinical trials (83). miR-122 is a micro-RNA that binds within the 5´-UTR of the viral genome, an interaction that is essential for efficient viral replication (85). Miravirsen (Roche), currently in Phase II trials has been shown to prevent this interaction between the micro-RNA and viral genome and inhibit viral replication (83, 85). There are also a number of small molecule inhibitors of viral entry under investigation (4). One compound, ITX 5061 (iTherx), currently in phase II clinical trials is an antagonist of a receptor involved in HCV entry, scavenger receptor BI, and has demonstrated an ability to prevent viral entry into the cell (83).

It is expected that by combining a number of these next generation antivirals that SVR rates will continue to get closer to 100% (82). As treatments continue to improve, a focus must be placed on eliminating barriers to treatment access as to ensure those who need treatment can access the best available options. This remains a challenge given the world-wide prevalence of HCV, the socio-economic issues involved and the extremely high cost of newly developed therapies.

1.1.4.5 Prevention / HCV vaccine

As there is no vaccine available for HCV current prevention efforts are aimed at preventing new infections and preventing disease in those already infected. One of the most successful prevention mechanisms is screening of the blood supply for the presence of HCV. Although such measures have been in place in developed countries since shortly after the discovery of the virus, this screening is lagging in developing countries (35, 38). Many of these regions either lack proper quality controls and/or focus primarily on HIV screening or, as is the case in 39 countries, do not perform routine testing for transfusion transmissible diseases (35, 38). Education and observance of infection control practices is key in preventing new infections, as unsafe medical procedures are the main route of HCV infection in developing countries (34, 38). In the developed world, much of the efforts at preventing new infections are targeted to intravenous drug users, as this is the population most at risk in these regions of the world (34, 35). Efforts include needle exchange programs in addition to educating and counselling both non-infected and HCV infected IDUs in an effort to eliminate transmission prone practices (35). Treatment uptake in IDU populations has been observed to be incredibly low, with estimates ranging between 1-6% (37). As such a lot of effort has been directed towards removing barriers to treatment that exist within these marginalized populations (37, 86). Additionally, medical organizations such as the Centers for Disease Control in the United States are now recommending that all individuals born between 1945 and 1965 be screened for HCV infection regardless of potential risk factors (87, 88). This population is estimated to include at least two-thirds of adults infected with HCV in the United States, many of who are unaware of their infection status (35). Once an infection has been identified, treatment can be initiated before the progression to severe liver disease such as cirrhosis and HCC. Such wide scale Identification and treatment will also help limit the spread of new infections.

In order to eradicate HCV infections an effective vaccine against HCV is required. The cost barriers associated with HCV treatment and potential for re-infection clearly demonstrate the continued need for a vaccine (89). Unfortunately the development of an HCV vaccine has proven elusive due to reasons such as the remarkable genetic diversity of the virus and the lack of a clear understanding of what constitutes a protective immune response to HCV (10, 89). Contributing to the difficulty in vaccine development is the lack of a small animal model in which to assess candidate vaccine safety and efficacy (10). The Identification of immune correlates in the approximately 25% of acute HCV infections that are spontaneously resolved is key to the development of an effective vaccine as this is the response vaccines should aim to reproduce (90). Furthermore, the fact that up to 80% of those who become re-infected

after this spontaneous clearance are able to clear the second infection suggests that the establishment of protective immune memory is possible (90, 91). Studies have suggested that a broad and multifunctional virus specific CD4+ and CD8+ T-cell response is associated with viral clearance (90, 92, 93). The role that an HCV specific antibody response may play in viral clearance is less clear, although recent findings suggest it may play a more predominant in viral clearance than previously thought (94-96). Therefore a successful HCV vaccine will likely have to stimulate both cellular and humoral immune responses in order to be protective (90, 94). A number of vaccine candidates are at various stages of development including phase II clinical trials (89, 90). One such candidate with promising results utilizes a prime-boost strategy with a replication defective simian adenovirus vector and a modified vaccinia Ankara vector encoding the NS3-NS5B HCV proteins (97). Results from the recently completed phase I clinical trial indicate that this vaccination strategy induced high levels of polyfunctional effector and memory T-cells, both CD4+ and CD8+, specific for multiple viral antigens (97). These results are in line what is known about a protective response *in vivo* and lead to high expectations for the current efficacy trial underway in a population of intravenous drug users.

1.1.5 Hepatitis C Virus Lifecycle

1.1.5.1 Viral entry

HCV particles circulating in the blood stream enter the liver after crossing though the fenestrated endothelium of the liver sinusoids (14). Once in the liver, the virus has direct access to the basolateral surface of hepatocytes within the space of Disse (14). The entry of virus particles is still not fully understood and appears to be a more complex process than observed for other viruses (11, 98). This involves sequential interactions with multiple cellular receptors (11). The implicated cellular receptors include; low-density lipoprotein receptor (LDLR), heparin sulfate proteoglycans (HSPGs), CD81, scavenger receptor B-1 (SRB1), tight junction proteins claudin-1 (CLDN1) and occludin (OCLN) and cholesterol absorption receptor Niemann-Pick C1-like 1 (NPC1L1) (99-105). Humanized mouse models show that transgenic mice, which stably express human CD81 and OCLN, are susceptible to HCV infection at a level similar to primary human hepatocytes (106, 107). These findings demonstrate a role for these receptors in governing the species tropism of HCV (106, 107).

Initial attachment of the virus particles to the hepatocyte surface appears to be mediated by low affinity interaction between the viral particle and glycosaminoglycans of HSPG, the LDLR and SRB1 (11,

14, 98). The receptors utilized in this initial step are proposed to be dependent upon the lipid make-up of the virus particles (14, 98). Initial contact is thought to occur independently of the viral glycoproteins and rely on the interaction between viral associated lipoproteins, most notably ApoE, and the cellular receptors (11, 98). However, the glycoprotein E1 may play a role through is interaction with apolipoproteins (108)

Following this initial attachment the virus particle then interacts with CD81 through the viral E2 glycoprotein (11). However, the region of E2 that interacts with CD81 is initially masked by its hypervariable region 1 region (11, 14). In addition to its role in initial attachment SRB1 is thought to be responsible for exposing the CD81 binding region of E2 (11, 14). This is proposed to occur through either modification of virion lipid composition by SRB1 lipid transfer activity and/or direct interaction with the hypervariable region of E2 (14, 109, 110). Once associated with CD81, the CD81-HCV complex undergoes lateral movement and interacts with the tight junction protein CLDN1 (111-114). The mechanism behind this migration remains unclear but several cellular signalling pathways have been implicated. This includes protein kinase A and two receptor tyrosine kinases; epidermal growth factor receptor and ephrin receptor A2 and downstream RAS and RHO GTPases (114-116). Activation of these pathways is thought to modulate cell surface trafficking of CD81 and possibly CLDN1 in order to promote the formation of the HCV-CD81-CLDN1 co-receptor complex at the basolateral surface (114-116). The formation of this complex then induces internalization of the virus-receptor complex by clathrin-mediated endocytosis (**Figure 1.2**) (115). The role that OCLN, another tight junction protein, plays in viral entry is unclear but it is essential for this process and thought to function at a late stage of entry after CD81 and CLDN1 interaction (99, 117).

After internalization, the acidic environment within the early endosome promotes fusion between the HCV envelope and endosomal membrane mediated by the viral glycoproteins (98, 118). The interaction with CD81 is thought to promote conformational changes in the E1 and E2 viral glycoproteins, priming them for pH-dependent membrane fusion (118). The CD81 and OCLN cellular factors that are endocytosed along with the virus particle are also thought to contribute to this membrane fusion (98). After this fusion event the viral nucleocapsid is released into the cytosol where it undergoes an uncoating process to release the viral genome (11, 14). The viral genome can then undergo translation to produce the viral proteins (11, 14). While the HCV E2 glycoprotein plays an essential role in mediating viral interaction with CD81 the role of E1 in entry less defined. However, E1 is proposed to be involved in modulating E2 structure affecting the accessibility of binding domains (119, 120). This was found to modulate binding of E2 to the cellular receptors SRB1, CD81 and permit an interaction between E1E2 with

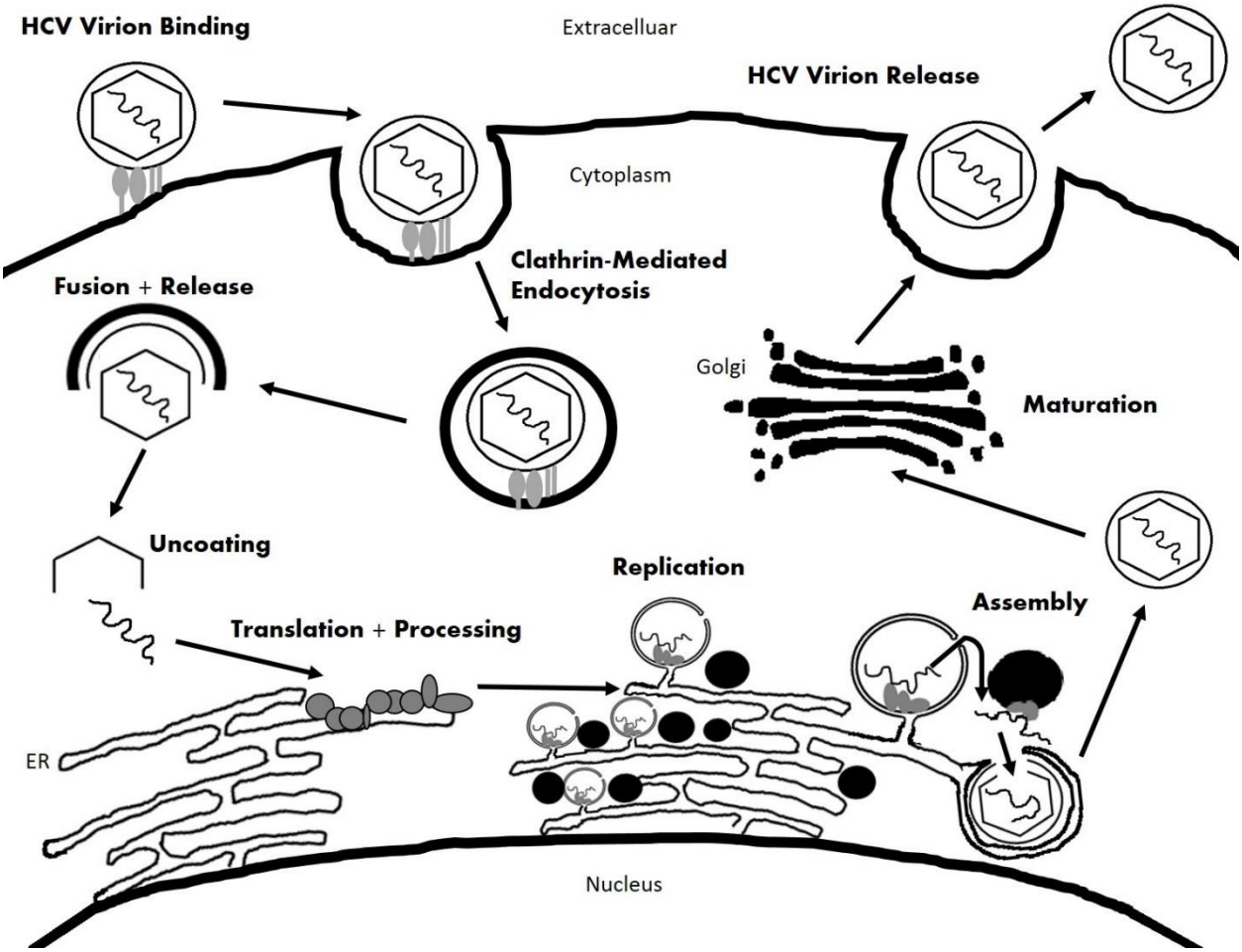

Figure 1.2 - The Hepatitis C virus lifecycle. The virus enters the cells via clathrin-mediated endocytosis after interacting with numerous cellular receptors including SRB1, CD-81 and CLDN1. pH-dependent fusion between the HCV envelope and endosome then releases the viral nucleocapsid. This is followed by capsid uncoating which releases the viral genome into the cytosol. The viral genome is then translated in association with the ER to produce the viral polyprotein. After cleavage of the viral polyprotein by both cellular and viral proteases the viral proteins induce the formation of the ER-associated membranous web. Viral replication is proposed to occur within DMVs in the membranous web. Viral RNA is thought to be transported to the surface of core/NS5A coated lipid droplets (black circles) and then to the assembling virion, which buds into the ER. The virion then acquires it envelope and associates with lipoproteins during transport through the secretory pathway and Golgi. The infectious HCV virion is then released from the cell.

CLDN1 (119). E1 also participates in mediating fusion between the viral envelope and endosomal membrane along with E2 (119, 121, 122). The role of the NPC1L1 cholesterol absorption receptor in viral entry is also unclear (14, 100). This cellular factor was found to be necessary for HCV infection but the mechanism remains to be determined (100).

While the above described route of viral entry is required to establish an infection there is accumulating evidence to suggest a mechanism of cell-to-cell spread of HCV between adjacent hepatocytes plays a role in propagation of the virus within the infected liver (123-126). Such a mechanism may promote viral persistence though evasion of neutralizing antibodies (124). While this route of entry is dependent upon the viral glycoproteins and may utilize many of the same host factors required for cell free transmission, the mechanism has yet to be defined (14). An additional mechanism involving virion independent transfer of HCV RNA between hepatocytes mediated by exosomes has also been described (127, 128). However, the physiological relevance of these alternative mechanisms of viral entry remains to be determined.

1.1.5.2 Translation

1.1.5.2.1 Permission to use

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1.1.5.2.2 HCV IRES and proposed mechanisms of HCV protein translation

As the HCV RNA genome does not possess a 5´-cap, the initiation of HCV protein translation occurs through a cap-independent mechanism mediated by an IRES (129, 130). The HCV 5´-UTR is a highly structured region that contains four distinct domains (I, II, III and IV) and is highly conserved among different viral strains (**Figure 1.3**) (131, 132). The IRES occupies the majority of the 5´-UTR spanning between nucleotides 40-372, encompassing domains II-IV (133). Domains II and III contain the structural

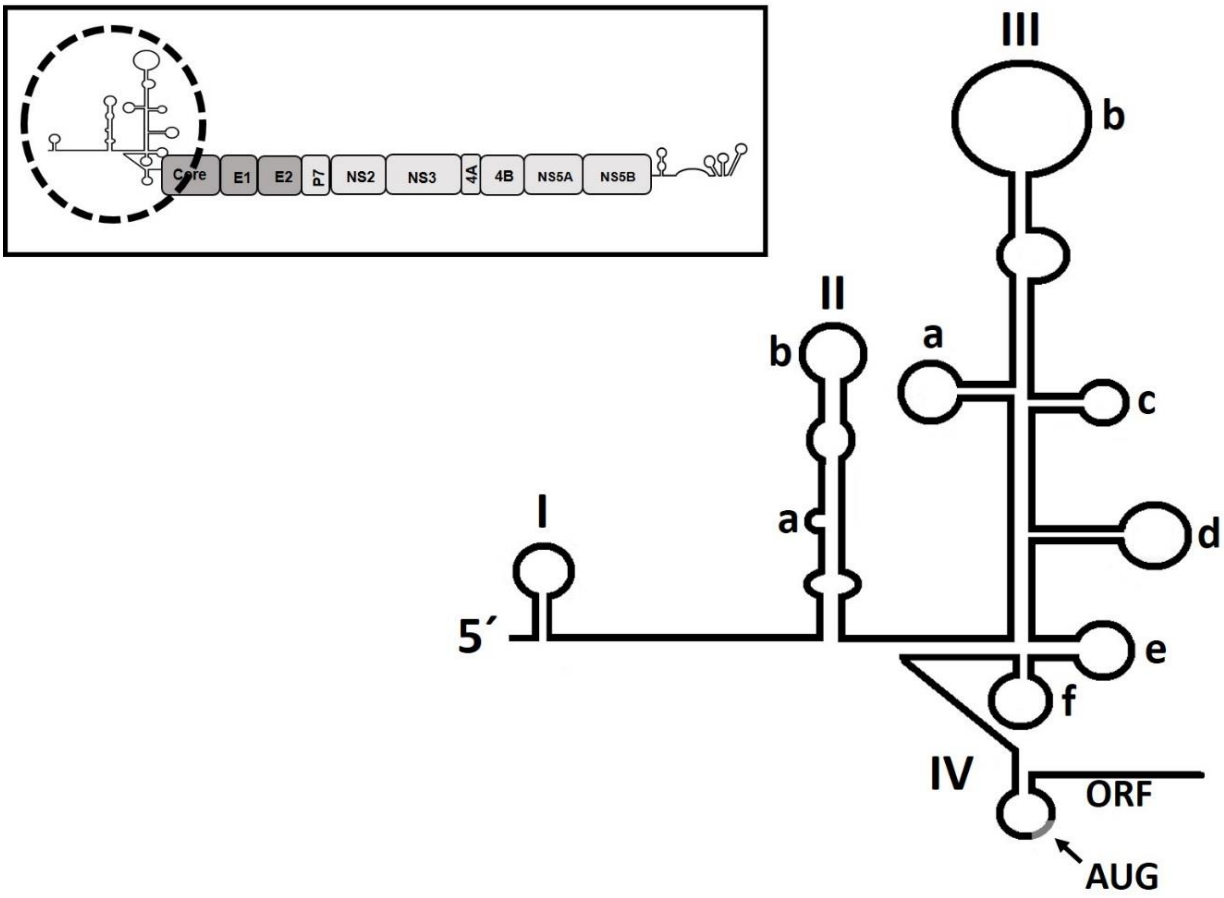

Figure 1.3 - Secondary RNA structure of the HCV 5´-UTR. The 5´UTR is composed of four domains labelled I-IV and numerous subdomains labelled a-f. The HCV IRES is composed of domains II-IV and the start codon is found within domain IV. The IRES functions to directly recruit the 40S ribosomal subunit during translation initiation. The stem-loop within domain I is required for viral replication.

elements crucial for the initiation of translation whereas domain IV contains the initiation codon and part of the core-coding sequence (131, 133-136). Domain II forms a long irregular stem-loop and is composed of two subdomains, IIa and IIb (137-139). Domain III, the largest and most complex of the IRES domains, contains six branching stem-loops that make up the domain III subdomains a-f (133, 137). Subdomains IIIabc make up a four-way junction at the apical portion of domain III while subdomain IIId is part of a three-way junction in the middle of domain III (133, 140). A four-way junction is present in the basal portion of domain III composed of sub-domain IIIe and a proposed pseudoknot formed by interactions between nucleotides present in domain IIIf and those in domain IV, upstream of the initiation AUG codon (133, 140, 141). The function of the HCV IRES is dependent upon both its conserved secondary structure as well as partly on the primary sequence as demonstrated by mutational analysis (142).

Translation initiation begins with the direct recruitment of the 40S ribosomal subunit to the HCV IRES in the absence of any host translation initiation factors, a single-step process that requires the basal portion of domain III (136, 137, 143-145). The 40S complex binds to the IRES in such a way that the ribosomal P-site is in the immediate vicinity of the initiation AUG codon, bypassing the need for scanning (136, 137, 144). 40S ribosomal subunit binding involves multiple sites of interaction with the IRES, this includes interactions between the IRES and ribosomal proteins and RNA (146-148). High affinity binding requires the junction formed between domains IIIabc as well as the junction formed by domains IIIe/f (137, 142). This binding additionally requires IRES domain IIId (137, 142). Furthermore, the proposed pseudoknot structure is also thought to be involved in the 40S-IRES interaction (141, 149). The interaction between the pseudoknot structure and the 40S ribosomal subunit is believed to contribute to the positioning of the AUG codon in the mRNA binding cleft of the 40S ribosome (141, 149). Disruptions of this pseudoknot structure show that it is required for efficient IRES-mediated translation initiation (141, 149). The 40S ribosomal subunit also interacts with domains II and IV, however these interactions do not add to the affinity between the IRES and the 40S subunit (142, 150). Domain II, particularly subdomain IIb, partially occupies the exit channel and extends towards the P-site in the ribosomal subunit and this interaction induces a conformational change in the 40S subunit that is thought to result in the opening of the mRNA entry channel of the ribosome to allow stable accommodation of the HCV RNA (133, 142, 150). Additionally, insertion of start codons upstream or downstream of the authentic start codon position is not recognized by the ribosome. This indicates that the IRES structure and the resultant multiple interactions between IRES domains and the 40S ribosomal subunit are involved in the correct placement of the 40S ribosome (142, 151-153).

Following the recruitment of the 40S ribosomal subunit, the eIF2-GTP-Met-tRNA$_i$ ternary complex and eIF3 translation initiation factor are recruited by domain IIIb and join the 40S-IRES complex, resulting in the formation of the 48S complex (137, 142). The ternary complex forms codon:anticodon base pairing interaction with the AUG start codon in the ribosomal P-Site (144). eIF3 interacts with the junction formed by domains IIIabc as well as loops in domains IIIa and IIIb (144). eIF3 is thought to stabilize the ternary complex thereby enhancing translation efficiency and initiation complex formation. This stabilization may be crucial for formation of the functional 80S ribosome complex (136, 142). Although this is the order of assembly suggested by assembly studies, it is possible that *in vivo* the 40S ribosomal subunit may already be associated with eIF3 and the ternary complex prior to IRES binding, nonetheless, these associations are not required for the recruitment of the 40S to the IRES (149). After the binding of the ternary complex and eIF3, GTP hydrolysis mediated by eIF5 and facilitated by HCV IRES domain II leads to the release of eIF2-GDP (137, 154). Further GTP hydrolysis by eIF5B is then required to release eIF3 during the joining of the 60S ribosomal subunit to form the 80S complex (154). During this process, eIF5B and domain II of the IRES are also thought to adjust the position of the initiator tRNA to facilitate 80S formation (155). This translationally competent 80S ribosome can then proceed with elongation, with efficiency of the first translocation event modulated by IRES domain II, and continue along the genome resulting in synthesis of the viral polyprotein (156) .

This mechanism of HCV IRES-mediated translation initiation allows HCV to translate its genome while utilizing only the limited set of host translation initiation factors described above. This is in contrast to cellular mRNA cap-mediated translation which requires at least nine initiation factors involved in such functions as ribosome recruitment and scanning for the start codon (142, 157). A wide array of additional cellular factors are also utilized to enhance the efficiency of this process (158). Notably the HCV IRES directly recruits the 40S ribosomal subunit independent of the eIF4F cap-binding complex required for cellular mRNA translation (142, 157). This direct recruitment is important as the formation of the cap-binding complex is a rate-limiting step of cellular cap-dependent translation initiation and, therefore, the virus does not have to compete with cellular mRNAs for the limited pool of eIF4F complexes (159, 160). Furthermore, formation of the eIF4F complex is antagonized during times of unfavourable physiological conditions such as low nutrient availability (160).

In contrast to the proposed mechanism described above, other studies suggest that the HCV IRES and HCV-like IRES (e.g., CSFV) are capable of initiating translation using an eIF2 independent mechanism during periods of cellular stress (161, 162). During times of cellular stress, such as during viral infection, the α-subunit of eIF2 undergoes phosphorylation, a modification which transforms eIF2 into an inhibitor

of eIF2B as opposed to a substrate (162, 163). eIF2B normally functions as a guanosine nucleotide exchange factor which promotes GDP/GTP exchange on eIF2 enabling the formation of the eIF2-GTP-Met-tRNAi ternary complex (164). The resulting inhibition of eIF2-GDP to eIF2-GTP exchange results in the global down-regulation of protein synthesis as eIF2 can no longer deliver the initiator tRNA to the ribosome (163). However, initiation of HCV translation has been found to be functional in the presence of reduced levels of the eIF2-GTP-Met-$tRNA_i$ ternary complex that effectively block cellular cap-dependent translation (165). Under conditions of induced eIF2α phosphorylation, HCV can initiate translation using only eIF3 and eIF5B/eIF2 and requires no GTP hydrolysis (161, 162). In one proposed model, eIF5B functions in a similar fashion as its bacterial homolog, IF2, in promoting initiator tRNA binding to the ribosomal P-site (161). Alternatively, eIF2A, which is thought to only play a minor role in cellular translation, may be involved in delivering Met-$tRNA_i$ through interactions with domain IIId of the HCV IRES (166, 167). The biological significance of these proposed alternative translation initiation mechanisms are not fully understood. However, the ability to accomplish protein translation during times of cellular stress, when cellular protein synthesis is down-regulated, would provide HCV with a selective advantage during such conditions.

1.1.5.2.3 Alternative reading frame products

The possibility of an alternative reading frame (ARF) within the HCV genome was first suggested by studies which found the HCV core-coding sequence to be far more conserved than necessary to preserve the amino acid sequence (168, 169). Subsequent studies proved this in fact to be true as an ARF that overlaps the core-coding sequence at +1 nucleotide was proposed to encode ARF proteins (ARFPs) (170, 171). Although never directly observed in a natural infection, antibody and T-cell responses directed towards potential ARFPs in HCV patients provide indirect evidence that ARFPs are expressed *in vivo* (170-175). Low expression levels may account for the lack of recovery of ARFPs from infected patients. The first proposed form of ARFP was the 16 kDa ARFP/F (F protein/core+1, core=21 kDa) that was found in HCV-1 replicating cells (176). This protein contains approximately 10 amino acid residues of the core protein attached to approximately 150 amino acids encoded in the ARF (176). Translation of the ARFP/F protein is thought to be initiated at the AUG of the main HCV ORF and translation occurs in this reading frame until an adenine-rich sequence is encountered at codons 8-11 which acts as a “slippery sequence” and stimulates a programmed +1 ribosomal frame-shift into the ARF (176, 177). Translation then proceeds in

the ARF until the first in-frame stop codon is reached (176). However, this adenine-rich sequence was found to be very rare among HCV variants and as such this mechanism alone cannot fully account for the observed ARFP-directed immune responses in HCV patients (176).

Another proposed ARFP product that was generated in *E. coli* from an HCV core encoding fragment referred to as the ARFP/DF is produced as a result of a double frame-shift (176, 178). ARFP/DF is a chimeric protein that contains 42 amino acids of core, 101 amino acids of ARFP and ends with another 30 amino acids of core (176, 178). The first frame-shift event is proposed to occur at a conserved RNA structure within the core-coding sequence referred to as stem-loop VI (176). The second frame-shift that shifts the ribosome back into the original ORF occurs when the ribosome encounters a stop codon located just upstream of another conserved RNA structure, the terminal stem-loop element (176). Other mechanisms reported to mediate ARFP expression in addition to ribosomal frame-shifting include internal initiation and transcriptional slippage (169). In the case of transcriptional slippage, in which nucleotides are added or deleted during RNA transcription at run of adenines or thymines, the cluster of adenines between codons 8-11 in HCV-1 was found to be prone to transcriptional slippage by both T7 and viral NS5B polymerases (177). Therefore the addition or deletion of an adenine in this region could lead to a frame-shift and result in production of ARFPs (177). However as mentioned above, this adenine rich sequence is very rare among HCV variants and in HCV strains containing the adenine-rich sequence, no heterogeneity has been reported. As such, although it is possible that transcriptional slippage may contribute to ARFP expression in the quasispecies variants within infected patients, this is likely not the lone or major mechanism for production of ARFPs (177). Consistent with this notion, an ARFP has been proposed to be initiated at internal methionine codons at positions 85/87 through an internal initiation mechanism (171). The internal initiation results in the production of a protein called ARFP/S (core +1/S) (171). In the absence of codons 85/87, codon 26 in the ARF has also been found to function as an internal translation initiation site (171). Only recently have ARFPs been shown to be expressed in the infectious cell culture system (169). Expression of a family of core isoforms ranging in size from 8 to 14 kDa has been observed (169). The 8 kDa isoform was subsequently determined to likely be the result of internal initiation at or near codon 91 (169). These investigations collectively suggest that there may be multiple mechanisms resulting in ARFP expression and these mechanisms may differ among HCV variants. However, the biological relevance of these mechanisms is not well understood as it is unclear if and how ARFP expression occurs *in vivo*.

The functions that ARFPs may play in the viral lifecycle remain unclear. Mutational studies suggest that these proteins play no critical role in the viral lifecycle as abolishing ARFP production had no

measurable effect on HCV replication in cell culture or in the uPA-SCID mouse model (179). Also MacMullan *et al.* found that the ARFPs F and DF were not essential for virus replication in a chimpanzee infected with an HCV clone containing four stop codons within the alternate reading frame (180). However, ARFPs have been associated with a variety of functions including suppression of cell cycle regulator p21 expression, disruption of transcription factor c-Myc activity, disruption of iron metabolism and enhanced hepatocyte proliferation (17, 181-183). ARFPs have also been implicated in the induction of various cytokines and chemokines such as interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1) (184). This is intriguing as it has been shown that large amounts of IL-6, IL-8 and MCP-1 are produced in HCV patients with advanced liver disease and that a higher prevalence of ARFP antibodies has been found in HCV patients that develop hepatocellular carcinoma suggesting that ARFP levels are higher in patients with advanced liver disease and HCC (171, 172). These findings point to a role in viral persistence and pathogenesis and/or modulation of host processes as possible functions of ARFPs.

1.1.5.2.4 Cis-modulation of HCV protein translation

1.1.5.2.4.1 Core coding sequence

As mentioned previously, domain IV of the HCV 5´-UTR is a component of the IRES and extends into the core-coding region. In addition to containing this 3´-terminal portion of the IRES, the core-coding region also contains a number of conserved RNA secondary structures that are postulated to be involved in HCV translation modulation (179, 185). Four highly conserved stem-loop (SL) structures have been predicted to be present in the core-coding region: from 5´- to 3´, SL47/SLV, SL87/SLVI, SL248, and SL443 (179, 185-187). It has been observed that disruption of the structural integrity of SL47 and SL87 but not SL248 or SL433 impairs translation and viral replication in cell culture and in the urokinase-type plasminogen activator-severe combined immunodeficiency (uPA-SCID) mouse model (179). It has been suggested that SL47 and SL87 enhance HCV translation through the binding of trans-activating factors or possibly through long-range RNA-RNA interactions with other parts of the HCV viral genome (179). On the other hand, a number of studies have identified a suppressive effect of a portion of the core-coding sequence on translation through a long-range RNA-RNA interaction between the 5´-UTR and nucleotides within the core-coding region (188-190). Kim *et al.* found that nucleotides 428-442 of the core-coding sequence, which is located at the 5´-end of SL87, can interact with nucleotides 24-38 in domain I of the

5′-UTR (188). Mutations that disrupted the stability of this interaction significantly enhanced HCV IRES-mediated translation while compensatory mutations that restored the RNA-RNA interaction lead to inhibition of translation in a bicistronic reporter system (188). It is worth noting that the bicistronic reporter system used included non-HCV sequences that may alter the secondary structure of RNA elements of the 5′-UTR of HCV. Perhaps the best evidence supporting a role of structured elements in the core-coding region modulating HCV lifecycle comes from the study by McMullen *et al.* mentioned in the previous section (180). In this study, the HCV clone that was modified through silent mutations to add four stop codons in the ARF resulted in low levels of viremia and minimal liver damage (180). However, it was determined that this effect was not the result of impaired ARFP expression but rather the disruption of RNA secondary structure. (180). The last two stop codons disrupted base-pairing within the predicted secondary structure of SL87 (SLVI) in the core-coding sequence, indicating the importance of maintaining this structured element in HCV lifecycle (180). These findings suggest that the core-coding sequence may be involved in modulation of HCV translation via long-range RNA-RNA interactions or perhaps though interactions with host or viral proteins.

1.1.5.2.4.2 NS5B coding sequence

In addition to the core-coding sequence, the 3′-end of the NS5B coding sequence is also predicted to form conserved RNA structures (185, 186, 191). One of these structures, a stem-loop referred to as 5BSL3.2 (SL9266) has been observed to be essential for viral replication as mutations disrupting its structure blocked RNA replication (191, 192). This stem-loop was found to interact with SL2 of the X-tail region of the viral 3′-UTR, forming a functional kissing-loop tertiary structure (192). This kissing-loop interaction is required for HCV replication (193, 194). In addition to the kissing-loop interaction with the 3′-UTR, the 5BSL3.2 structure is proposed to interact with a non-structured conserved sequence about 200 nucleotides upstream within the NS5B coding sequence (195). Disruption of this interaction was found to inhibit viral replication in the Con-1 replicon system (195). However, it is not known whether this interaction occurs simultaneously with the interaction between 5BSL3.2 and the X-tail region or at different time points during the viral lifecycle (195). Since these two interactions involve distinct regions of 5BSL3.2, it is possible that the upstream interaction functions to stabilize the kissing-loop interaction (195). In addition to the above interactions that are proposed to play a role in viral replication, 5BSL3.2 has also been proposed to participate in a long-range interaction with the IRES found in the 5′-UTR (196).

The apical loop of domain IIId of the IRES and the internal loop of 5BSL3.2 were found to be essential for this interaction (196). The interactions between the UTRs and this structured region of the NS5B coding sequence has also been implicated in modulating the structure of both the IRES and 3´-UTR (197, 198). Although the biological significance of such a long-range interaction remains to be clearly determined, it is possible that this end-to-end communication between the IRES and NS5B coding sequence could play a role in the circularization of the viral genome and/or the switch between different stages of the viral lifecycle (196). Furthermore, cellular or viral proteins that interact with these regions of the viral genome could be involved in mediating this RNA-RNA interaction and its potential effects on the viral lifecycle (196).

1.1.5.2.4.3 The 3´-UTR

In cap-dependent translation, translation is stimulated by poly(A)-binding protein (PABP) bound to the 3´-poly A region of mRNA transcripts interacting with eIF4G of the eIF4F cap-binding complex (199, 200). This interaction has been suggested to promote the circularization of the transcript (199, 200). The interaction between PABP and eIF4G has been shown to increase the affinity of the eIF4F complex for the 5´-cap potentially resulting in increased 40S recruitment. The circularization of the transcript may also enhance translation by promoting ribosome recycling (199, 200). In a conceptually similar fashion, the HCV 3´-UTR has been shown to have stimulatory effects on HCV translation (200-205). The 3´-UTR of HCV is composed of three distinct regions: the variable region, the poly-U/UC tract and the X-tail, a region with highly conserved secondary structure (**Figure 1.4**) (201). The X-tail forms three stem loop structures, named SL1, SL2, and SL3 (205).

Although clearly essential for replication as it serves as the initiation site for negative strand synthesis, the exact role of the 3´-UTR in HCV translation remains unclear (200, 201, 205). For many years the role of the 3´-UTR in the modulation of HCV translation was a contentious issue with published reports suggesting that the region down-regulates, enhances or has no effect on translation (200-204, 206-211). However, as observed in a study by Song *et al.*, these discrepancies may have been partly due to the reporter design (201). Only when monocistronic RNA reporters with precise 3´-ends or complete RNA genomes are utilized is efficient translation enhancement observed (201). The use of bicistronic reporters and the transfection of DNA reporters either encoded on circular or linearized plasmids or PCR fragments may not accurately reflect the role of the 3´-UTR (201). In the same study, the authors found that the

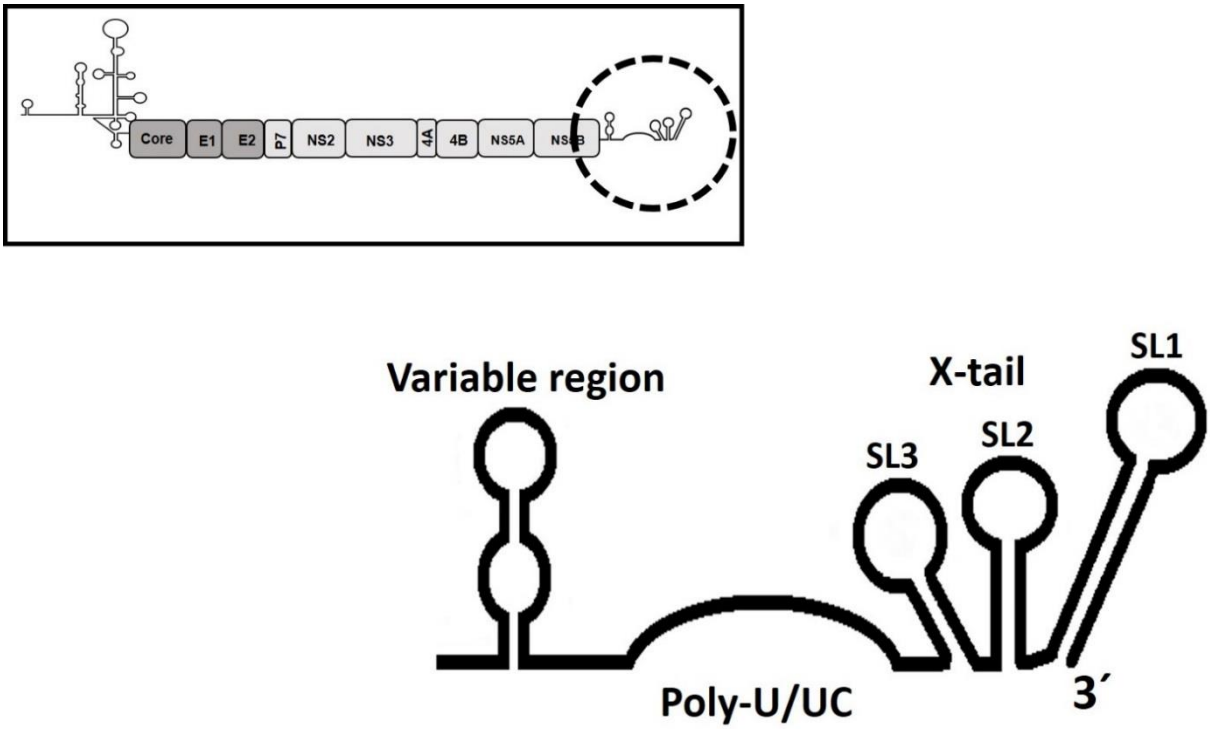

Figure 1.3 - Secondary RNA structure of the 3´-UTR. The 3´-UTR is composed of four regions; the variable region, poly-U/UC region and the x-tail region, which contains 3 highly conserved stem-loops. The 3´-UTR is essential for viral replication as it serves as the initiation site of negative strand synthesis. The 3´-UTR also functions to significantly enhance viral translation. A wide array of cellular proteins has been found to bind to the 3´-UTR and modulate viral translation and replication.

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 while SL2 and SL3 play only minor roles (201).

The mechanism of 3′-UTR enhancement of translation remains to be clearly defined. Possible mechanisms include cellular or viral factors binding to the 3′-UTR that may promote circularization of the viral genome as well as potential long-range RNA-RNA interactions between regions of the 3′-UTR and IRES or other regions (200, 201). The poly-U/UC region is proposed to act as a molecular interaction platform for recruitment of host and viral proteins including the viral NS3, NS5A and NS5B proteins (212-215). Host proteins interacting with this region such as HuR and Lsm1-7 will be described in upcoming sections (216, 217). Furthermore, interactions between the IRES and the 3′-UTR have been found to alter the structure of essential IRES subdomains IIIb and III and domain IV (197). Conversely the 5′-UTR has been observed to be involved in modulating the structure of the 3′end of the viral genome, particularly the 3′Xtail (198). These results suggest a complex network of long-range RNA-RNA interactions that may modulate different steps of the viral lifecycle (197, 198). The 3′-UTR is also proposed to function in the recycling of the ribosome back to the IRES following translation termination, facilitating efficient initiation of subsequent rounds of translation (200, 218).

In a study aimed at determining the specificity of the enhancement of translation by the 3′-UTR, Bung *et al.* utilized chimeric monocistronic reporters with 5′- and 3′-UTRs from different viruses (205). They found the HCV 3′-UTR is capable of stimulating not only HCV IRES-mediated translation but also translation mediated by the structurally similar porcine teschovirus IRES to a comparable extent (205). Porcine teschovirus does not possess a structured 3′-UTR similar to HCV but rather is polyadenylated at its 3′-end (205). Additionally, the HCV 3′-UTR was found to stimulate both the translation of the ECMV IRES which is structurally and functionally different than the HCV IRES as well as the translation of a capped cellular mRNA (205). However, this results in only a moderate stimulation of translation and is only observed in certain cell culture systems suggesting the importance of both UTRs in modulating this effect (218). These results suggest that the mechanism of stimulation by the HCV 3′-UTR may not be solely based on the recognition or interaction of specific RNA structure or sequences present within the HCV genome such as IRES by the 3′-UTR (205). Adding to this view is the fact that in the same study, the substitution of the HCV 3′-UTR with a poly (A) tract also lead to the stimulation of HCV IRES translation, albeit to a lower extent (205). These results therefore suggest that the stimulation of protein translation by HCV 3′-UTR may occur through multiple mechanisms such as RNA-RNA interactions with HCV IRES structure and recruitment of cellular factors bound to the 3′-UTR that interact with components of the translational

machinery present on the IRES in a fashion similar to the PABP-eIF4G interaction found on cellular mRNAs (205). The latter might be a more generalized mechanism which may explain why HCV 3´-UTR can enhance protein translation directed by a non-HCV IRES. Another potential role of HCV 3´-UTR in HCV lifecycle is that this region may be important for the switch between viral protein translation and RNA replication as this site is essential for both replication and translation (201). This switch could involve the sequential interactions of cellular and/or viral factors with the 3´-UTR or RNA-RNA interactions.

1.1.5.2.5 Trans-modulation of HCV protein translation

1.1.5.2.5.1 Cellular factors/ ITAFS

In addition to the limited number of canonical translation initiation factors (eIF2, eIF3 and eIF5/B) required for HCV translation, a number of non-canonical cellular factors have been shown to interact with the HCV IRES and/or 3´-UTR and potentially modulate translation (**Table 1.1**). These IRES-trans-acting factors (ITAFs) have been proposed to function through multiple mechanisms such as enhancing the recruitment of translational machinery, as well as other trans-acting factors, or act to stabilize the IRES structure to facilitate efficient translation. This section will give an overview of some of the ITAFs listed in Table 1.

The La autoantigen has been shown to interact near the initiator AUG codon and a GCAC motif within stem-loop IV appears to be important in this interaction (219-221). This interaction was determined to be facilitated by the RNA recognition motif (RRM) 2 RNA binding domain present with La autoantigen (221). The interaction between the viral RNA and La autoantigen appears to enhance IRES-mediated translation as mutations in this GCAC motif that alter the primary sequence while maintaining the overall secondary RNA structure affect the binding of La autoantigen and result in a significant impairment of IRES-mediated translation (220-222). Additionally the seclusion of La autoantigen results in inhibition of HCV IRES- mediated translation (223). In terms of the mechanism, it was observed that such mutations to this GCAC motif in addition to affecting the binding of La autoantigen also appeared to impair the binding of the S5 ribosomal protein, which is a component of the 40S ribosomal subunit known to interact with the HCV IRES, suggesting a role for La autoantigen in enhancing 40S ribosome recruitment (221). Furthermore, a synthetic peptide derived from the RRM2 domain of La autoantigen, which mediates its interaction with the GCAC motif within the IRES, competes with La protein for IRES binding and interferes with the assembly of the 48S ribosomal complex, resulting in inhibition of translation (222). These

Table 1.1 Cellular trans-modulators of HCV translation				
Cellular factors	**Effect on HCV translation**	**Proposed mechanisms**	**Cellular functions**	**References**
Gemin5	**-**	Binds to IRES and forms complex with eIF3a, b, c. Addition of purified Gemin5 resulted in down-regulation of HCV IRES activity, while shRNA knockdown of Germin5 resulted in increase of HCV IRES activity.	snRNA binding protein of SMN complex, which is involved in biogenesis of snRNPs ,a component of mRNA slicing machinery. Also involved in down-regulation of cellular translation in which a complex containing eIF4E is formed.	**(224)**
hnRNP D (AUF1)	**+**	Interacts with SLII of HCV IRES. Knockdown inhibits translation but increases replication suggesting a role in balancing viral translation and replication.	Involved in mRNA decay, telomere maintenance and translation initiation.	**(225)**
hnRNP L	**+**	Binds to 3´ end of HCV IRES and 5´- terminal region of 5´-UTR. hnRNP L specific RNA aptamers inhibited IRES function in dose dependent manner. Also found to promote efficient replication	Role in mRNA processing including alternative splicing, mRNA export and mRNA stability.	**(226-228)**
Hu antigen R (HuR)	**+**	Binds to poly-U/UC region of 3´-UTR. Over-expression enhances HCV IRES activity while knockdown down-regulates HCV IRES activity.	Selectively binds and stabilizes AU rich element containing mRNAs.	**(217, 229, 230)**
IGF2BP1 (IMP-2)	**+**	Binds to 5´- and 3´ UTRs (poly-U/UC). siRNA knockdown down-regulates HCV IRES activity. May stimulate translation by promoting RNA circularization and/or recruitment of eIF3.	Binds to and regulates the translation of certain mRNA such as insulin-like growth factor 2 and beta-actin.	**(227, 231)**

La auto-antigen	**+**	Binds near the initiation AUG codon of IRES. Facilitates formation of initiation complex and stimulates internal initiation of translation.	Multifunctional protein with roles in RNA biogenesis. Binds to the 3´termni of many newly synthesized RNAs, particularly those made by RNA pol III, protecting the 3´ ends from exonucleases. Autoantigen in systemic autoimmune diseases.	**(219, 221-223, 232-235)**
LSm1-7	**+**	LSm1-7 ring binds to 5´-UTR dependent on SLIII. Also binds to poly-U/UC tract of 3´-UTR. Silencing of LSm1 selectively down-regulates HCV translation.	Component of P-bodies. Involved in mRNA turnover. Binds to short oligo (A) tracts at 3´ end of deadenylated mRNA and inhibits 3´ degradation while promoting decapping and 5´ to 3´degradation.	**(216)**
miR-122	**+**	Binding to S1 and S2 sites in 5´-UTR upstream of the IRES enhances translation. Thought to enhance the association of 40S ribosomal subunit and HCV RNA.	Well-conserved, highly abundant, liver specific microRNA. Binding to 3´-UTR of cellular mRNA encoding cationic amino acid transporter CAT-1 results in down-regulation of CAT-1 protein levels.	**(236-240)**
miR-199a*	**? (-)**	Target sequence in domain II of the HCV IRES. Over-expression results in down-regulation of viral replication.	Implicated in the post-transcriptional regulation of gene expression for various genes such as Ceruloplasmin (CP).	**(241, 242)**
mIR-196	**-**	Potential target sequence present in HCV NS5A coding region. Over-expression results in down-regulation of HCV protein expression and replication.	Implicated in the post-transcriptional regulation of gene expression for various genes such as Bach1.	**(242-244)**
Nucleolin	**? (+)**	No data using HCV reporter. Identified by mass spectrometry as a component of HCV IRES bound 40S ribosomal subunit. Bound by yeast inhibitor RNA (IRNA). Stimulates poliovirus IRES.	Implicated in a variety of processes including rRNA maturation, and ribosome assembly.	**(245-247)**

NSAP1 (SYNCRIP, hnRNP Q)	**+**	Binds to adenosine rich region downstream of AUG start codon in core coding sequence. Facilitates 40S ribosome binding and enhances 80S ribosome formation	A member of hnRNP family of proteins implicated in mRNA processing mechanisms. Component of the spliceosome.	**(248, 249)**
PTB	**?**	Interacts with both 5´- and 3´ UTRs of HCV RNA. Role in HCV protein translation is unclear with contradictory reports suggesting stimulation, inhibition, or no effects on HCV protein translation.	Implicated in the regulation of pre-mRNA splicing.	**(250-256)**
PSMA7	**+**	Knockdown by siRNA or ribozymes results in significant inhibition of HCV translation.	Human 20S proteasome α-subunit type 7 is a component of 20S core structure of proteasome.	**(230, 257)**

observations combined with the observation that La autoantigen binding appears to alter IRES conformation strongly suggest a mechanism through which La autoantigen binding induces a conformation change that enhances the recruitment of the 40S ribosomal subunit to the IRES and/or is involved in ensuring the proper positioning of the 40S subunit on the IRES (221, 222). Furthermore, the La autoantigen is purposed to contribute to the host tropism of HCV as expression of human La autoantigen significantly enhanced both viral translation and replication in mouse cell lines (232).

Other host proteins shown to modulate HCV IRES-mediated translation are heterogeneous ribonucleoprotein D (hnRNP D) and hnRNP L (225, 226, 228). hnRNPs are a diverse family of RNA-binding proteins that are involved in wide range of functions related to cellular nucleic acid metabolism, such as the splicing and packaging of nascent transcripts as well as translational regulation (258). In regards to HCV translation, hnRNP D was found to bind to the stem-loop II region within the IRES (225). Over-expression of this cellular protein enhances viral translation while siRNA knockdown of hnRNP D inhibits viral translation suggesting that hnRNP D is a viral translation enhancer (225). It was also observed that an increase in HCV RNA replication occurs when hnRNP D expression is knocked down (225). This leads to an interesting possibility that hnRNP D acts to balance translation and replication and may be even involved in the switch from translation to replication (225). In regards to hnRNP L, Hahm *et al.* found that it binds to the HCV RNA genome within the 3´ border region of the HCV IRES which includes part of the core-coding sequence (228). This study also showed that hnRNP L binding increased translational efficacy of translation driven by the HCV IRES (228). In agreement with this model, a dose-dependent inhibition of HCV IRES function occurs when hnRNP L is depleted using RNA-aptamers (226). These results suggest an important role of hnRNP L in the modulation of HCV IRES-mediated translation. hnRNP L has also recently been shown to interact within the 5´- terminal region of the 5´-UTR and promote efficient replication (227). Another member of the hnRNP family, NSAP1 (also referred to as hnRNP Q and SYNCRIP) has also been shown to interact with and modulate the HCV IRES (248). NSAP1 binds within an adenosine rich region in the N-terminal region of the core-coding region (248). Same as with hnRNP D and L, over-expression of NSAP1 enhances HCV IRES-dependent translation whereas small interfering RNA (siRNA) knockdown or mutation of its binding site results in inhibition of HCV IRES-mediated translation (248). In terms of a mechanism NASP1 has been found to also interact with the 40S ribosomal subunit and is proposed to aid in correctly positioning the viral RNA thereby enhancing 80S complex formation (249).

Another cellular protein proposed to be involved in the modulation of HCV IRES-mediated translation is polypyrimidine tract-binding protein (PTB), another hnRNP protein family member. Contradictory results have been published and as a result the role for PTB in HCV translation remains

unclear (256). PTB has been found to interact with both the 5′- and 3′-UTRs, a finding that lead to the speculation that PTB may be involved in 5′-3′ crosstalk resulting in the circularization of the viral genome (252, 259). However, subsequent studies by various groups have had different results, reporting that PTB stimulates, inhibits or has no effect on HCV translation (250, 251, 253-256).

Interestingly, a number of cellular proteins implicated in modulating viral translation are capable of interacting with each other (15). For example, hnRNP D interacts with hnRNP L, IGF2BP1 and NSAP1 whereas the La protein can interact with LSm1-7 (260-263). This suggests that cellular ITAFs may act as a larger complex bridging different regions of the IRES and/or regions of the 3′-UTR to enhance translation initiation (15). However, this concept and the significance of these interactions on viral translation remains to be investigated.

Recent research has led to the suggestion that the autophagy pathway may be involved in HCV translation (264). Autophagy is an evolutionary conserved process that functions to maintain cellular homeostasis by removing damaged organelles and protein aggregates from the cytoplasm through the formation of autophagosomes which facilitate delivery to the lysosome for degradation (265). In some viral infections, autophagy performs an antiviral function by targeting viral proteins and/or viral particles for lysosomal degradation and as such viruses have developed mechanisms to evade the autophagic response (265). In other cases, however, viruses appear to have evolved to utilize the autophagic machinery in their viral lifecycle (265). Poliovirus is thought to induce the formation of autophagosome and utilize this structure as scaffold for viral replication and for use in non-lytic viral release (265, 266). In the case of HCV knockdown of key autophagic proteins (Beclin-1, Atg4B, Atg5, and Atg12) results in the suppression of productive HCV infection (264). By analyzing the effect of this knockdown on the various stages in the viral lifecycle it was determined that the autophagy machinery is required for the translation or delivery of incoming RNA to the translation machinery but is not required once translation/replication is established (264). The mechanism of this involvement remains to be clarified but it has been speculated that HCV may utilize autophagosome membrane as a site of initial translation prior to viral induced cellular modifications (264). However, where in the viral lifecycle autophagy acts remains controversial.

While the above discussed cellular modulators of HCV translation are expressed in a wide range of tissues, a recently identified modulator of HCV translation, miR-122, is a well conserved, highly abundant liver-specific micro-RNA (miRNA) (267). miRNAs are 21-23nt long non-coding RNAs that play a key role in post-transcriptional regulation of gene expression (268, 269). miRNAs modulate gene expression by binding to complementary sequences typically found within the 3′-UTR of cellular mRNA transcripts (269). As part of an RNA-induced silencing complex containing Argonaute and other proteins,

miRNAs act to determine target specificity (269). The binding of the RNA-induced silencing complex to complementary sequences within the 3′-UTR of cellular transcripts mediates translational repression by a number of different mechanisms including cleavage of the transcripts and steric interference of initiation complex formation (268, 269). miR-122 functions to down-regulate the expression levels of cationic amino acid transporters and has been implicated in modulation of genes involved in lipid and cholesterol metabolism within the liver (237). The HCV RNA genome contains three potential miR-122 binding sites (237). Two sites (S1 and S2) are located between stem loops I and II of the 5′-UTR (237). The third potential site (S3) is located within the 3′-UTR (237). The function of miR-122 in the HCV lifecycle was first identified by Jopling *et al.* who observed that sequestration of miR-122 resulted in reduced accumulation of HCV RNA in cell culture, suggesting a role in viral replication (267). Additional studies have determined that miR-122 binding to sites S1 and S2 in the 5′-UTR positively affects HCV RNA levels and virus yields with the S1 site having the dominant effect (237, 239). The ability of miR-122 binding to the S1 and S2 sites to modulate viral RNA levels and infectious yields was shown to be position-dependent (239). When the S1 binding site was placed in the 3′-UTR of a reporter construct it was observed to mediate down-regulation of reporter gene expression (239).

In many of the first studies on miR-122, viral translation did not appear to be modulated by miR-122 binding. However, more recent studies have found that miR-122 binding to both S1 and S2 sites in the viral 5′-UTR has a positive effect on IRES-mediated translation (237, 240, 270, 271). Mutations in either S1 or S2 result in a 50% reduction in HCV IRES-mediated translation and this reduction is partially restored by provision of complementary miR-122 mutants (237). This stimulation was proposed to be the result of enhanced association between the 40S ribosomal subunit and the HCV IRES (270). Another study suggested that the stimulation of HCV translation by miR-122 occurs through a conformation change of the IRES (236). It was found that the binding of miR-122 to the 5′-UTR shifts the conformation of the IRES from a closed to an open conformation by preventing the formation of the inhibitory long-range annealing motif between nucleotides 438-442 within the core-coding region and nucleotides 24-38 within domain I of the 5′-UTR (236). However, a role for this mIR-122 induced conformational change in IRES modulation remains controversial (236, 237, 240). Additionally, it has been observed using IRES mutants that the stimulation of translation by miR-122 binding cannot alone account for the effect on RNA levels observed in other studies (237). Recent data suggests that miR-122 may play a major role in stabilization of the viral genome by protecting the 5′-end of HCV RNA from degradation by cellular exoribonucleases XRN1 and XRN2 (272-275). mIR-122 binding may also serve to block recognition of the 5′-end of the viral genome

by cellular RNA sensors and prevent induction of an innate immune responses (238, 272). As such it appears that miR-122 may have multiple positive effects on HCV lifecycle (237, 276).

Recently, two other miRNAs, miR-199a* and miR-196, have been identified to potentially modulate the HCV lifecycle (241, 243). miR-199a* has a target sequence present in IRES domain II and over-expression of miR-199a* results in suppression of HCV replication (241). The effect of miR-199a* on HCV protein translation has yet to be determined but with a target sequence found within the IRES it is likely miR-199a* has an effect on protein translation. The other miRNA, miR-196, has been observed to down-regulate HCV translation and replication when over-expressed and a potential binding site for miR-196 has been found within the NS5A-coding sequence (243). These findings suggest that miRNAs may play a larger role in the HCV lifecycle beyond the effects of miR-122.

1.1.5.2.5.2 Viral factors

The majority of the research into the modulation of HCV IRES-mediated translation by trans-acting factors has been focused on determining the effects of non-canonical cellular factors. In the meantime, there have been numerous studies on the potential roles of the viral proteins in this process (277). It is important to point out that most of the results generated thus far remain controversial, likely due to the variety of reporter systems used (277). The viral NS5A protein, which is essential for the viral replication and particle assembly, has been implicated in the modulation of viral translation (207, 277-282). NS5A is capable of binding to the poly-U/UC region within the HCV 3´-UTR as well as to regions within the IRES in the 5´-UTR, leading to speculation about a role in genome circularization and/or in the switch between translation and replication (214, 279, 283). However, the role NS5A plays in translation remains unclear, as contradictory studies have been published suggesting that NS5A stimulates, inhibits or has no effect on viral translation (207, 277, 280-282). One possible reason for this discrepancy is the use of reporters that lack the 3´-UTR where NS5A binds, as well as the use of plasmid encoded reporters that may not accurately reflect the role of the 3´-UTR in HCV translation (201).

In addition to the core-coding sequence, the core protein has also been implicated in the modulation of HCV IRES translation. However, as with NS5A, the results are unclear as core has also been shown to stimulate, inhibit or have no effect on HCV IRES-mediated translation (190, 277, 284-287), although the consensus opinion seems to be leaning more towards an inhibitory function. In this regard, the core protein has been observed to bind to regions within the 5´-UTR/IRES and inhibit viral translation (285-289). The mechanism of the proposed inhibition is unclear, however a potential interaction between

core and a GGG triplet within domain IIId of the IRES could lead to a disruption of the interaction between the 40S ribosomal subunit and the IRES (285).

The NS3 protease has also been implicated in the modulation of viral translation. In addition to cleaving the viral polyprotein, a single study has observed NS3 binding within the IRES, predominantly in stem-loop IV (234). This interaction was found to compete with La autoantigen for binding to this region of the IRES, downregulating viral translation (234). Furthermore, overexpression of NS3 in a viral replicon system was found to decrease viral translation but significantly enhance viral replication, suggesting a role in the switch between these stages in the viral lifecycle (234).

Other HCV proteins such as NS2, NS4A, NS4B, and NS5B have also been proposed to be involved in modulating HCV IRES-mediated translation (277, 290, 291). However, as with both NS5A and core, there is no definitive answer as to the role these proteins play in viral translation. The discrepancies in these studies again emphasize the crucial importance of the use of appropriate translation reporter systems. Other potential factors include the cell lines and concentrations of viral proteins used in each study.

Despite a fair amount of research into how cellular ITAFs and individual viral proteins modulate viral IRES-mediated translation, it remains unclear how all of these factors coordinate to modulate this process. Further research into the interplay between IRES modulating factors will be required in order to obtain a clear overall model of how HCV IRES translation is controlled.

1.1.5.2.6 Targeting HCV protein translation as a therapeutic approach

HCV translation represents a target in the development of novel antiviral therapeutics as this process is mediated by a distinct mechanism that relies on highly conserved sequences and secondary structures in addition to utilizing different modulating factors than typical eukaryotic cap-dependent translation (292, 293). It is speculated that by targeting such highly conserved regions and functions, the risk of the emergence of resistant viral variants over the course of treatment may be decreased. Another way to minimize the development of viral resistance is through the combination of treatments with multiple targets, such as combining treatments that target viral protease with viral translation inhibitors (294, 295).

A number of different approaches have been investigated to target viral translation as a therapeutic approach. The direct targeting of the IRES as well as therapeutics targeting host cellular factors involved in viral translation are two strategies under investigation. These approaches are used in

an attempt to block the translation of the viral RNA and/or to accelerate breakdown of the viral RNA (296). However, it is important to note that the strategies presented in this section, with the exception of mir-122 targeting, represent proof of principle experiments performed in cell culture. Therefore, the major challenges of drug delivery, final formulation and the optimizing of pharmacokinetics have not been addressed in these studies. In terms of targeting the HCV IRES, many researchers have utilized oligonucleotide based components such as antisense oligonucleotides, RNA interference (RNAi), ribozymes/DNAzymes, as well as DNA/RNA aptamers (293, 296-302). Common targets for such oligonucleotide based strategies which significantly inhibit viral translation *in vitro* include IRES domain III and IV (293).

In addition to strategies using oligonucleotides, a number of small molecule inhibitors that target the IRES have also been studied (296, 299). Two classes of small molecules, benzimidazoles and diaminopiperidines, have been found to interact with the IRES subdomain IIa and inhibit viral translation (292, 303, 304). These molecules are thought to function by interfering with conformational changes of the secondary RNA structure within this essential IRES region (292, 304, 305). Benzimidazoles restrict the IRES subdomain IIa to an extended state which may interfere with 40S ribosome binding while diaminopiperidines confine this domain in a bent conformation possibly preventing ribosome release (292, 304, 306)

An alternative therapeutic strategy to targeting the RNA genome of HCV is to target essential non-canonical translation initiation factors involved in the initiation of HCV translation. One potential advantage of this strategy is the thought that it is less likely that the virus can develop resistance to such indirect treatment. This can be approached using some of the above mentioned strategies such as therapeutic RNAi to block the expression of the host factors. One host factor that has been targeted in such a fashion is mIR-122 as previously discussed. However, extreme caution must be used when targeting host factors as to not cause cellular toxicity by interfering with potentially essential cellular functions of such factors. Additionally, decoy RNA or small peptides can be utilized to sequester cellular HCV IRES trans-acting factors. An approximately 60 nucleotide long RNA isolated from yeast, termed IRNA, has been found to specifically inhibit viral translation while having little to no effect on the translation of cellular mRNA by sequestering trans-acting factors such as La autoantigen that modulates HCV translation (307, 308). Furthermore, small RNA molecules corresponding to domain III and subdomains IIIe and IIIf have been used to effectively and selectively inhibit HCV IRES-mediated protein translation in a similar fashion (309).

One final strategy that involves utilizing HCVs requirement for ITAFs is to design synthetic peptides corresponding to regions of such ITAFs as inhibitors of translation (222, 255, 310). Peptides composed of the RRM2 motif of La autoantigen, which mediates the interaction between the protein and a GCAC motif near the initiator AUG codon, have been found to inhibit viral translation (233, 311). These peptides were shown to compete with cellular La autoantigen protein for binding to the HCV IRES and selectively inhibit viral protein translation by interfering with 48S ribosome complex assembly (233, 311).

Although many of these therapeutic strategies appear to be promising in cell culture based studies, one major problem is efficient and targeted delivery. Recent advances in the use of liposomes, nanoparticles, polymers, exosomes and viral/bacteriophage vectors as delivery agents have improved the delivery efficacy and specificity of oligonucleotide based treatments (301, 312). In the case of peptide based treatments strategies such as Pegylation, hyperglycosylation, polymeric nanospheres and fusion to cytoplasmic transduction peptides such as HIV trans-activator of transcription peptide have been found to enhance peptide stability and/or delivery (313, 314). As these delivery methods are further improved, such potential treatment approaches may be further studied in animal models to test for toxicity and to investigate the pharmacokinetics.

1.1.5.3 Replication

1.1.5.3.1 Viral proteins required for replication

Following translation of the viral genome and polyprotein processing, the viral replicase complex is formed within a viral induced membranous web. The molecular switch which signals for the transition between viral translation and replication is unclear at this point but a number of potential viral and cellular proteins involved have been previously discussed. The viral proteins NS3, NS4A, NS4B, NS5A and NS5B are the minimal requirements for this complex formation as subgenomic replicons containing these viral proteins flanked by the 5´- and 3´ UTRs are sufficient for viral replication (315). For unknown reasons these viral proteins are largely required to be expressed in cis as a polyprotein from the RNA that undergoes replication (316, 317). Only a limited number of mutations in NS5A and NS4B can be rescued by transcomplementation (316, 318, 319). This requirement has complicated the investigation into the role that each of these viral proteins in replication (320).

1.1.5.3.2 The membranous web

To facilitate viral replication, HCV induces extensive rearrangements of intracellular membranes (321-323). HCV, like all positive stranded RNA viruses, replicates its genome in these distinct membranous compartments, the formation of which are induced by the action of viral proteins in concert with cellular proteins (323-327). These altered membrane structures provided a scaffold on which viral replication complexes assemble (323, 328). This structure is referred to as the membranous web and is composed of an accumulation of various vesicle structures, including single membrane vesicles (SMVs), double membrane vesicles (DMVs) and multiple membrane vesicles (MMVs) (322, 329-331). The membranous web appears to be primarily derived from the ER but also contains markers of early and late endosomes, secretary pathway vesicles, mitochondria and lipid droplets (322, 323, 328). It was originally thought that NS4B was the viral protein mainly responsible for the induction of the membranous web formation (321, 332, 333). Expression of NS4B was sufficient to induce massive membrane rearrangements that were characteristic of the membranous web observed during viral replication (321). NS4B contains 4 transmembrane segments and amphipathic helices (AHs) within its N and C-termini (321). Due to this membrane localization and its ability to self-oligomerize NS4B is predicted to be a major scaffold protein within the membranous web (333, 334). However, it has recently been observed that each viral replicase factor is capable of inducing distinct membrane alterations individually (322). While NS4B induces the formation of primarily SMVs, NS5A expression leads to the formation of DMVs and MMVs (322, 330). NS3/4A expression leads to the accumulation of complex large SMVs distinct from the smaller SMVs induced by NS4B (322). Finally, expression of the viral polymerase NS5B induced enlargements of the ER (322). These observations suggest that the proper formation and organization of the membranous web requires the collaborative action of all the viral replicase components (322).

The role that the various vesicle structures in the membranous web play in viral replication remains controversial. The MMVs that are observed within the membranous web are not thought to be directly involved in the replication process as they appear late after infection (322). This suggests that they may form as a result of stress response due to the massive membrane alterations induced by viral infection (322). Mutations in NS4B that prevent self-oligomerization are detrimental to viral replication and disrupt the formation of NS5A induced DMVs (332, 333). This suggests that the DMVs are crucial to viral replication and supports the notion that NS4B serves as a scaffold protein to membrane alterations primarily induced by NS5A (320). In further support of DMVs serving as the site of viral replication, viral replicase components have been observed to colocalize with DMVs within the membranous web in close

association with the ER and lipid droplets (322, 329). Furthermore, upon extraction and purification of NS4B containing membrane structures from HCV infected cells it was found that DMVs were dominant (331). Additionally, these purified DMVs were found to contain NS3 and NS5A and were capable of *de novo* HCV RNA synthesis (331). Host proteins found to be required for replication were also located within these DMVs (331). Finally, DMVs were shown to be associated with active viral replication though metabolic labelling of nascent viral RNA (331). Replication is thought to occur within the lumen of these DMVs, as this process is detergent resistant and protected from protease and nuclease activity (322, 328, 335, 336). However, it remains possible that replication occurs on the surface of the DMVs similar to what it believed to occur in poliovirus replication (322). In this case, the replication complex may be protected by the tight clustering of the replication vesicles (322).

These DMVs appear to be protrusions of the ER into the cytosol and frequently remain connected to the ER (322). Some of these DMVs have also been observed to possess an opening to the cytosol, which may function to allow the transport of components required for replication, such as viral proteins, viral RNA and nucleotides, from the cytosol to the lumen of the DMV (322). Alternatively, various nuclear pore complex proteins may be involved such transport (337). Nuclear pore complexes (NPCs) are normally found within the nuclear envelope and regulate the transport of molecules between nucleus and cytoplasm (337). NPC proteins have been found to be required for HCV infection where they are recruited to the membranous web and interact with viral proteins (337). Interference of the interaction between nuclear transport machinery and viral proteins inhibits viral replication (338). These findings suggest that NPCs may contribute to membranous web architecture and facilitate transport of viral and cellular proteins from cytosol into viral replication sites within the membranous web (337, 338).

The biogenesis of these DMVs remains unclear. However, a number of interactions between viral and host proteins have been implicated in this process. For example, NS5A and NS5B lead to the activation of the host protein phosphatidylinositol 4-kinase IIIα (PI4KIIIα) during viral infection, activation which appears to play a key role in membranous web production (330, 339, 340). Active PI4kIIIα results in the substantial production and redistribution of phosphatidylinositol 4-phosphate (PI4P) lipid to the membranous web (330, 341). Inhibition of PI4KIIIα impedes HCV replication and disrupts membranous web structure (330, 339). PI4P may contribute to curvature or integrity of the membranous web vesicles in addition to recruiting other cellular factors to the replication site (340, 341). One such factor, four-phosphate adaptor protein 2 (FAPP2) a glycosphingolipid transport protein required for HCV replication, contains a Pleckstrin homology (PH) domain which binds to PI4P and mutation of this PH domain impedes viral replication (341). This suggests that PI4P binds to FAPP2, recruiting glycosphingolipids to the

developing replication complex (341). Localized membrane accumulation of glycosphingolipids may stimulate membrane curvature, potentially representing the first step in membranous web vesicle and HCV replication complex formation (341). PI4P also functions to recruit oxysterol-binding protein (OSBP), which is required for DMV formation, to the developing membranous web (342). OSBP normally functions to exchange PI4P and cholesterol at ER-Golgi contact sites and is speculated to perform a similar function on the HCV induced membranous web (340, 342). Additionally, NS5A has been implicated in utilizing the host protein ADP-ribosylation factor GTPase-activating protein 1 to remove the PI4P phosphatase Sac1 from the membranous web (343). Therefore, NS5A both promotes PI4P synthesis at site of replication and actively displaces the PI4P phosphatase to maintain high levels of PI4P (330, 341, 343). Another example of a host protein implicated in membrane reorganizations during membranous web formation is proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2), which belongs to the Pombe Cdc15 homology (PCH) family of proteins involved in generating membrane curvature during vesicle formation (344). Both NS4B and NS5A interact with PSTPIP2 and recruit it to the membranous web where it is involved in DMV formation dependent upon its membrane-deforming activity (344). From findings such as those discussed here it is apparent that the formation of the membranous web is accomplished through the action of both viral and co-opted host factors.

In addition to the rearrangement of cellular membranes, HCV infection also results in the accumulation of lipids through the up-regulation of genes involved in lipid metabolism (345-347). HCV leads to the increased expression of multiple lipogenic proteins such as acetyl CoA carboxylase and fatty acid synthase (345, 347). Inhibition of these enzymes is detrimental to virus replication (347). The increase in cellular lipid levels may be required for the membrane proliferations characteristic of the membranous web and for the modification of viral proteins (320, 348, 349)

1.1.5.3.3 Viral replication complex and RNA synthesis

Within this membranous web, the membrane associated viral replication complex containing the NS3, NS4A, NS4B, NS5A and NS5B viral proteins replicates the viral genome via a negative strand intermediate (320). In addition to acting as a viral protease in association with co-factor NS4A during polyprotein processing, the NS3 protein possess NTPase/helicase activity required for replication that may be involved in unwinding the viral RNA (350-352). This may function during replication initiation by resolving the stem loops found in the 3´-UTR and/or during the elongation phase by separating the double-stranded replication intermediates and removing proteins bound to the viral RNA (22, 320, 351).

Furthermore, there appears to be significant coordination between the NS3 helicase activity and the viral polymerase NS5B(22, 353-355). These proteins have been observed to modulate each other activities (22, 353-355). NS4A, in addition to acting as a co-factor for NS3 protease activity, mediates membrane association of NS3/4A (22, 356). As discussed NS4B appears to be a main factor in membranous web formation but is also speculated to be involved in the assembly of the viral replication complex itself. NS4B may serve as an interaction platform within the replication complex. NS4B as it has been observed to interact with multiple non-structural viral proteins in addition to the viral RNA (334, 357-359). These interactions are critical to viral replication as demonstrated by the inhibition of viral replication upon disruption of the interaction between NS4B and NS5A (360). Also heavily involved in the formation of the membranous web, NS5A performs essential but still unclear functions in viral replication which may be modulated by its phosphorylation status (26, 361, 362). NS5A has been observed to modulate NS5B activity and bind to the viral RNA (363-367). Within the replication complex NS5A and NS5B interact with different domains of the host protein hVAP-33, which is typically involved in intracellular vesicle trafficking (368-370). The interaction between NS5A and hVAP-33 is dependent upon the phosphorylation status of NS5A and disruption of this interaction or silencing of hVAP-33 inhibits viral replication (369, 370). These findings suggest hVAP-33 may play an important role in the assembly of the replication complex by bringing together NS5A and NS5B (369). NS5A also interacts with a wide array of cellular proteins many of which are central to cellular signalling pathways suggesting NS5A may play a key role in inducing a cellular environment that favours the virus (26, 27). The role of NS5A in the viral lifecycle will be discussed in greater detail in a later section.

The NS5B protein is the viral RNA-dependent RNA polymerase which catalyzes the synthesis of new viral genomes via a negative stand intermediate (320). Synthesis of the negative strand intermediate is initiated presumably by NS5B binding to the extreme 3′ end of the 3′-UTR of the viral genome (320). In agreement with this model, purified NS5B has been shown to be capable of *de novo* initiation *in vitro* (371-373). However, *de novo* initiation at the 3′ end of the positive strand appears to occur with low efficiency, possibly due to the presence of a stem loop structure, suggesting that other viral and/or host factors are involved in regulating this process (320, 374). After synthesis of the negative strand NS5B can then initiate production of new positive viral genomes through *de novo* initiation at the 3′ end of the negative strand (374). This end consists of a stem loop with a single strand overhang which facilitates efficient *de novo* initiation by the NS5B polymerase (374, 375) These nascent viral positive strands can then presumably be utilized for either negative strand synthesis, polyprotein translation or be packaged into new viral particles (320). Synthesis of the negative stand is detectable 4-6h after introduction of the viral genome in cell

culture (320, 375, 376). Asymmetric RNA synthesis occurs and plateaus 24-48h after infection with a 10:1 ratio of positive to negative stranded viral RNA (320, 375, 376). Due to the lack of proofreading ability RNA synthesis by NS5B is highly error prone with an error rate of approximately 10^{-3} per nucleotide site observed which facilitates the genetic variation of the virus and development of resistance mutations (377).

A wide array of cellular factors has also been implicated in modulating viral replication beyond those involved in membranous web formation (26, 378-380). A number of these proteins were described in the section on viral translation such as miR-122 and hnRNP D. Furthermore, over 70 cellular proteins have been found to interact with the viral genome within the 3´-UTR and a role in modulating viral replication has been ascribed to some of these RNA binding proteins, though the mechanism remains largely unclear (381). This includes the RNA helicase DDX5, Nuclear factor proteins such as NF90 and the far upstream element binding protein (381-383). Additionally, numerous cellular proteins have been found to interact with viral proteins and modulate viral replication such as FK506-binding protein 8 (FKBP8) and Cyclophilin A and B (CypA/CypB) (384-386). FKBP8 interacts with NS5A and recruits the heat shock protein 90 (Hsp90) to the replication complex (386, 387). The mechanism is unclear but Hsp90 is a chaperone protein involved in protein folding and stabilization and disruption of this NS5A-FK506-Hsp90 complex impairs viral replication (386, 387). CypA and CypB are cellular peptidyl isomerases that facilitate protein folding and CypB interacts with NS5B and stimulates its polymerase activity whereas an interaction between CypA and NS5A is essential for viral replication (84, 384, 385, 388).

1.1.5.3.4 Modulation of viral replication by cis-acting RNA elements

In addition to acting as the site for replication initiation the 3´-UTR also contains a number of cis-acting RNA elements which modulate viral replication (212). The variable region, although not absolutely required for replication, contributes to efficient replication (389, 390). In the poly-U/UC region a minimal 26-33 stretch of consecutive uridines is required for replication, although the precise position is variable, supporting its proposed role as a platform for molecular interactions described previously (212, 389, 391). The threes stem-loop structures within the x-tail region are all essential for replication (389, 390, 392). Both the sequence and structure of this x-tail region appear to be critical for viral replication (320, 390, 392). In addition to the 3´-UTR RNA, structures found within the coding region have also been found to modulate viral replication. As discussed in the section on HCV translation both, the core and NS5B coding regions contain regions of secondary RNA structure implicated in modulating HCV replication. In regards

to positive strand synthesis, the 3´-UTR of the negative RNA strand intermediate contains secondary structures which differ significantly from that of the complementary 5´-UTR region of the positive viral RNA genome which contains the IRES (393, 394). The negative strand intermediate 3´-UTR contains seven stem loop structures (395). However, only the terminal 125 nucleotides, containing two of these structures, are required for viral replication (395).

1.1.5.4 Virion assembly and release

The assembly and secretion of HCV particles remains poorly defined. However, the process has been found to be linked to cellular VLDL synthesis (396, 397). Accordingly, viral particles have been observed to circulate within infected patients as low-density lipoviral particles resembling VLDL associated with lipoproteins such as ApoB and ApoE resembling VLDL (398-401). Additionally, multiple interactions between various viral proteins and viral or cellular protein have been observed to be involved in modulating viral assembly (402). However, how these interactions coordinate to assemble and secrete the virus particle remains largely unclear. Contributing to the lack of a precise working knowledge of viral assembly and release is that Huh7 derived cells, which are utilized for infectious HCV cell culture (HCVcc), are defective in the VLDL secretion pathway (403). These cells produce mainly intermediate density lipoprotein (404). This results in physical differences between viral particles produced in HCVcc and those isolated from infected patients or primary cell culture (405, 406). Viral particles produced in HCVcc have a higher density and lower infectivity compared to those produced *in vivo* (398, 401, 405, 407). Despite these shortcomings, a model for viral assembly and release has emerged and is described below.

After synthesis and prior to assembly, Core is localized to the surface of lipid droplets (LDs), a cellular lipid storage organelle derived from the ER and primary source of lipids for incorporation into VLDL (408-410). This interaction results in accumulation of LDs in the perinuclear region, compared to their dispersed distribution in uninfected cells (411). This localization of core to the surface of LDs is required for viral assembly and may act to sequester core prior to initiation of assembly, potentially preventing premature nucleocapsid formation (402, 412-414). Efficient recruitment of core to LDs requires at least two host factors involved LD biosynthesis, the triglyceride synthesizing enzyme diacylglycerol acyltransferase 1 (DGAT1) and cytosolic phospholipase A2 (PLA2G4A) (415, 416). NS5A plays an essential role in assembly through co-localization with core on the surface of LDs and this localization

is also dependent upon DGAT1 (417-420). The precise role NS5A plays in assembly is unclear. However, NS5A is a member of the replication complex and binds to the viral RNA (410). It may thus play a role in the transition from replication to assembly by transporting nascent viral RNA from the ER associated replication complex RNA to LDs to interact with core (410). In support of this model, mutations which impair NS5A association with LDs also impairs the recruitment of viral RNA to the surface of LDs (413). Furthermore, two cellular proteins Rab18, a lipid droplet associated protein and regulator of vesicular traffic, and tail-interacting protein 47 (TIP47), which is involved in LD biogenesis, bind to NS5A and promote association between LDs and the site of viral replication (421-424). Interestingly, it was observed that TIP47 interacts with NS5A in complex with viral RNA and is also involved in regulating viral replication (421). NS5A also interacts with the cellular trafficking protein annexin A2 (425, 426). An interaction that has been observed to stimulate both viral replication and assembly, possibly though modifications of the ER membrane structures involved in these processes (425, 426). An additional interaction between NS5A and ApoE is also required during virus assembly though the mechanism is unclear (427-429). The VLDL-associated molecular chaperone ApoJ interacts with both NS5A and core on LDs, stabilizing this interaction and facilitating viral assembly (427-429).

The viral glycoproteins E1 and E2 are translocated into the ER where they interact to form heterodimers during translation (430, 431). Early in viral assembly NS2, is involved in organizing structural and non-structural proteins on the ER (432). This function is independent of the protease activity of NS2 (433). NS2 interacts with p7 and this interaction is required for the further recruitment of the E1/E2 glycoproteins and the NS3/4A complex by NS2 to the sites of assembly on the cytosolic ER membrane (432, 434-437). These non-structural proteins are thought to interact in close proximity to the core/NS5A coated LDs (434). The core protein is then recruited from the LD to the ER-complex site of assembly (438-440). This recruitment is dependent upon the interaction between p7-NS2 and NS3/NS4 (438-440). An interaction between core and the cellular protein AP2M1, which is involved in modulating intracellular trafficking, is also required for the recruitment of core from LDs (441). A transient and weak interaction is also observed between NS5A and the p7-NS2 complex at this stage of viral assembly (434, 436, 437). It remains unclear how the RNA enters the site of assembly. It may be recruited along with LD associated core or it may be recruited directly from the replication complex. An interaction between core and the RNA helicase domain of NS3 is essential for virus assembly, suggesting that NS3/4A may be involved in packing the viral RNA during this process (402, 433, 442). Nucleocapsid formation is proposed to occur on the surface of the ER at this site concurrent with budding into the ER lumen (402).

The results of this process is the production of a virus particle surrounded by a lipid envelope, with the viral glycoproteins on the surface, and containing the viral nucleocapsid that encapsidates the viral RNA (434). Intracellular virus particles are of significantly higher density than those secreted from the cell, indicating that viral particles go through a maturation process in which they acquire low-density properties prior to release (397, 443). This process may begin in the ER lumen through association of the viral particles with VLDL precursors, luminal lipid droplets (434). In support of this model, it was found that blocking microsomal triglyceride transfer protein, which has a role in VLDL biogenesis through transporting lipids to ER, inhibits viral particle production (396, 397). Unlike VLDL biogenesis, however, HCV assembly has been observed to be dependent upon ApoE rather than ApoB in some studies (440, 444). Virus particles associated with ApoE are then proposed to be exported through the secretory pathway and pass through the Golgi complex similarly to VLDL. Within the Golgi, the viral glycoproteins E1/E2 undergo modifications and further lipidation of the viral particle may occur similar to VLDL (438, 440, 445). The mechanism of viral egress through the secretory pathway to the plasma membrane is largely unknown, but it appears to be associated with endosomal trafficking (446). Components of the endosomal sorting complex required for transport pathway have been implicated in this process as have early, late and recycling endosomes (440, 446-449). During viral maturation and egress, the viral p7 protein functions to protect nascent virions by regulating pH gradients (450). P7 prevents the acidification of compartments of the secretory and endosomal pathways, presumably by functioning as ion channels (450). Upon arrival at the cell surface, vesicle-associated membrane protein 1 mediated fusion between the HCV containing vesicle and the plasma membrane results in the release of the viral particle into the extracellular environment (440).

1.2 HCV Non-Structural Protein 5A

The HCV non-structural 5A protein (NS5A) is multifunctional phosphoprotein and as discussed in preceding sections is essential for viral replication and assembly, although the exact role the protein plays in the viral lifecycle remains unclear (361, 418, 419, 451-455). NS5A interacts with numerous cellular and viral proteins and as such has been implicated in a multitude of functions such as modulation of cellular signalling pathways and interferon resistance (27, 456, 457). In fact, NS5A has been found to potentially interact with over 130 host proteins including many that are central to cellular signalling pathways (27, 456, 458). This suggests NS5A plays a key role in modulating the cellular environment to favour viral

pathogenesis. Despite being implicated in such a diverse range of functions no enzymatic activity has been found for NS5A (459). NS5A is composed of an N-terminal AH and three domains that are separated by two regions of repetitive low complexity sequences (LCS) (**Figure 1.5**) (456). The AH is necessary and sufficient for membrane localization of NS5A, at the site of the viral replication complex, and is essential for replication as mutation of this region leads to a diffuse cytoplasmic localization of NS5A and inhibition of viral replication (460-462). The AH has also been found to mediate the interaction between NS5A and TIP47, which is involved in the interaction between the replication complex and LDs (422). The LCS-I is a serine rich region while the LCS-II is proline rich (463). The individual domains of NS5A have been implicated in various aspects of the viral lifecycle and will be discussed in detail below.

1.2.1 Phosphorylation

NS5A has been found to be present in two differently phosphorylated forms within infected cells (456). A basally phosphorylated form and a hyperphosphorylated form are resolved, based on the presence of 56 kDa and 58 kDa bands, upon SDS-PAGE resolution (456). Hyperphosphorylation, but not basal phosphorylation, is thought to be mostly dependent upon expression of NS5A in cis with NS3 and NS4A/B (464, 465). Phosphorylation has been implicated in the regulation of multiple functions of NS5A. It may regulate the transition between viral replication and viral assembly, possibly by modulating the conformation of the viral protein or the interaction with cellular or viral proteins (361, 451, 456, 466). However, both the exact sites of phosphorylation and the kinases responsible for it remain unclear. It is thought that NS5A is basally phosphorylated on residues within the central and C-terminal regions, whereas residues responsible for hyperphosphorylation reside mainly within the LCS-I region (370, 466-468).

Residues S222, S225, S229 and S232 within the LCS-I have been implicated in hyperphosphorylation and cell culture adaptive mutations that reduce hyperphosphorylation often affect residues within this region (20, 361, 467, 469). Adaptations that reduce hyperphosphorylation generally increase replication of HCV replicons, although this effect differs between viral genotypes (451, 468, 470, 471). Suppression of NS5A hyperphosphorylation by kinase inhibitors in non-adapted HCV replicons also leads to enhanced replication (472, 473). In support of this model the previously described interaction between NS5A and VAP-A, which is essential from viral replication, is blocked by hyperphosphorylation, leading to disruption of the replicase complex and inhibition of viral replication (369, 370). Furthermore,

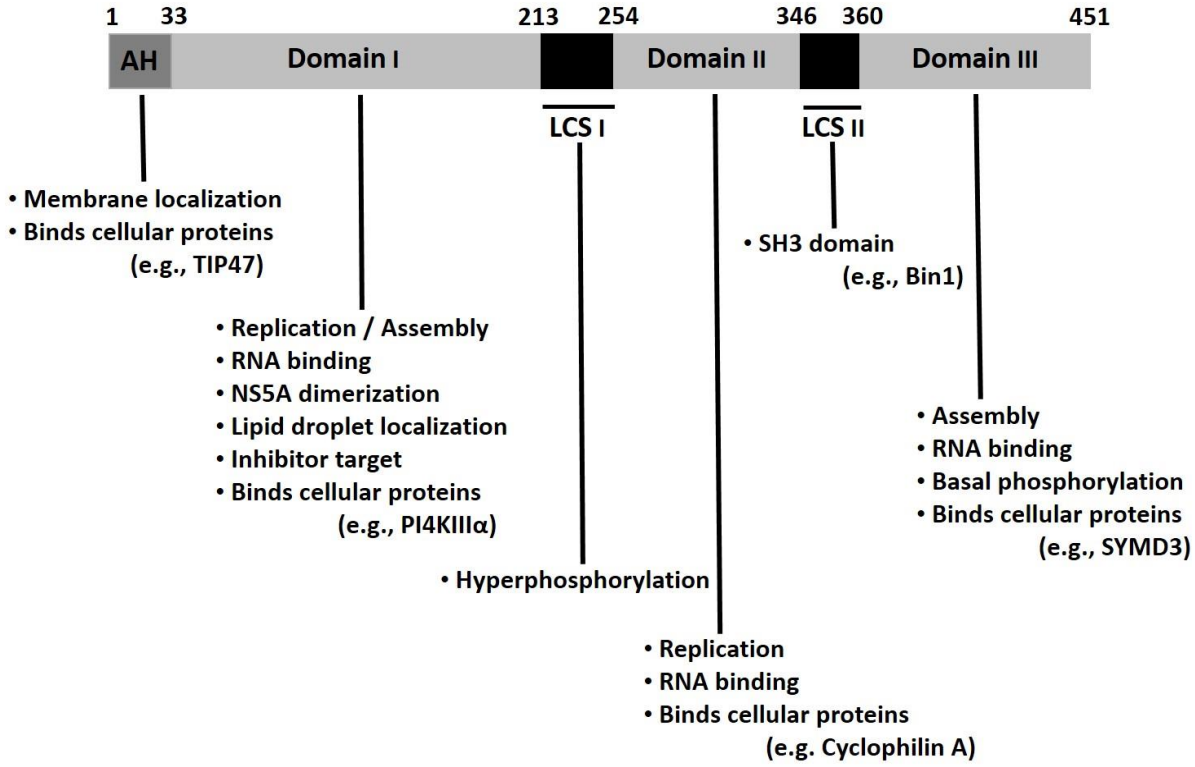

Figure 1.5 - NS5A domain organization and proposed functions in the viral lifecycle. Numbering corresponds to 1b N HCV NS5A protein. NS5A is composed of an N-terminal amphipathic helix and three domains separated by low complexity sequences (LCS). Various functions attributed to the regions of NS5A are indicated. See text for details.

basally phosphorylated NS5A mainly localizes in high-density fractions along with replicase components NS3 and NS5B in addition to ER markers, consistent with a key role of basally phosphorylated NS5A within the replication complex (466). In contrast, hyperphosphorylated NS5A is predominantly located within low-density fractions where it interacts with lipid droplets, an interaction essential for viral assembly (466, 474). Our understanding of NS5A phosphorylation is further complicated by the observations that the combination of multiple adaptive mutations that reduce hyperphosphorylation, or the treatment of adapted viral replicons with kinases inhibitors that further reduce hyperphosphorylation, inhibits HCV replication (451, 472, 473). This suggests a complex differential modulation of NS5A activity by hyperphosphorylation that has both positive and negative effects on RNA replication.

Seven serine residues within the LCS-I region have so far been identified as phosphorylation sites (361, 463, 466, 469). Consistent with the contrasting effects of hyperphosphorylation on viral replication, phosphorylated serine residues within this region have been observed to have differing effect on viral replication. Some phosphorylation events enhance replication whereas others are inhibitory (370, 463, 469). For example, mutation of S225 or S232 to alanine, which prevents phosphorylation, results in a 10-fold reduction in replication of JHF-1 (genotype 2a) HCV (463). However, mutation of S229 or S235 to aspartic acid, which mimics a constitutively phosphorylated state, results in a similar reduction in viral replication (463). These mutations were all found to negatively affect the establishment of viral replication complexes by disrupting the cellular distribution of NS5A and other replicase components, such as NS3, PI4P lipid and the viral dsRNA intermediate (463). The mechanism remains to be defined but could result from a reduced motility of NS5A or the disruption of interactions between NS5A and cellular or viral proteins (463). Interestingly, mutation of S229 to alanine is also lethal, again demonstrating the complexities in the roles of NS5A phosphorylation in regulating the viral lifecycle (361, 455).

A cluster of serine residues within the C-terminal region of domain III undergo basal phosphorylation and play a key role in modulating viral assembly (419, 420, 452). These residues, and the phosphorylation of threonine 356 (T356) will be discussed in detail below when describing NS5A domains II and III.

Regulation of NS5A phosphorylation appears to be tightly controlled but the mechanism remains elusive. A number of cellular serine/threonine protein kinases have been implicated in this process, such as casein kinase I (CKI-α), casein kinase II (CKII), Polo-like kinase I, testis-specific serine kinase, protein kinase A (PKA) and mitogen-activated protein kinases (362, 466, 475-477). CKI-α may be one of the best-characterized examples. It has been implicated in a hyperphosphorylation event that may regulate the recruitment of NS5A to lipid droplets, recruitment which is required for viral assembly (466). Recent

observations suggests that hyperphosphorylation may also occur in a sequential manner (361). As mutations which mimic phosphorylation within the serine cluster in LCS-I (S222-S238) approach the C-terminus of this region a progressive increase in the apparent molecular weight of the basally phosphorylated species is observed (361). These findings suggest that the phosphorylation of a residue within this region would promote the phosphorylation of upstream residues possibly by acting within the kinase recognition motif of the neighbouring residue (361). Furthermore, phosphorylation of S146 within domain I appeared to negatively impact hyperphosphorylation, perhaps through intra-protein interactions, suggesting an additional mechanism that regulates NS5A phosphorylation (26, 361). Other viral proteins also appear to be involved in regulating NS5A phosphorylation. Such regulation is suggested by the requirement of NS5A expression in cis with NS3 and NS4A/B for hyperphosphorylation. Adaptive mutations that reduce NS5A hyperphosphorylation have also been mapped to NS4B, and mutation of residues implicated in mediating the interaction between NS4B and NS5A abolished NS5A hyperphosphorylation (360, 473).

1.2.2 NS5A Domain I: Crystallization and dimerization

NS5A domain I (aa. 33-213) is highly conserved and has been found to function in multiple essential roles assigned to NS5A in the viral lifecycle, including genome replication and virion assembly. Domain I contributes to the RNA binding activity of NS5A and mediates NS5A dimerization, functions that will be discussed in detail below. Domain I also interacts with multiple host proteins including the previously described PI4KIIIα and FK506-binding protein 8 which play key roles in membranous web biogenesis and viral replication (26, 386, 387, 478). In regards to assembly, the amino acid (aa.) 99-104 sequence within domain I has been implicated in mediating the recruitment of NS5A to the surface of LDs (413). Mutation of this region inhibits not only the recruitment of NS5A but also of the viral RNA to LDs (413)

Due to the essential role of NS5A in viral replication, and therefore its attractiveness as a drug target, a number of studies have been performed to determine its structure of NS5A. Such information on the structure of NS5A would help guide the design and development of NS5A targeting antivirals. To date only domain I of NS5A has been successfully crystallized. In one such study by Tellinghuisen *et al.* a genotype 1b (Con1) domain I fragment made up of residues aa. 25-215 was found to form a homodimer composed of two subdomains, the N-terminal subdomain IA where contact between the molecules

primarily occurs, and a C-terminal subdomain IB (479). A zinc-binding motif containing the previously identified essential Cys-57, Cys-59 and Cys-80 residues was identified within subdomain IA, the location of which suggested a structural role of this zinc molecule in maintaining the NS5A fold (479, 480). The molecular surface of this domain I dimer was found to have uneven charge distribution with the N-terminal subdomain IA displaying a primarily basic surface and the C-terminal subdomain IB containing a largely acidic surface (479). Additionally, a surface exposed region spanning subdomains IA and IB was found to contain a high number of conserved residues the authors propose may represent an interaction surface (479). The dimer contact interface was found to be composed of two regions with the larger region found primarily in subdomain IA containing several conserved residues in close proximity to the zinc binding motif (479). The second and smaller dimer interface region was found in subdomain IB and displays lower sequence conversion (479). Lastly, a large basic groove was formed between the subdomain IB regions that was predicted to have sufficient dimensions to bind either single or double stranded RNA and as such was identified as a potential site of NS5A/RNA interaction (479). This crystal structure identified a number of intriguing structural elements of a domain I dimer but it has, however, proven difficult to reproduce, leading to the possibility of the existence of alternative form of NS5A domain I dimers (481, 482).

Accordingly, an alternative domain I dimer crystal structure has been obtained by Love *et al.* using a genotype 1b (Con1) domain I fragment containing aa. 33-202 residues (482). In this structure, the zinc binding motif displayed the same organization as the Tellinghuisen dimer (479, 482). Also in a similar fashion to the Tellinghuisen *et al.* dimer, an uneven surface charge distribution was observed with a predominantly basic N-terminus and a more acidic C-terminal portion (479, 482). However, the association of the domain I monomers differed substantially between the two different crystal structures and there was no overlap between the dimer contact interface regions as the contact interface of the Love *et al*. dimer was fully exposed in the Tellinghuisen *et al.* dimer (479, 482). Conceptually the Tellinghuisen *et al.* dimer can be described as a face to face interaction with the N-termini facing inward whereas the Love *et al*. dimer was organized back to back with the N-termini facing outward (479, 481, 482). In the Love *et al.* dimer there was an extensive buried surface area and the dimer contact interface was composed of approximately 20 residues from each monomer covering the residues; 92-99, 112-116, 139-143, 146-149 and 160-161 (482). This represents the most highly conserved surface region within domain I (482). Within this contact region an intermolecular salt bridge, a combination of a hydrogen bond and electrostatic interaction, was formed between the highly conserved Arg-112 and Glu-148 and a hydrogen bond was formed between the conserved Ala-92 and Gly-96 (482). Notably absent in this crystal

structure was the positively charged cleft that was proposed to be an RNA binding site in the Tellinghuisen *et al*. dimer (479, 482). One potential implication of the non-overlapping dimerization interfaces of the two different domain I dimers is the assembly of domain I proteins via interactions of alternating interfaces which would result in a superhelical appearance and sterically accommodate domains II and III of NS5A (482).

To further add to the complexity of our understanding of domain I dimerization, a third study by Lambert *et al.* has obtained crystals of a genotype 1a (H77) domain I aa. 33-202 fragment which reveled two novel domain I dimer structures (481). The domain I crystal structures obtained in this study consisted of 4 monomers, termed A, B, C and D, with two dimeric interfaces forming an AB dimer and a CD dimer (481) . The exposed surfaces of these molecules appeared similar to those found within the Love *et al.* study, whereas the zinc binding motif appeared similar in organization to both the previously identified dimers (479, 481, 482). The contact interface within the AB dimer was found to be similar, although smaller, to that reported for the Love *et al*. dimer (481, 482). The residues that composed the contact interface were 97-99, 112-115, 149 and 160-161 (481). One notable difference, however, was that the orientation of one of the monomers in the AB dimer was reversed, creating a head to tail (N-terminus to C-terminus) interaction as opposed to the back to back interaction observed in the Love *et al*. dimer (479, 481, 482). Also differing from the Love *et al*. dimer was the lack of the salt bridge between Arg-112 and Glu-148 and the hydrogen bond between Ala-92 and Gly-96 (481, 482). The second domain I dimer in this study, dimer CD, was aligned in a head to head conformation (N-terminus to N-terminus). The contact interface of dimer CD was composed of highly conserved residues 74-78 and 83-84 (481). Within this region, intermolecular hydrogen bonds were formed between Ile-74 and Arg-78 and between Gly-76 and Gly-76 (481). It was also observed that copies of dimer CD interacted together utilizing the same interface as the AB dimer, suggesting the possibility of higher order multimers (481). The authors speculated that such an extended multimeric network could interact with the membranous web and therefore have implications in viral replication (481). Common to the different domain I crystal structures identified thus far, the overall polypeptide fold of each NS5A monomer was similar (479, 481, 482). The finding of such distinct domain I dimers is perplexing. However, it is possible that all are physiologically relevant and may represent different dimers that have distinct functions within the viral lifecycle. The regulation of the conformations may be influenced by such factors as phosphorylation or interaction with viral or cellular proteins, or viral RNA (361).

Results from NS5A inhibitor studies suggest that NS5A dimers are physiologically relevant as they may represent the target for high potency NS5A inhibitors. Highly potent NS5A inhibitors such as LDV and

Daclatasvir (DCV) (Bristol-Myers Squibb) are symmetrical in nature (483). Based on selection for resistant mutations in the N-terminus of NS5A domain I, and models of NS5A structure, the inhibitors are predicted to interact at the NS5A dimer interface (483-486). Resistance mutations tend to cluster around the dimeric interfaces, most predominantly at Tyr-93 (487). Furthermore, recent studies have demonstrated specific high affinity binding by symmetrical inhibitors to NS5A and provided evidence for the interaction of the drug compound with NS5A dimers and possibly higher order multimers (484, 487). Additional evidence comes from the finding that NS5A inhibitor candidate compounds lead to disruption of both intra- and intermolecular NS5A conformations (488). These compounds were found to alter the conformations of domains I and III within a single NS5A molecule, and to increase the distance between the N-termini of NS5A molecules found in multimeric conformation (488). However, treatment actually leads to the formation of large aggregates of NS5A, rather than disrupting NS5A multimer formation, a finding which may help explain the high potency of such NS5A inhibitors (488). The relevance of multiple NS5A dimer conformations within infected cells is also suggested by the finding that these NS5A inhibitors cannot be observed to interact with purified NS5A *in vitro* (481, 484). These inhibitors have been observed to physically interact with NS5A only in HCV replicon cells (481, 484). An observation which suggests that NS5A may only transiently be in the correct conformation for drug compound interaction (481).

1.2.3 RNA-binding by NS5A

NS5A has been observed to possess RNA binding activity towards G/U rich RNA and binds with high affinity directly to the poly-U/UC region found within the 3′-UTR of the viral genome (363-365). An interaction of significantly lower affinity has also been observed between NS5A and the 5′-UTR (364). Potential sites within the 5′-UTR for this interaction, based on presence of G/U rich sequences, include multiple sites within domain III of the IRES (365). However, NS5A binding to these regions remains to be determined. The interaction between NS5A and both the 5′- and 3′-UTRs suggests that NS5A may play a role in modulating the transition from translation to replication given the roles these regions play in the viral lifecycle (365)

NS5A lacks similarity to any of the previously identified RNA-binding motifs including the RRM common to cellular proteins that preferably bind G/U rich RNA (363, 365). This suggests NS5A utilizes a novel mechanism for RNA binding (363, 365). In a study by Foster *et al.* aimed at defining the region of NS5A which mediates RNA binding it was found that all three of the NS5A domains are capable of

specifically interacting with the 3´-UTR independently (363). However, while domains I and II bound the 3′-UTR RNA efficiently domain III demonstrated lower, but still specific, RNA binding affinity suggesting RNA binding is primarily mediated thought domains I and II (363). In support of this model, expression of domains I and II as a single fragment displayed an additive effect on RNA binding efficiency which was not seen when domains II and III were expressed together as a single fragment (363). However, in the context of the full NS5A protein, domain III does appear to contribute to the RNA binding ability (363). While NS5A and the domain I/II fragment display similar RNA binding affinities, NS5A binds with greater efficiency (363). A separate study by Hwang *et al.* obtained results suggesting that NSA RNA binding activity is primarily mediated by the domain-LCS I region (364). In this study, the deletion of domain I negated the ability of NS5A to bind to rU7 RNA and domain I individually bound this RNA with much lower affinity than NS5A (364). However, when the LCS I region was added to the domain I fragment, RNA binding was restored to levels similar to NS5A indicating the importance of this region in the RNA binding ability of NS5A (364). Differing experimental conditions could possibly explain the different RNA binding profiles observed in these two studies. This study also found that both NS5A and domain I-LCS I fragments were able to from dimers and that the presence of U-rich RNA stimulated the formation of these dimers (364). This observation suggests that NS5A dimers bind to the viral RNA (364). However, a role for the LCS I in contributing to NS5A dimerization is unclear as all of the NS5A domain I crystallization studies lacked this region (479, 481, 482). A subsequent study by the Cameron group found that four cysteine residues, Cys-39, -57, -59 and -80, which were found to form a zinc binding motif in the domain I crystallization studies were critical for NS5A dimerization (489). Mutation of any of these cysteine residues lead to a loss of NS5A dimerization in pull down assays (489). These mutations were also found to result in a significant decrease in the RNA binding efficiency of NS5A, supporting the importance of dimerization in this activity (489). Furthermore, the mutation of any one of these four cysteine residues in NS5A within a subgenomic viral replicon inhibited viral replication (489). This study therefore identified a link between NS5A dimerization, NS5A RNA binding and HCV replication (489).

1.2.4 NS5A domains II and III

While NS5A domain I has been has been fairly well characterized, much less is known about NS5A domains II and III, which are significantly less conserved than domain I (26). Domain II (aa. 254-346) appears to be largely dispensable for viral replication. Whereas deletion of the entire domain lead to a

complete loss of viral replication, mutagenesis studies have identified between 12-23 amino acids within the C-terminal portion of domain II that was essential for viral replication (418, 454, 490). Unlike domain I, domain II has been found to be largely disordered and natively unfolded, and as such, no crystal structure has been obtained for this domain (491, 492). Potential advantages of unstructured proteins include a larger accessible surface for molecular interactions compared to folded proteins and the flexibility to bind to many different targets without sacrificing specificity (491). However, it has also been found that although highly unstructured, domain II is capable of adopting different transient conformations that may play a role in modulating domain II function and protein interactions (492, 493). Domain II is postulated to interact with several proteins, including PKR, phosphatidylinositol 3-kinase (PI3K) and CypA and CypB (458, 494-496). The interaction between domain II and CypA, a protein that possesses peptidyl-prolyl isomerase activity, has been fairly well characterized. This interaction was essential for viral replication and treatment with CypA inhibitors blocked viral replication (497, 498). The isomerase activity of CypA was essential for its role in modulating viral replication (498, 499). The resulting cis-trans peptide bond interconversion modulated by this isomerase activity is speculated to cause a structural shift in domain II towards a more extended form (500, 501). Mutations within domain II that confer resistance to CypA inhibitors lead to structural modifications that appear to mimic the effect of CypA isomerization, thereby reducing the dependence upon CypA to induce such structural change (500, 501). It remains unclear how the interaction between CypA and NS5A domain II modulates HCV replication. However, it has been shown that CypA binding and isomerization activity enhance the RNA binding activity of domain II (84).

NS5A has also been observed to function as a transcriptional trans-activator and viral replication was found correlates with this function (502-504). An acidic region located within Domain II was found to be the main driver of NS5A transcriptional activity and its role in modulating viral replication (504). This transcriptional trans-activation function may represent a mechanism by which the virus can alter the cellular environment in order to modulate the viral lifecycle.

Domain III (aa. 360-451) is similar to domain II in that it appears to be natively unfolded as it lacks significant secondary structure (479). However, domain III has also been proposed to possess transient secondary structure (505). Domain III exhibits a tendency to form α-helices at both its N- and C-terminal regions and such transient structures may play a role in the interaction of NS5A domain III with viral or host factors (505). Although domain III has been shown to be completely dispensable for replication, this region may have role in establishing early replication efficacy. Deletion of this domain leads to delayed

replication kinetics in the JFH-1 HCV (genotype 2a) (419). On the other hand this region plays a critical role in viral assembly.

Efficiency of viral assembly correlates with the levels of NS5A-Core interaction on the lipid droplets associated with the membranous web (452). A number of mutagenesis studies have identified an important role for the C-terminal region of domain III in this interaction (418-420, 452). In particular, a cluster of serine residues within this region of domain III have been found to play a key role in viral assembly (419, 420, 452). Mutation of these serine residues to alanine impairs basal phosphorylation and results in the loss of NS5A-Core co-localization on the surface of LDs, resulting in an inhibition of virion assembly (419, 452). Additionally, these mutations inhibited the interaction of core with viral RNA, supporting a role for NS5A in delivering the viral RNA to the surface of LDs during the assembly process (452). In contrast, mutation of these serine residues to aspartic acid, which mimics a phosphorylated serine, restores this interaction and viral assembly, suggesting a key role for the phosphorylation in modulating viral assembly (452). Domain III has also been found to interact with multiple cellular proteins including CypA and SET and MYND domain containing protein 3 (SMYD3) (26, 506). SMYD3 is a lysine methyltransferase involved in transcriptional regulation though the methylation of histones (506). It also modulates the kinase activity of the vascular endothelial growth factor receptor 1 (506). SMYD3 co-localizes with NS5A around LDs and appears to act as a negative regulator of viral assembly (506). Overexpression of SMYD3 reduced intracellular infectious virus titer and resulted in an accumulation of core protein (506).

Domain III also contains a nuclear localization signal (NLS) that plays a role in the transcriptional activation properties of NS5A described above (503). Due to the presence of the amphipathic helix at the N-terminus, NS5A is typically associated with ER-derived membranes (460). However, N-terminal truncated versions of NS5A have been found during the viral lifecycle, in a caspase dependent manner (503). These truncated NS5A molecules are transported into the nucleus, by virtue of the NLS, where they can then function as transcriptional activators (504).

One theme that emerges when looking into the roles of NS5A domains II and III in the viral lifecycle is the array of proposed functions and interactions ascribed to these regions despite both being natively unstructured. Although this may appear counterintuitive, it is a trait shared by numerous cellular proteins often referred to as intrinsically disordered proteins (IDP), such as p53 and BRAC1 (507, 508). IDPs are very abundant and often function as signalling and regulatory hubs (508). IDPs are also characterized by significant binding promiscuity (508). IDPs often adopt transient structured elements that are utilized to carry out functions (508). These are all characteristics that have been also described for domains II and III

of NS5A that are thought to function as a complex molecular interaction platform (362, 493). Structural changes within IDPs can be induced by interactions with nucleic acids, other proteins, membranes and post-translational modifications such as phosphorylation (508). An example of this modulation within NS5A has recently been identified (362). Phosphorylation of NS5A at Thr-360 in JFH-1 HCV (genotype 2a) by PKA leads to conformational change within a neighbouring poly-proline-II motif within LCS-II region resulting in a stabilized conformation adequate for binding to host proteins containing SH3 domains (362). NS5A has been shown to interact with multiple SH3 containing cellular proteins, such as the pro-apoptotic Bin1 protein, and mutation of this Thr-360 residue was lethal in JFH-1 HCV (158, 362, 509).

1.2.4 NS5SA direct-acting antivirals

Even though it lacks enzymatic activity, NS5A represents an attractive drug target as it has multiple essential roles in the viral lifecycle. As discussed previously, DAAs targeting NS5A have been developed and are currently utilized in the treatment of HCV infection. However, the precise mechanism of action of NS5A inhibitors remains unclear. A common feature of highly potent NS5A inhibitors such as LDV and DCV is their symmetrical nature and apparent interaction with NS5A dimers at the dimer interface as discussed earlier (483-486, 510). Interaction with NS5A dimers, perhaps in multimeric formation, may explain the incredibly potency displayed by symmetrical NS5A inhibitors (488). Furthermore, the inhibitors may impede the conformation or flexibility of the adjacent AH region and thus may affect NS5A membrane interaction (486). The phenotypes associated with NS5A inhibitors include reduced NS5A motility, redistribution of NS5A either from the ER to lipid droplets or into large cytoplasmic aggregates, an alteration in the ratio of basal to hyperphosphorylated NS5A in favour of the basal phosphorylated form and the inhibition of HCV induced PI4P accumulation (72, 455, 474, 486, 510-512). Furthermore, symmetrical NS5A inhibitors have been observed to prevent NS5A domain I binding to poly-U RNA and, conversely, poly-U RNA binding prevents interaction with the inhibitor (487). This supports a model in which these inhibitor compounds bind to an NS5A dimer that does not bind RNA, potentially preventing the transition to a conformation compatible for RNA binding (487).

Modelling of HCV replication kinetics upon LDV or DCV treatment suggest that these inhibitors have two distinct modes of action, inhibiting both viral replication and viral assembly (72, 513, 514). This characteristic has been observed both *in vitro* and in infected patients (72, 513, 514). Independently of increasing compound concentration, the inhibition of viral assembly occurs almost immediately after LDV

treatment whereas the inhibition of RNA synthesis occurs early but it is only partial (72). This observation suggests that NS5A inhibitors prevent the formation of new replication complexes but do not affect RNA synthesis in those already formed (72, 511). Another study found that a DCV derivative blocked NS5A-mediated membrane rearrangements and therefore replication complex formation (486). Treatment lead to the collapse of the membranous web independent of RNA replication, leading to both a reduction in DMV diameter and number of DMVs in a concentration dependent manner (486). In disagreement with the kinetics studies, this study observed a rapid loss of DMVs upon NS5A inhibitor treatment (486). The reasons for these discrepancies remain unclear.

Regardless of the precise mechanisms the interference with multiple functions in the viral lifecycle likely contributes to the high efficacy of symmetrical NS5A inhibitors. As investigation into determining the exact mechanisms of these inhibitors continues, it is clear that NS5A inhibitors will continue to pay a key role in the treatment of HCV.

2.0 HYPOTHESES AND OBJECTIVES

2.1 Rationale

The HCV NS5A protein appears to play a role in virtually every aspect of the viral lifecycle beyond entry and has been found to be essential for viral replication and particle assembly. A role for NS5A in modulating viral translation is strongly indicated by its ability to interact with both the 5´- and 3´ UTRs. The 5 ´UTR contains the HCV IRES which directly recruits the 40S ribosome to initiate HCV translation whereas the 3'UTR functions to stimulate translation through unknown mechanisms. However, the role NS5A and its association with the poly-U/UC region play in modulating viral translation remains unclear. Contradictory studies have been published suggesting that NS5A either stimulates, inhibits or has no effect on viral translation. We contend that the possible reasons for these discrepancies include reporter design. A number of previous studies utilized reporter constructs that lacked the HCV 3´-UTR where NS5A binds with high affinity to the poly-U/UC region. Other studies, however, utilized reporter systems that have been shown to not accurately reflect the role of the 3´-UTR in modulating HCV translation. This includes DNA based or bicistronic RNA reporter systems. Only when monocistronic RNA reporters containing the authentic 5´ and 3´UTRs are utilized is the stimulatory role of the 3´UTR in viral translation realized.

2.2 Hypotheses

(i) NS5A modulates HCV IRES-mediated translation

(ii) The poly-U/UC region of the 3´UTR is required for the modulation of HCV IRES-mediated translation by NS5A

(iii) The modulation of viral translation by NS5A plays an important role in the viral lifecycle and will thus have effects that extend to viral replication

2.3 Objectives

i. (a) Examine the effect of NS5A on the translation of a monocistronic HCV RNA reporter which contains both the 5´- and 3´-UTRs

(b) Determine the role of the poly-U/UC region in such an effect by utilizing the above described monocistronic HCV RNA reporter containing deletions in this region

ii. (a) Determine which domain of NS5A is responsible for the modulation of viral translation
(b) Attempt to map the residues/regions involved within the implicated domain(s)

iii. Examine the effect of the implicated residues/regions on viral replication

iv. Investigate the mechanism of translational modulation by NS5A domain I by examining the effect of the R112A mutation on domain I dimerization and RNA binding activity

3.0 DOWN-REGULATION OF VIRAL PROTEIN TRANSLATION BY HEPATITIS C VIRUS NONSTRUCTURAL 5A PROTEIN (NS5A) REQUIRES THE POLY-U/UC SEQUENCE IN THE 3´UNTRANSLATED REGION

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3.1 Authors' contribution

All the experiments within this chapter were performed by Brett Hoffman with the exception of the RT-PCR which were performed in collaboration with Zhubing Li. The manuscript was written by Brett Hoffman and edited by Qiang Liu.

3.2 Abstract

Hepatitis C virus nonstructural protein 5A (HCV NS5A) is essential for viral replication and assembly, however, its effect on HCV RNA translation remains controversial. This is partially due to the use of reporters lacking the 3'untranslated region (UTR) which functions to stimulate viral translation and contains the poly-U/UC sequence to which NS5A binds. Various studies have also utilized plasmid based or bicistronic reporters, neither of which accurately reflect the role of the 3´-UTR in stimulating viral translation. Thus we investigated the role of NS5A in HCV translation using monocistronic RNA containing the *Renilla* luciferase gene flanked by both HCV UTRs. We found that NS5A down-regulates viral protein translation in a dose-dependent manner. HCV genomic RNA translation is also down-regulated by NS5A. This inhibitory effect of NS5A requires the poly-U/UC sequence as NS5A does not affect translation when this region is deleted. In addition, we showed that three domains of NS5A can inhibit translation independently. These results suggest that NS5A down-regulates HCV RNA translation through a mechanism involving the poly-U/UC sequence in the 3´-UTR.

3.3 Introduction

Hepatitis C virus (HCV) is a serious global health issue. The World Health Organization estimates there are currently 170 million infected individuals worldwide, representing approximately 2% of the global population. HCV was first discovered in 1989 as the agent responsible for non-A, non-B post-transfusion hepatitis and chronic HCV infection is now recognized as a major factor in the development of severe liver damage such as decompensated cirrhosis, hepatic steatosis and hepatocellular carcinoma (1, 50, 515). Chronic infection is estimated to develop in 75 to 80% of those infected (50). There is no vaccine available for HCV and current treatment varies as to its effectiveness against different viral

genotypes, although newly released direct acting antivirals are significantly improving treatment outlook (516-518).

HCV is a small, enveloped, single-stranded positive-sense RNA virus and belongs to the *Flaviviridae* family as the only member in the genus *Hepacivirus* (519). The viral genome is 9.6 kb in size and contains a single open reading frame (ORF) which encodes for a polyprotein of approximately 3000 amino acids (515). 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, E2, and seven non-structural proteins, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (519). The single ORF is flanked by highly conserved 5′- and 3′-untranslated regions (UTRs) which are involved in the control of viral translation and replication (516). The 5′-UTR contains an internal ribosome entry site (IRES) which initiates the cap-independent translation through the direct recruitment of the 40S ribosomal subunit (19). While the 3′-UTR is essential for replication, as it serves as the initiation site for negative strand synthesis, the exact role of the 3′-UTR in HCV translation remains unclear (520-522). For many years the role of the 3′-UTR in the modulation of HCV translation was a contentious issue with published reports suggesting that the region down-regulates, enhances, or has no effect on IRES-mediated translation (389, 390, 520, 521, 523-529). However these discrepancies are thought to be at least in part due to reporter design (521). Efficient translation enhancement by the 3′-UTR is observed only when monocistronic RNA reporters with precise 3′ ends or complete RNA genomes are utilized is (521). It is now generally accepted that the 3′-UTR serves to stimulate translation.

The 3′-UTR of HCV is composed of three distinct regions: the variable region, the poly-U/UC tract and the X-tail, a region with highly conserved secondary structure (**Figure 3.1a**) (521). The X-tail forms three stem loop structures, namely SL1, SL2 and SL3 (522). 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 while SL2 and SL3 play only minor roles (521). The mechanism of 3′-UTR enhancement of translation remains to be clearly defined but possible mechanisms include RNA-RNA interactions with HCV IRES structure and recruitment of cellular factors to the 3′-UTR that interact with components of the translational machinery present on the IRES in a fashion similar to the PABP-eIF4G interaction found on cellular mRNAs (522). A recent study by Bai *et al.* found that the 3′UTR is cabaple of binding to the 40S ribosomal subunit and enhancing not the first but subsequent rounds of translation (218). This led the authours to propose a model in which the 3′UTR may function to recycle ribosomes from the 3′-UTR to the IRES within the 5′UTR (218).

HCV NS5A is a multifunctional protein essential for viral replication and assembly (419, 456). NS5A has also been found to interact with multiple viral and cellular proteins (456). Despite the numerous functions attributed to NS5A, the protein has no known enzymatic activity (459). NS5A contains a conserved N-terminal amphipathic α-helix that serves as a membrane anchor for NS5A and is both necessary and sufficient for mediating association between the protein and cellular membranes (460, 461). This α-helix plays an unknown but essential role in viral replication. Disruption of this region, which leads to a diffuse cytoplasmic localization of NS5A, prevents viral replication (460, 480). Following this N-terminal amphipathic α-helix, NS5A is separated into three domains which are separated by two regions of repetitive low complexity sequences (480). Domain I is the most conserved and best characterized of the three domains across all HCV genotypes (480). Four distinct crystal structures have been obtained for domain I, all demonstrating the ability of this domain to dimerize (480, 482). In the Tellinghuisen *et al.* study this dimerization results in the formation of a large, basic groove at the interface that is speculated to be involved in RNA binding activity (480). Domain I also contains an unconventional zinc binding motif which is composed of four conserved cysteine residues the mutation of which is lethal to virus replication (480).

NS5A domains II and III are much less characterized than domain I. Domain II was been shown to be required for viral replication in an early study (454). However, subsequent studies showed that only 23-35 amino acids are essential for replication (418, 454, 530). Recent work has further narrowed this essential region down to 12 residues absolutely required for viral replication of JFH-1, with several of these residues mapping to a region involved in binding to a host factor implicated in viral replication, cyclophilin A (490). Domain II has also been implicated in the interaction between NS5A with the viral RNA-dependent RNA polymerase, NS5B, and the modulation of NS5B activity (367, 531). Other activities in which domain II has been implicated in are activation of the PI3K signaling pathway and the modulation of the antiviral response as this region contains the interferon sensitivity-determining region and binds to PKR (496, 532). Domain III has been found to be completely dispensable for viral replication (418). However, this domain has been implicated in viral particle assembly (418, 419, 454). Both domains II and III appear to be largely disordered and are natively unfolded which may contribute to the ability of NS5A to play a role in so many diverse functions as these regions are capable of adopting different transient conformations (491, 493, 533, 534). Due to the numerous roles that NS5A appears to play in the viral lifecycle, it is a very attractive antiviral target and high potency drug compounds have recently been discovered to target NS5A (485).

Additionally, NS5A has been found to bind to the poly-U/UC region of the viral 3´-UTR with high affinity (364). Binding was found to be a result of direct interaction between NS5A and the HCV RNA (364).

It was subsequently found that all three domains of NS5A were able to independently bind to the 3´-UTR (363). However, while domains I and II showed strong binding, domain III exhibited weaker binding affinity (363). This suggests that domains I and II are the major contributors in the binding of NS5A to the viral 3´-UTR (363). A recent study has found a correlation between viral replication and the ability of NS5A to bind both synthetic poly-U RNA and HCV RNA in cell culture (489). Other potential binding sites for NS5A i include sites within each of the three domains which make up the HCV IRES (364). The fact that NS5A binds to regions within both the 5´- and 3´-UTRs suggests that NS5A may function in the modulation of HCV translation and could perhaps be involved in genome circularization or regulating the switch from translation to replication (363, 364, 535). Accordingly, NS5A is highly likely to be implicated in the modulation of viral translation, although its exact role remains uncertain. Contradictory studies have been published suggesting that NS5A either stimulates, inhibits, or has no effect on viral translation (527, 536-539). The possible reasons for this discrepancy include the use of reporters that lack the 3´-UTR, where NS5A has been shown to bind to the poly-U/UC region, as well as the use of plasmid-based reporters which fail to accurately reflect the role of the 3´-UTR in HCV translation (521).

In this study we investigated the effect of NS5A on modulating viral translation using monocistronic reporters by focusing on understanding the role the poly-U/UC region in the 3´-UTR in the modulation of viral translation by NS5A. We found that NS5A down-regulates viral translation in a dose dependent manner and that this modulation is dependent upon the presence of the poly-U/UC region in the viral 3´-UTR. Additionally, we found that each domain of NS5A is capable of modulating viral translation independently.

3.4 Results

3.4.1 NS5A down-regulates HCV protein translation in a dose-dependent manner in the presence of the poly-U/UC region of the viral 3´-UTR

For this study we utilized a monocistronic RNA reporter containing the HCV 5´-UTR plus the first 16 amino acids of the core gene, an internal *Renilla* luciferase gene fused to the last 5 amino acids of the NS5B gene, and the 3´-UTR (520). Reporters that contained deletions of the poly-U/UC region or the entire 3´-UTR were also utilized. To investigate the role of the HCV 3´-UTR in modulating viral translation, Huh7 cells were transfected with these RNA reporters. As shown in Fig. 1A, deletion of the poly-U/UC region decreased viral translation by approximately 50% whereas when the entire 3´-UTR was removed viral translation was decreased by approximately 90% compared to the wild-type 3´-UTR (**Figure 3.1b**).

These results showed the importance of the 3´-UTR in the modulation of viral translation and were similar to those obtained by others (218, 521, 522). The mechanism behind the stimulatory effect of the individual 3´-UTR regions on viral translation remains to be clearly defined. 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 (218, 521, 522).

The effect of NS5A on HCV translation is controversial in part due to use of non-reliable reporter systems. As we and others have validated the monocistronic RNA reporter as a reliable system, we sought to investigate the role of NS5A in HCV protein translation. For this purpose, Huh7 cells were transfected with HCV-1b NS5A expression plasmid and followed by the transfection of the monocistronic *Renilla* luciferase HCV-1b RNA reporter. Luciferase assay was performed 4 h after RNA transfection. As shown in **Figure 3.2a**, the expression of NS5A resulted in significant decrease in luciferase activity, representing HCV IRES-mediated translation, compared to vector control. On the other hand, expression of green fluorescent protein (GFP) did not affect HCV translation suggesting this is a specific effect of NS5A expression (**Figure 3.2f**). These results suggested an inhibitory effect of NS5A on HCV translation.

Although it has been shown that NS5A preferentially binds to the poly-U/UC region in the 3´-UTR, the function of this RNA binding is not clear (363, 364). To test whether the poly-U/UC region plays a role in protein translation modulation by NS5A, we utilized an RNA reporter without the poly-U/UC in the 3´-UTR. **Figure 3.2a** showed that NS5A had no significant effect on the level of translation in the absence of the poly-U/UC region. The overall translation activity was significantly decreased in the absence of the poly-U/UC region as expected (**Figure 3.1b**). These data suggested that the poly-U/UC region of the 3´-UTR was necessary for the down-regulation of viral translation by NS5A. These experiments were repeated using RNA reporters and NS5A of HCV-1a to determine if the observed effect was restricted to HCV-1b. In the 1a experiments the expression of NS5A led to a similar level of down-regulation of viral translation and again the absence of the poly-U/UC region negated the down-regulation caused by NS5A expression (data not shown).

In order to demonstrate that the down-regulation of translation observed was not due to NS5A expression negatively affecting the stability of the RNA translation reporters, we performed RT-PCR. We found that NS5A expression did not affect the RNA levels of these HCV RNA translation reporters as similar levels were observed in the presence or absence of NS5A (**Figure 3.2b**)

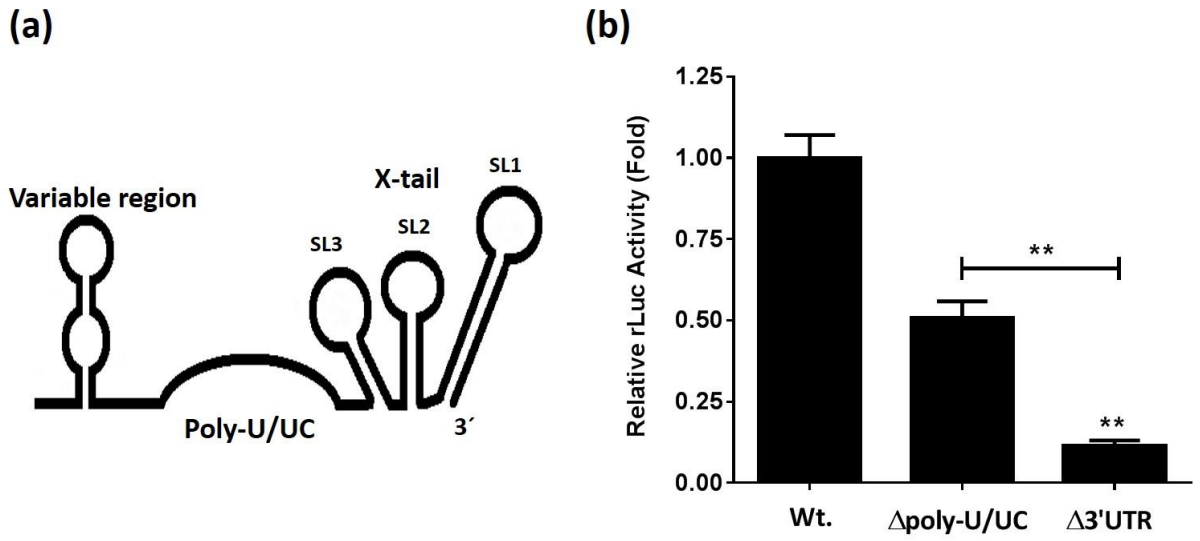

Figure 3.1 - The 3´-UTR modulates viral translation. (**a**) Diagram of the HCV 3´-UTR. (**b**) Huh7 cells were transfected with a monocistronic HCV RNA translation *Renilla* luciferase (rLuc) reporter RNA containing either wild-type or Δpoly-U/UC 3´-UTR. Luciferase assay was performed 4 hr after RNA transfection. The rLuc value after vector transfection was set to 1 and error bars indicate mean ± SD. Statistical differences were analyzed by Student *t* test and indicated as * if $p \leq 0.05$ or ** if $p \leq 0.01$ compared to wildtype unless otherwise indicated. Error bars indicate mean ±SD

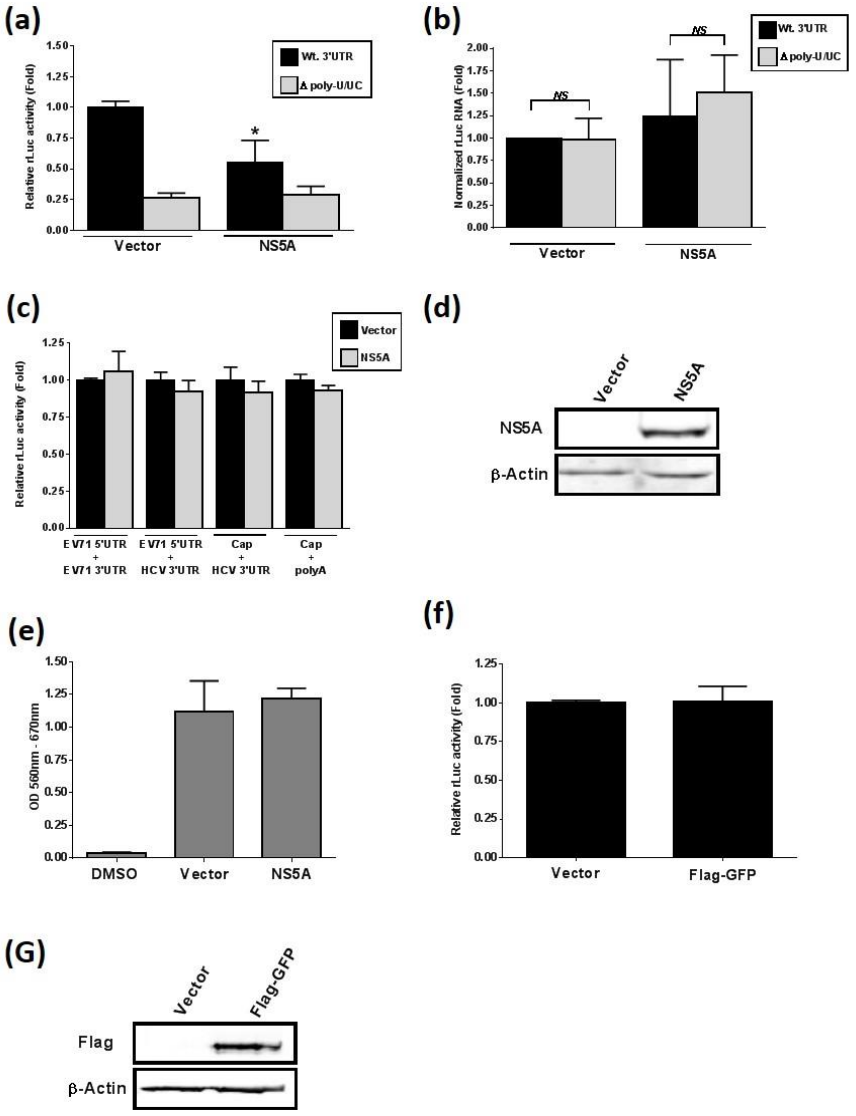

Figure 3.2 - NS5A down-regulates HCV protein translation in the presence of the poly-U/UC region in the 3´-UTR. (**a**). Huh7 cells were transfected with plasmid expressing HCV NS5A or plasmid vector. At 24 hr later, cells were transfected with a monocistronic HCV RNA translation *renilla* luciferase (rLuc) reporter RNA containing either wild-type or Δpoly-U/UC 3´-UTR. Luciferase assay was performed 4 hr after RNA transfection. The rLuc value after vector transfection was set to 1. Statistical differences were analyzed by Student *t* test and indicated as * if $p \leq 0.05$ compared to vector (**b**). The levels of rLuc RNA in cells in (**a**) were determined by real-time PCR. The RNA levels of a housekeeping gene GUSB were used for normalization. (**c**). Huh7 cells were transfected sequentially with HCV NS5A expressing plasmid or plasmid vector and RNA translation reporter RNAs as indicated. Luciferase assay was performed 4 hr after RNA transfection. (**d,g**). Expression of (d) NS5A or (g) GFP was demonstrated by Western blotting using either an (d) NS5A or (g) flag specific antibody. The levels of β-actin were also determined using a β-actin specific antibody, as a loading control (**e**) MTT assay for cell viability was performed 24 hr after transfection of Huh7 cells with plasmid vector or HCV NS5A expression plasmid. DMSO treatment was used as a control (**f**) Huh7 cells were transfected sequentially with Flag-GFP expressing plasmid or plasmid vector and wild-type HCV RNA translation reporter. Luciferase assay was performed 4 hr after RNA transfection.

To investigate whether the effect of NS5A on translation was HCV-specific, we measured the RNA translation driven by EV71 5′-UTR. As shown in **Figure 3.2c**, HCV NS5A did not affect EV71 RNA translation. Furthermore, no effects were observed by NS5A on the translation of RNA with EV71 5′-UTR and HCV 3′-UTR, or with 5′- cap and HCV 3′-UTR, or with 5′- cap and poly-A sequence (**Figure 3.2c**). These results indicate that the expression of NS5A does not result in a general decrease in global translation. This effect is specific to the HCV reporter. Additionally, these results demonstrate that NS5A only inhibits translation when both HCV 5′- and 3′-UTRs are present. This is consistent with a current model for HCV translation which postulates that the UTRs are adjacent to each other in a closed loop conformation during translation (218). A number of cellular factors such as HuR, LSm1-7 and IGF2BP1 have been identified to bind to both UTRs and modulate viral translation (216-218, 231). Such interactions with both UTRs could function to mediate an interaction between the two UTRs. Furthermore, as these cellular proteins bind to the poly-U/UC within the viral 3′-UTR, it is conceivable that the high affinity binding of NS5A in this region could disrupt such interactions and in turn disrupt the interaction between the two UTRs. This possibility remains to be investigated. Finally, MTT assay was performed to investigate the potential effects of NS5A expression on cell viability. NS5A expression had no effect on cell viability (**Figure 3.2e**)

Next, we performed a dose response experiment to further characterize the effect of NS5A on HCV protein translation. Transfection of increasing amounts of NS5A expression plasmid led to increased and statistically significant reductions in viral translation (**Figure 3.3a**). This effect was again found to be dependent upon the presence of the poly-U/UC region of the 3′-UTR as the increasing NS5A expression had no significant effect on the level of translation of the reporter lacking this region (**Figure 3.3a**). The increasing expression of NS5A protein was demonstrated by Western blotting (**Figure 3.3b**). The down-regulation 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 (**Figure 3.3c**). Increasing levels of NS5A did not induce measurable changes in cellular viability (**Figure 3.3d**). These results suggested that NS5A dose-dependently down-regulates HCV RNA translation in the presence of the poly-U/UC region.

3.4.2 NS5A binds to the poly-U/UC region of the HCV 3′-UTR

The above results indicated a role of the poly-U/UC sequence in HCV protein translation inhibition by NS5A protein. As such, we hypothesized that binding of NS5A to the poly-U/UC sequence might play a

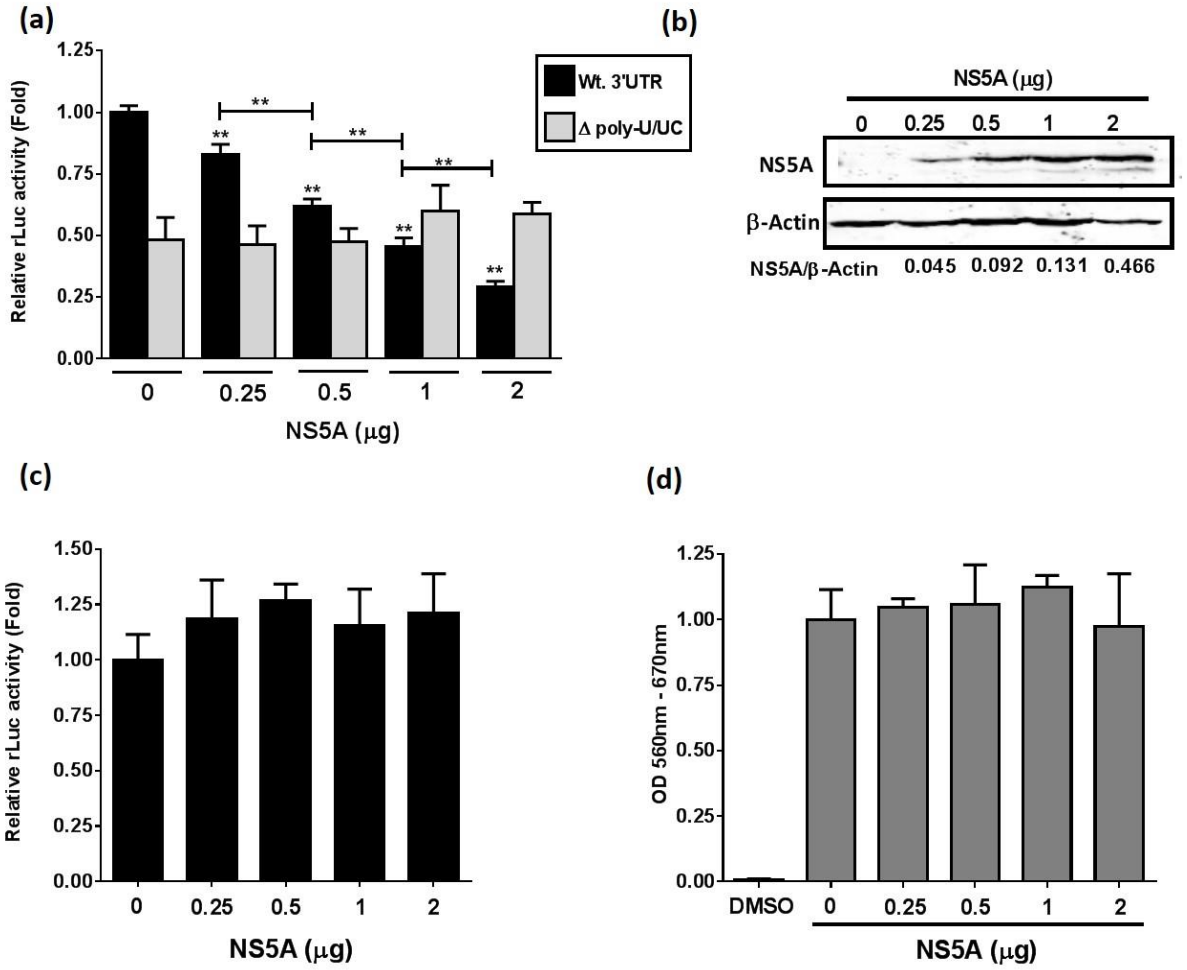

Figure 3.3 - NS5A down-regulates HCV protein translation in a dose dependent manner in the presence of the poly-U/UC region in the 3´-UTR. (**a**). Huh7 cells were transfected with 0, 0.25, 0.5, 1, or 2 μg of HCV NS5A expression plasmid for 24 hr. Total amounts of DNA transfected were kept consistent by adjusting the amounts of vector. Cells were then transfected with an HCV monocistronic translation *renilla* luciferase (rLuc) reporter RNA, containing either wild-type or Δpoly-U/UC 3´-UTR. Luciferase assay was performed 4 hr after RNA transfection. (**b**) The levels of NS5A and β-actin were determined by Western blotting. (**c**) Huh7 cells were transfected with increasing amounts of HCV NS5A plasmids as in (**a**), followed by a capped mRNA expressing *renilla* luciferase. Luciferase assay was performed 4 hr after. The rLuc value after vector plasmid or control mRNA transfection was set to 1. Statistical differences were analyzed by Student *t* test and indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ compared to 0 μg unless otherwise indicated (**d**). Cell viability was determined by MTT assay 24 hr after transfection with increasing amounts of HCV NS5A expressing plasmids. DMSO treatment was included as a control.

role. To demonstrate the binding of NS5A within this region we performed NS5A-RNA binding experiments. For this purpose, purified NS5A protein and Cy5-labeled HCV 3′-UTR with and without the poly-U/UC RNA were used in a gel shift assay. BFP protein was used as a control. As shown in **Figure 3.4a**, a shift in the RNA band was visible at 21.36 pmol NS5A and the shift became more pronounced at 37.38 pmol NS5A relative to the RNA band when no protein was added, indicating NS5A-RNA binding. No significant RNA band shift was observed when the poly-U/UC was deleted (**Figure 3.4b**), indicating no RNA binding of NS5A. The control BFP protein did not cause an RNA band shift in both cases indicating the specific nature of the NS5A-RNA interaction. These experiments demonstrated that NS5A bound to the poly-U/UC sequence in the 3′-UTR, confirming the findings of other groups (84, 365). Together with our previous results, these results suggest that NS5A binding within the poly-U/UC region might play a role in HCV protein translation inhibition by NS5A.

3.4.3 NS5A domains modulate HCV protein translation

NS5A consists of an N-terminal amphipathic α-helix (AH), domains I, II, III with two inter-domain low complexity sequences (LCS) (**Figure 3.5a**). To map the domain(s) of NS5A responsible for this modulatory effect, plasmids encoding NS5A with 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 mutant was capable of downregulating viral translation similar to Wt. NS5A (**Figure 3.5b**). Furthermore each domain of NS5A was capable of significantly down-regulating of HCV translation independently (**Figure 3.5b**). These inhibitory effects again were dependent upon the poly-U/UC sequence (**Figure 3.5b**). None of the NS5A domain proteins had an effect on cap translation or cell viability (**Figures 3.5d and 3.5e**). The expression of NS5A and mutant proteins were demonstrated in Western blotting (**Figure 3.5b**). The apparent differing levels of expression may be in part due to the use of a polyclonal antibody. The fact that each individual domain was capable of down-regulating viral translation may not be completely surprising since it has been shown that each domain is capable of binding to the 3′-UTR independently (363).

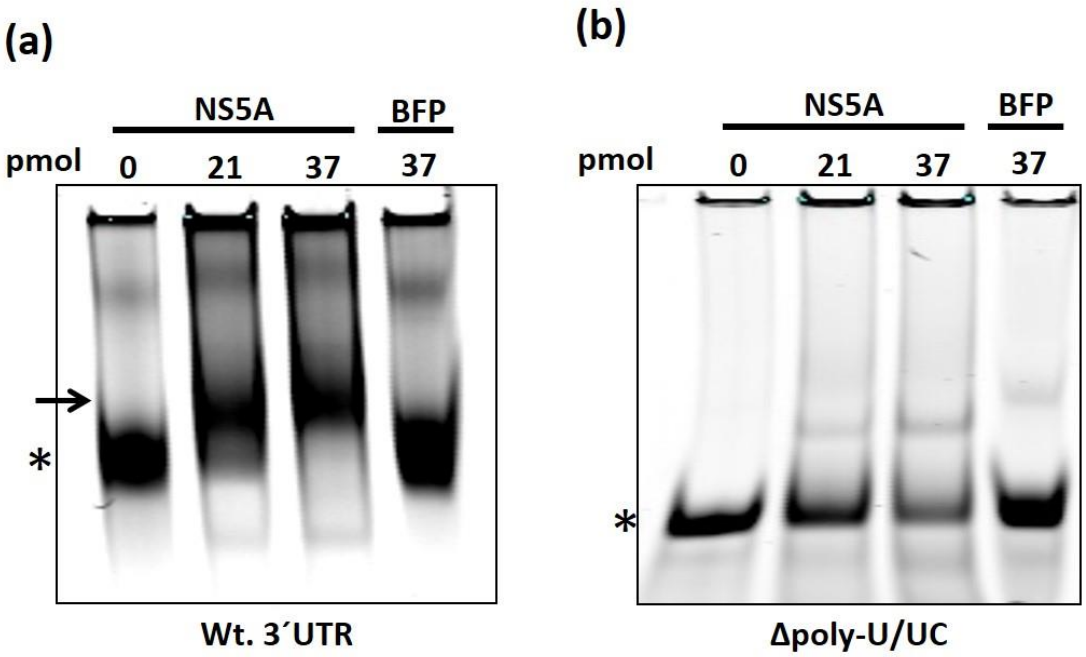

Figure 3.4 - NS5A binds to the poly-U/UC region in the HCV 3´-UTR. (**a,b**) Cy5-3´-UTR RNA (**a**) or Cy5-3´-UTR Δpoly-U/UC RNA (**b**) (1.25 pmol) was incubated with the indicated amount of NS5A protein or blue fluorescent protein (BFP) control protein in a binding buffer (25 mM MOPS, 10 mM NaCl, pH 4) for 1 h at 4°C. The binding reactions were then run into a 6% native polyacrylamide gel after addition of 6X loading buffer (50% sucrose). Cy5-RNA was visualized with a Li-Cor Odyssey scanner. Asterix indicates unbound RNA and arrow indicates NS5A/RNA complexes. Representative of multiple independent experiments.

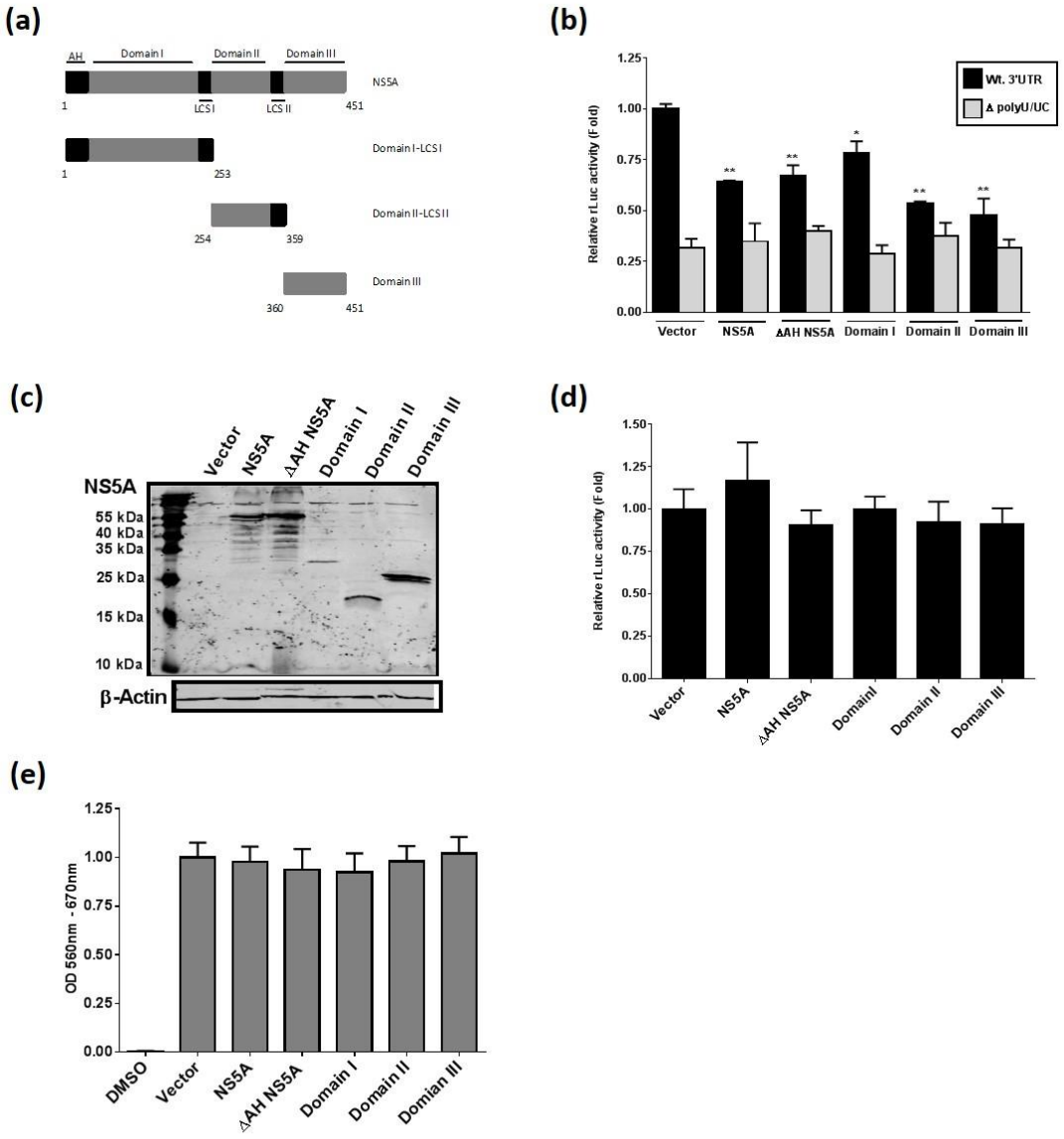

Figure 3.5. - Individual NS5A domains down-regulate HCV RNA translation in the presence of the poly-U/UC region in the 3´-UTR. (**a**). Diagram of NS5A domains and the domain fragments utilized. (**b** and **d**). Huh7 cells were transfected with plasmid vector, plasmids expressing HCV NS5A or mutants for 24 hr. Cells were then transfected with monocistronic HCV translation *renilla* luciferase (rLuc) reporter RNAs, containing either a wild-type 3´-UTR or Δpoly-U/UC 3´-UTR (**b**), or a capped mRNA expressing *renilla* luciferase (**d**). Luciferase assay was performed 4 hr after RNA transfection. The rLuc values after vector plasmid or control mRNA transfection was set to 1. Statistical differences were analyzed by Student *t* test and indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ compared to vector (**c**). Expression of NS5A and domains was demonstrated by Western blotting using a polyclonal NS5A antibody (**e**). Cell viability was determined by MTT assay 24 hr after plasmid transfection or DMSO treatment.

3.4.4 NS5A expression in trans down-regulates the translation of full length HCV ΔGDD viral RNA

To substantiate the results obtained using the monocistronic RNA reporters, we wanted to measure protein translation from a full length HCV genomic RNA. For this purpose, we generated a full length HCV-1b genomic reporter with an insertion of *Renilla* luciferase gene and the foot and mouth disease virus 2A peptide between p7 and NS2 as described for HCV-2a genome previously (**Figure 3.6a**) (433). The GDD sequence was deleted in the NS5B to render this viral RNA replication deficient, so the level of *Renilla* luciferase could be utilized as a measurement of viral translation only.

When the full length HCV genomic RNA containing the wild-type 3´-UTR was utilized, additional NS5A expression led to decreases in viral translation compared to control at 2 hr and 4 hr post-electroporation, respectively (**Figure 3.6b**). However, when the poly-U/UC was deleted from the 3´-UTR, additional NS5A expression did not affect viral translation (**Figure 3.6c**). NS5A expression at 2 hr and 4 hr time points was demonstrated by Western blotting although the expression at 2h is low (**Figure 3.6d**). NS5A expression from the genomic RNA reporter was not detected in this assay, presumably due to the low level of expression from this reporter. These results suggested that NS5A expression *in trans* could down-regulate viral translation from HCV genomic RNA dependent upon the presence of the poly-U/UC sequence in the 3´-UTR.

3.5 Discussion

NS5A plays key roles in the HCV lifecycle as it is essential for viral replication/assembly and has been found to be involved in numerous other processes such modulation of cellular signaling pathways and inhibition of interferon responses (419, 456). NS5A has also been found to interact with multiple viral and cellular proteins (456). A role for NS5A in the modulation of viral translation has however, been unclear as numerous contradictory studies have been published (527, 536-539). In this study, we utilized monocistronic RNA reporters with the precise 3´-UTR as well as HCV genomic RNAs to investigate the role that NS5A plays in modulating viral protein translation.

We first set out to determine the effect of NS5A on HCV protein translation using the monocistronic HCV translation reporter RNA. We found that NS5A specifically down-regulated viral protein translation in a dose dependent manner. The effect of NS5A on viral translation was then investigated using a full length viral RNA to test this effect in a situation more relevant to HCV infection.

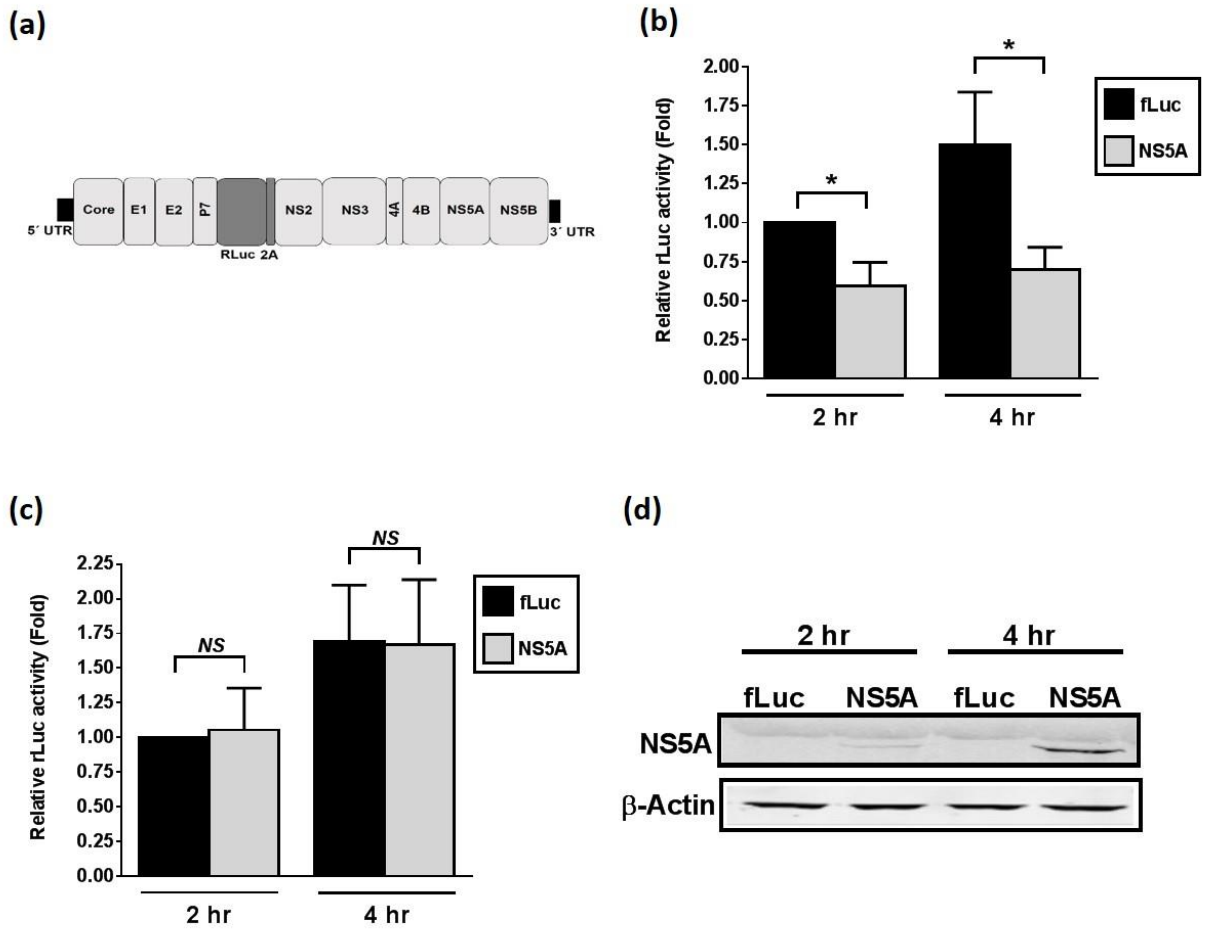

Figure 3.6 - NS5A down-regulates translation of full length HCV genomic RNA in the presence of poly-U/UC region in the 3´-UTR. (**a**). Diagram of HCV reporter utilized. (**b** and **c**) Huh7.5 cells were electroporated with HCV (p7-rLuc2A) ΔGDD full length viral RNA containing either the wild-type 3´-UTR (**b**) or Δpoly-U/UC 3´-UTR (**c**) together with mRNA expressing NS5A mRNA or control fLuc mRNA. Cells were harvested at 2 and 4 hr after electroporation for luciferase assay. The rLuc values were normalized to the total cell number in each sample by quantitating total protein concentration. Normalized value of control mRNA at 2 hr time-point was set to 1. Statistical differences were analyzed by Student *t* test and indicated as * if $p \leq 0.05$. (**d**). Expression of NS5A was demonstrated by Western blotting using an NS5A antibody. The levels of β-actin were determined by a β-actin antibody as a loading control.

Using the full length HCV RNA, which is replication deficient, we observed that viral translation was again down-regulated when NS5A was expressed *in trans*. Importantly, in all of the above experiments, NS5A did not have an effect on HCV protein translation when the poly-U/UC sequence of the 3′-UTR was deleted, suggesting that the poly-U/UC sequence was required.

Having established that NS5A functions to down-modulate viral translation, we then set out to determine which regions of NS5A were responsible for this effect. NS5A protein has an amphipathic α-helix (AH) of approximately 30 amino acids at its N-terminus followed by three domains (480). When the AH sequence was deleted, NS5A could still down-regulate protein translation, indicating that the AH did not have an effect on viral protein translation. Interestingly, the AH region of NS5A was previously been found to be necessary and sufficient for membrane localization of NS5A and be required for viral replication (460, 461). Next, we investigated the effect of NS5A domains on viral protein translation. It was observed that each individual NS5A domain was able to down-modulate viral translation independently. This effect was again dependent upon the presence of the poly-U/UC region in the 3′-UTR. On one hand, the fact that each individual domain was capable of down-regulating viral translation may not be completely surprising since it has been shown that each domain is capable of binding to the 3′-UTR independently (363). On the other hand, however, since domains I and II can bind to 3′-UTR RNA to a stronger degree than domain III, it begs the question as to whether 3′-UTR RNA binding is the only mechanism for the observed translation inhibition by NS5A and its domains (363).

The mechanisms of how NS5A down-regulates HCV RNA translation are not clear. The requirement of the poly-U/UC region in the 3′-UTR for this effect suggests that NS5A, potentially through binding to this region, may interfere with the stimulatory functions exerted by the poly-U/UC region. It has been shown that long range interaction between the 5′-UTR and 3′-UTR may act as molecular switches for different steps in HCV lifecycle (197, 198, 383). We showed that NS5A specifically down-regulates RNA translation when both HCV 5′-UTR and 3′-UTR 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 or the recycling of ribosomes between the 3′-UTR and IRES (15, 218). A model of this theory is presented in **Figure 3.7**. While the biological relevance and mechanism remains to be elucidated, data presented here as well as those already published collectively suggest that NS5A may play a role in the switch from viral translation to viral replication

In conclusion, we have shown that NS5A down-regulates viral translation though 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 HCV lifecycle.

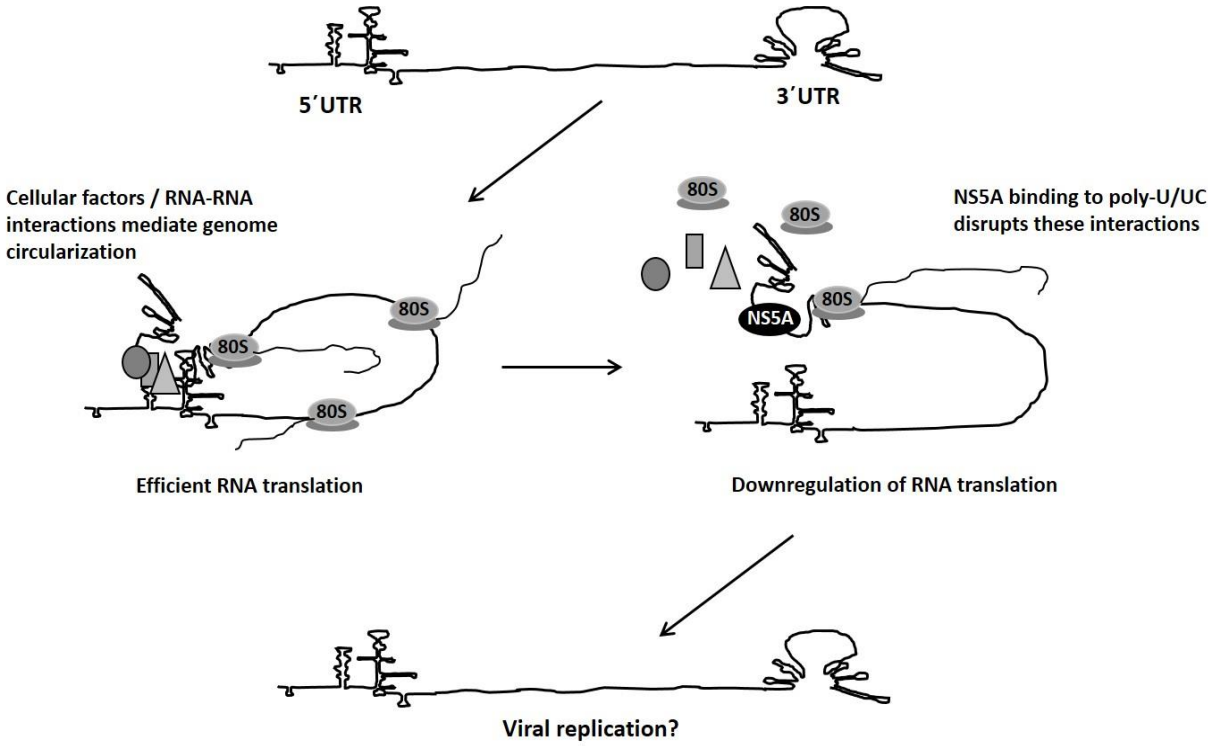

Figure 3.7 - NS5A down-regulates viral translation. The binding of NS5A to the poly-U/UC region of the 3′UTR may disrupt the binding of cellular factors and the interaction between the 5′and 3′UTRs that is thought to stimulate viral translation. This disruption may account for the down-regulation of viral translation observed within this study and may also be involved in the switch between viral translation and replication.

3.6 Materials and Methods

3.6.1 RNA reporters and expression plasmids

All plasmids were constructed as per standard methods and confirmed by DNA sequencing. HCV monocistronic RNA translation reporter contained the HCV 5′-UTR, the sequence encoding the first 16 amino acids of the core protein, an internal *Renilla* luciferase (rLuc) gene, the sequence encoding the last five amino acids of the NS5B and the 3′-UTR of HCV-1b N (540). This reporter was used to develop additional reporters with 5′-UTR, the poly-U/UC region or the entire 3′-UTR deleted. A monocistronic EV71 (enterovirus 71) RNA translation reporter was generated. This reporter contained the rLuc gene flanked by the 5′-UTR and 3′-UTR sequences of EV71 Anhui1 (541). In another construct, the EV71 3′-UTR was replaced by HCV 3′-UTR. A restriction enzyme recognition sequence (*Xba*I for HCV and *Xma*I for EV71) was engineered around the very 3′ end of the 3′-UTR sequences. Plasmid DNAs digested with *Xba*I or *Xma*I were treated with mung bean nuclease (New England Biolabs) to allow generation of RNA transcripts with authentic 3′ ends (542). The HCV-1b (p7-rLuc2A) reporter is a full length viral genome which contains an insertion of rLuc and the foot and mouth disease virus 2A peptide located between p7 and NS2 of the HCV genome as per a previously described strategy (433). The GDD sequence within the NS5B viral polymerase was deleted rendering this viral reporter replication deficient. We also generated an HCV-1b (p7-RLuc2A) GDD reporter 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 at 5′- ends (no cap) and viral 3′-UTR sequences at 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 (540) as template respectively and cloned into pEF/cyto/myc vector (Invitrogen) with a stop codon or in-frame with the myc tag sequence. These plasmids were used to express NS5A proteins upon transfection. 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 cap structure at 5′- ends and poly-U sequence at 3′ ends.

3.6.2 Cell lines, transfections and luciferase assay

Human hepatoma cell lines HuH-7 and HuH-7.5 were kindly provided by Bartenschlager (315) and Rice (543) , respectively. HuH-7 and HuH-7.5 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% gentamicin and cultured at 37°C and 5% CO_2. In sequential transfection experiments, HuH-7 cells in 24-well plates were transfected with 1 µg of expression plasmid using TransIT-LT1 transfection reagent according to the manufacturer's protocol (Mirus Bio). In NS5A dose experiments, cells were transfected with increasing amounts of NS5A expressing plasmid and the total amounts of plasmid DNA for transfection were kept to 2 µg by adjusting the amounts of vector. The next day the cells were transfected with 1 µg of reporter RNA using DMRIE-C (Invitrogen) and incubated for 4 hr. In HCV genomic RNA experiments, HuH-7.5 cells in PBS were electroporated (270 V, 950 µF, 4 mm cuvette) with the indicated amounts of RNA using an electroporator (Bio-Rad) before they were harvested at the indicated time points. Following incubation, the cells were lysed with Passive Lysis Buffer and rLuc activity was determined according to the manufacturer's instructions (Promega). Luciferase data in these electroporation experiments was normalized to protein concentration that was determined by Bradford assay (BCA Protein Assay kit, Thermo Scientific). For luciferase experiments vector control was set to 1 (100%) and error bars represent mean ± SD. All luciferase experiments are the average of three independent experiments performed in triplicate.

3.6.3 SDS-PAGE and Western blot

Western blots were performed as previously described (544). 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 hr 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 Biosciences) for 1 hr at room temperature. Blots were then washed with PBST (PBS+0.1% Tween-20) before a final wash with PBS. Blots were scanned using a Li-Cor Odyssey scanner at the appropriate wavelengths (Li-Cor Biosciences). A monoclonal anti-NS5A antibody (Virogen) and a polyclonal NS5A antibody (545) were used. Anti-β-actin antibody was purchased from Cell Signaling Technology. Western blots shown are representative of multiple independent experiments.

3.6.4 MTT assays

Cell viability was investigated using an MTT assay. Cells in a 96-well plate were added with 20 µL of MTT reagent (5 mg/mL) and the plate was placed on a shaker for 5 minutes at room temperature. The cells were then incubated for 4 hr to allow the MTT to be metabolized. The media were removed from the cells and 200 µL DMSO added to each well. After 5 minutes at room temperature on a shaker, the optical density was read at 560 nm and background at 670 nm was subtracted using a Spectra Max 340PC plate reader (Molecular Devices). Data represents average of three independent experiments performed in triplicate.

3.6.5 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´- TTGTTGAAGGTGCCAAGAAG 3´) and rLuc-rev (5´- TGAGAACTCGCTCAACGAAC 3´) using SYBR green based detection system. The transcript levels of β-glucuronidase (GUSB) determined in parallel with primers GUSB-FD (5´- GGTGCTGAGGATTGGCAGTG 3´) and GUSB-rev (5´- CGCACTTCCAACTTGAACAGG 3´) were used for normalization.

3.6.6 Protein expression and purification

A poly-histidine tagged NS5A protein for RNA binding experiments was expressed in *E. coli* using the Expresso T7 SUMO cloning and expression system according to the manufacturer's protocol (Lucigen). NS5A protein was purified through affinity chromatography under native conditions with the Ni-NTA purification system (Qiagen). Expression and purification of His_6-tagged NS5A protein was confirmed by both Coomassie blue staining and Western blotting. Purified proteins were aliquoted and stored at -80˚C. Blue fluorescent protein (BFP) was produced in a similar fashion to be used as a control in RNA binding experiments.

3.6.7 RNA binding assays

Cy5 labeled HCV 3´-UTR RNA with or without the poly-U/UC region was produced using the MEGAscript T7 *In Vitro* Transcription reagent (Ambion) with Cy5 CTP (GE Healthcare). For the RNA binding experiments, Cy5-3´-UTR RNA or Cy5-3´-UTRΔpoly-U/UC RNA was incubated with purified NS5A protein or BFP protein in a binding buffer (25 mM MOPS, 10 mM NaCl, pH4) for 1 h at room temperature. The RNA-protein mixture was loaded onto a 6% native polyacrylamide gel and the Cy5 labeled RNA was visualized with a Li-Cor Odyssey scanner (Li-Cor Biosciences). Data representative of multiple independent experiments.

3.6.8 Statistical analysis

All the experiments were performed a minimum of three times in triplicate. The experimental data were analyzed by Student's *t* test and reported as the Mean ± SD using Microsoft Excel software. A *p* value of ≤ 0.05 was considered statistically significant.

3.7 Acknowledgments

We would like to thank Drs. Matthias Gromeier, Shelton Bradrick, Stan Lemon, Charles Rice, Deborah Anderson, Michael Niepmann, Craig Cameron and Linda Chelico for providing reagents and/or help with the experiments. This work was supported by a Discovery Grant from Natural Sciences and Engineering Research Council of Canada to QL. BH is a recipient of a Ph.D. training scholarship from the National CIHR Research Training Program in Hepatitis C, University of Saskatchewan CGSR and Vaccinology and Immunotherapeutics Graduate Student scholarships. This article is published with the permission of the Director of VIDO-InterVac, journal series no. 702.

4.0 LINKER BETWEEN CHAPTERS 3 AND 5

In the previous chapter, we attempted to determine which region of NS5A was responsible for the observed modulation of viral translation. We observed that each domain of NS5A was capable of downregulating viral translation independently through a mechanism requiring the poly-U/UC region. Each domain has been found to bind within this region, suggesting a possible mechanism for this modulatory effect. In the following chapter we set out to determine the region/residues involved in this modulation by domain I and investigated possible mechanisms of action.

5.0 ARGININE 112 IS INVOLVED IN THE MODULATION OF HCV TRANSLATION AND RNA BINDING ACTIVITY EXHIBITED BY NS5A DOMAIN I

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Keywords: hepatitis C virus (HCV); HCV NS5A; HCV RNA translation modulation; HCV 3´-UTR; poly-U/UC sequence

Running title: HCV NS5A domain I R112 modulates viral translation

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5.1 Authors' contribution

All the experiments within this chapter were performed by Brett Hoffman. The manuscript was written by Brett Hoffman and edited by Qiang Liu.

5.2 Abstract

HCV NS5A is essential for viral replication but the mechanisms remain unclear. However, viral replication has been correlated to NS5A dimerization and RNA binding activity. We previously found that NS5A is able to down-regulate viral translation through a mechanism requiring the poly-U/UC region within the viral 3´-UTR to which NS5A binds. In this study, we have further investigated the role of domain I in modulating viral translation. Using a series of deletion and substitution mutants we identified a number of positively charged residues that played a role in this modulatory effect, most prominently R112. We found that mutation of R112 or E148, which form a hydrogen bond in a domain I dimer, negated the ability of domain I to modulate viral translation. Additionally, we found that this R112A mutation impeded both domain I dimer formation and binding to poly-U/UC RNA, suggesting a mechanism for the down-regulatory effect. Finally, when this R112A mutation was introduced into an HCV subgenomic replicon, the replicon was rendered replication deficient. These results collectively point to a crucial role for the NS5A arginine 112 residue in the modulation of HCV lifecycle by NS5A.

5.3 Introduction

Hepatitis C virus remains a serious global health concern with upwards of 150 million infected individuals worldwide (28). Furthermore, HCV results in chronic infections in approximately 75% of those infected and is associated with the development of significant liver diseases such as steatosis, decompensated liver cirrhosis and hepatocellular carcinoma (62). Accordingly, HCV infection is one of the major causes of end-stage liver disease and the primary indication for liver transplantation (13, 485, 546). Recent years have seen the development and approval of new direct-acting antivirals, such as viral protease inhibitors, which have substantially increased treatment efficacy (547). Although treatment outlook is promising, the high cost of the new antivirals, the potential for selection of resistance, limited efficiency in certain populations and the lack of a vaccine highlight the need for continued research.

Tremendous progress has been made in the understanding of the virus since its discovery in 1989 as the cause of non-A non-B post-transfusion hepatitis (1, 2). HCV, as member of the *Flaviviridae* family, is a small positive sense single-stranded RNA virus (293). The 9.6 Kb viral genome contains a single open reading frame (ORF) which is flanked by highly conserved 5′ and 3′ untranslated regions (UTRs) which are involved in the modulation of viral translation and replication (4, 19). Translation of this single ORF, mediated by an internal ribosomal entry site (IRES) located within the 5′-UTR, results in the production of the viral polyprotein (4). The polyprotein is cleaved co- and post-translationally by both cellular and viral proteases into at least 10 viral proteins: three structural proteins (core, E1, and E2) and seven non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The IRES, located in the 5′UTR, directly recruits the 40 S ribosomal subunit to initiate viral translation in a cap-independent manner (293). Viral polyprotein translation is also modulated by viral cis-acting factors and viral and cellular trans-acting factors (15, 19). One of the viral cis-acting factors that modulates translation is the viral 3′UTR (15, 19). While the 3′UTR serves as the initiation site for negative strand synthesis and as such is essential for viral replication, it also functions to stimulate viral translation (200, 201, 205). This is comparable to cap translation in which the polyA binding protein bound to the 3′polyA tail interacts with the cap binding complex enhancing translation by increasing initiation factor recruitment and possible circularization of the mRNA transcript (15). The 3′-UTR is composed of three regions: the variable region, a poly-U/UC tract and an X-tail region which is composed of three stem loops; SL1, SL2 and SL3 (15). The variable region, poly-U/UC region and SL1 have been found to significantly contribute to the enhancement of viral translation mediated by the 3′UTR (201). The mechanism of this enhancement is not clearly defined. The proposed mechanisms include the recruitment of viral and/or cellular factors which may mediate 5′ and 3′ UTR interaction similar to the function of PABP in capped translation (293). Additionally, it has been observed that the 3′UTR may function in ribosome recycling between the two UTRs, in that the 3′UTR is able to bind the 40S ribosomal subunit and enhance successive rounds of translation (218).

HCV non-structural 5A protein (NS5A) is essential for viral replication and assembly (26, 361, 418, 419, 451-453, 455). However, although its exact role in the viral lifecycle remains unclear. NS5A is composed of an N-terminal amphipathic α-helix (AH) and three domains which are separated by two regions of low complexity sequences (LCSs) (548). The AH is necessary and sufficient for membrane localization of NS5A, at the site of the viral replication complex, and is essential for replication (549-551). NS5A domains I and II are essential for viral replication (361, 454, 480, 490). Domain III, while largely dispensable for viral replication, plays an essential role in modulating viral assembly (278, 552, 553). NS5A is an RNA binding protein that binds with high affinity to the poly-U/UC region in the viral 3′ UTR (214).

All three NS5A domains are capable of specifically interacting with the 3´-UTR independently (279). However, while domains I and II bound the 3′UTR RNA efficiently, domain III demonstrated lower, albeit still specific, RNA binding affinity suggesting RNA binding is primarily mediated through domains I and II (279, 554, 555). Additional studies have suggested that domain I is the key mediator of NS5A RNA binding (554, 555). Due to the essential roles of NS5A in the viral lifecycle and its attractiveness as a drug target, a number of studies have been carried out in order to determine the structure of NS5A. Four different domain I structures, all in dimer formations, have been observed (479, 481, 482). Domains II and III have been found to be natively unfolded and largely disordered, capable of adopting different transient conformations (492, 493, 505, 533).

In a previous study, we have shown that the viral NS5A protein functions to down-regulate viral translation through a mechanism that requires the poly-U/UC region of the 3′UTR, where the protein has been shown to bind (chapter 3). We have also shown that domain I is able to modulate viral translation as effectively as the full NS5A protein. Given the role of domain I in modulating viral replication and NS5A dimerization, we further investigated the ability of domain I to modulate viral translation. Using a series of deletion and substitution mutants, we identified a number of positively charged residues that play a role in this modulatory effect, most prominently R112. We found that substitution of either R112 or E148, which form a hydrogen bond in one dimer structure (556), with alanine negates the ability of domain I to modulate viral translation. We also observed that the presence of low complexity sequence I (LCS I) can compensate for the E148A mutation but not the R112A mutation. Additionally we found that this R112A mutation impedes domain I dimer formation and reduces the ability of domain I to bind to poly-U/UC RNA, suggesting a mechanism for this down-regulatory effect. Finally, the R112A mutation renders an HCV subgenomic replicon replication deficient. These results point to a crucial role for the arginine 112 residue in the modulation of the viral lifecycle by NS5A.

5.4 Results

5.4.1 NS5A Domain I down-regulates viral translation in a manner dependent upon the presence of the poly-U/UC region in the viral 3´-UTR

To substantiate our previous findings regarding domain I using a co-transfection method, we utilized a set of monocistronic HCV RNA reporters in combination with plasmids expressing NS5A or NS5A domain I (**Figure 5.1a**). The monocistonic HCV RNA reporters contain the viral 5´- and 3´-UTRs, an internal

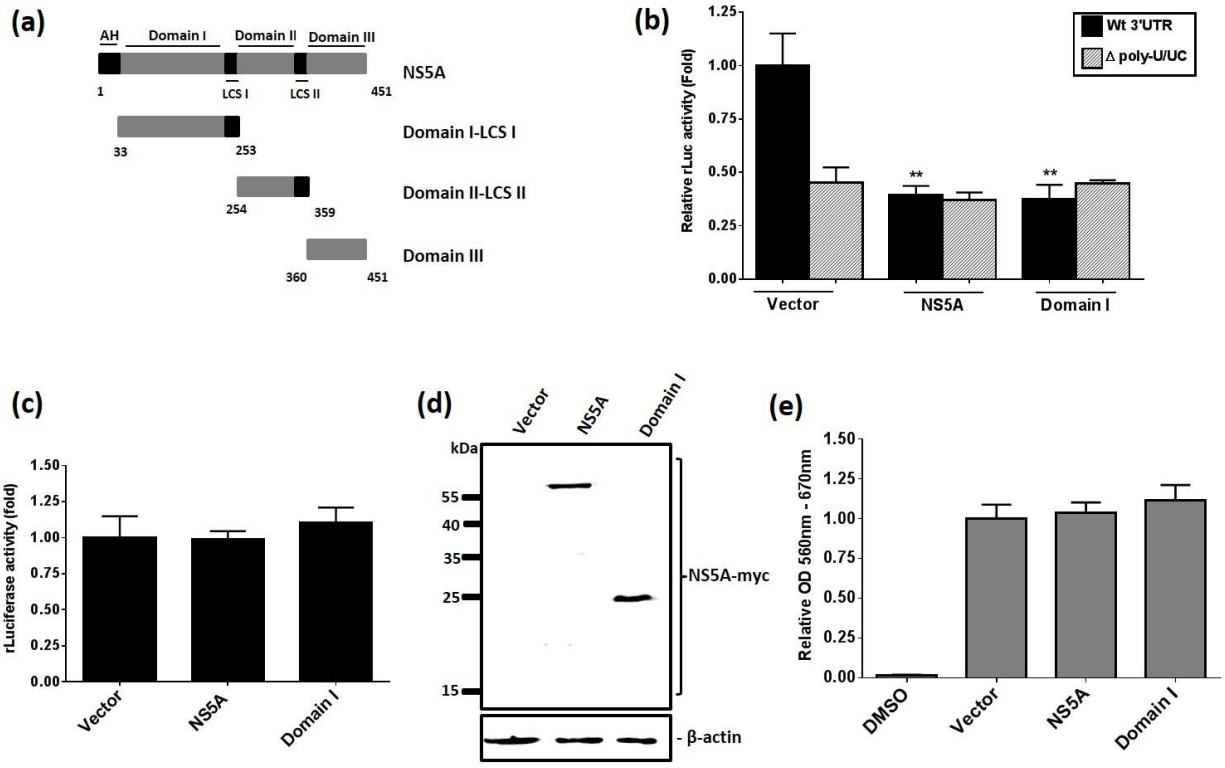

Figure 5.1 - NS5A domain I down-regulates HCV translation though a mechanism dependent upon presence of poly-U/UC region. **(a)** Diagram of NS5A domains with the amphipathic helic (AH), domains and Low complexity sequences labelled. Residues are number according to 1b N NS5A protein. **(b,c)** Huh7 cells were transfected with **(b,c)** plasmid vector or the indicated 1b NS5A expression plasmid and a monocistronic RNA 1b HCV luciferase reporter, containing either a (**b**) wt 3´-UTR, (**b**) Δpoly-U/UC 3´-UTR or **(c)** a capped mRNA Luciferase reporter. Luciferase assay was performed 24 hr after transfection. **(d)** Expression of NS5A and domain I were detected by Western blot using a Myc specific antibody. **(e)** MTT assay for cellular viability 24 hr. after transfection of indicated expression plasmid. DMSO treatment was used as a control. Mean of vector set to 1. Error bars indicate mean ±SD, **=p<0.01 relative to vector.

Renilla luciferase flanked by the first 16 amino acids of the HCV core gene and the last 5 amino acids of the HCV NS5B gene. The viral 5´-UTR contains the viral internal ribosome entry site (IRES), which drives the translation of the internal Renilla luciferase gene, which can be quantified as a measurement of viral translation. We also utilized a monocistronic HCV RNA reporter containing a deletion of the poly-U/UC region within the viral 3´-UTR in order to study the role of this region in modulation of viral translation by NS5A domain I. Monocistronic HCV RNA reporters such as the ones used in this study have been shown to more accurately reflect the key role of the viral 3´-UTR in modulating viral translation as compared to plasmid encoded or bicistronic reporters (521).

When Huh7 cells are co-transfected with the monocistronic HCV RNA reporters and plasmids expressing either wt NS5A or NS5A domain I the levels of HCV IRES mediated translation are significantly reduced compared to vector control (**Figure 5.1b**). This modulatory effect on viral translation again required the poly-U/UC region of the viral 3´-UTR as previously reported. This effect was specific as translation of a capped RNA luciferase reporter was unaffected by either NS5A or domain I expression (**Figure 5.1c**). Expression of the NS5A fragments was confirmed by western blot (**Figure 5.1d**). Expression of NS5A domain I did not result in a change in cellular viability as determined by an MTT assay (**Figure 5.1e**). Additionally, we have previously demonstrated that the down-regulation of viral translation by NS5A is not a result of RNA degradation (chapter 3). These results confirm our previous findings that domain I is able to down-regulate viral translation independently and that this effect requires the poly-U/UC region of the 3′UTR. The mechanism of this down-regulatory effect on viral translation is unclear as is the mechanism behind the stimulatory function of the 3´-UTR on viral translation. One possibility is that through binding to the poly-U/UC region within the 3´-UTR domain I is interfering with the stimulation of viral translation by this region. This could be the result of interfering with such processes as the binding of stimulatory cellular proteins to this region, ribosome recycling or genome circularization.

5.4.2 Amino acids 100-161 of domain I are sufficient for the modulation of viral translation

To determine which region of this domain is involved in modulating viral translation, we generated a series of NS5A domain I deletion mutants (**Figure 5.2a**). The AH and LCS-I regions were also included to determine if these regions had an effect on the modulation of translation by domain I. We utilized four domain I fragments as shown in **Figure 5.2a**: aa. 1-253 (AH-domain I-LCS I), aa. 1-161, aa. 1-99, and aa. 100-161. These truncation expression plasmids were co-transfected along with the

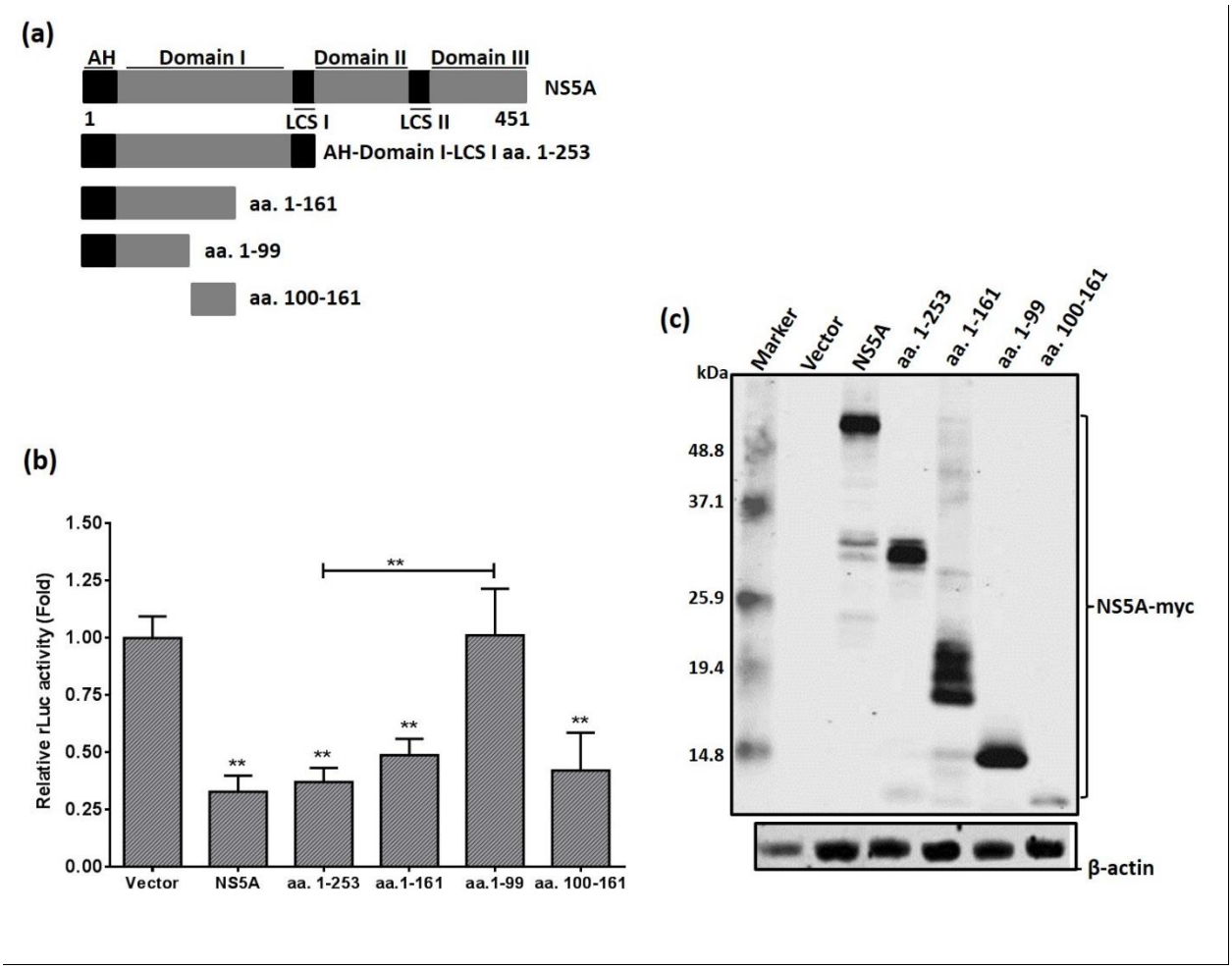

Figure 5.2 - NS5A domain I region aa. 100-161 is sufficient for the down-regulation of viral translation. **(a)** Diagram of domain I deletion fragments used. **(b)** HuH-7 were co-transfected with vector, plasmids expressing myc-tagged NS5A or the domain I deletion mutants indicated, and HCV RNA translation rLuc reporter RNA. Luciferase assay was performed 24 hr after transfection. Statistical differences compared to vector unless otherwise indicated are indicated as ** if $p \leq 0.01$. **(c)** Expression of the NS5A and domain I deletion mutants in (**b**) was demonstrated by Western blotting using a myc-tag specific antibody. As a loading control, the level of β-actin was determined using a β-actin antibody.

monocistronic HCV RNA translation reporter RNA into HuH-7 cells. Plasmid vector and plasmid expressing the full-length NS5A were included as controls. As shown in **Figure 5.2b** expression of the AH-domain I-LCS I (aa. 1-253) was able to down-regulate HCV RNA translation to the same degree as full-length NS5A. While aa.1-161 of domain I retained the ability to down-regulate viral translation, this modulatory function was completely lost by the aa. 1-99 fragment (**Figure 5.2b**). These results indicated the importance of the C-terminal 62 amino acids of the aa. 1-161 fragment in down-regulating translation. To further investigate the significance of this 62-amino acid region, the N-terminal 99 residues of the aa. 1-161 fragment were removed to produce the aa. 100-161 fragment. Indeed, this fragment retained the ability to down-regulate viral translation to similar levels as seen with both the aa. 1-161 and full AH-domain I-LCS I fragments (**Figure 5.2b**). These results indicated that the aa. 100-161 of domain I is sufficient for the modulatory function of domain I on viral translation. The expression of these NS5A mutants was demonstrated by Western blot (**Figure 5.2c**). There were additional protein bands for NS5A and the aa. 1-161 truncation (**Figure 5.2c**), whose identities were not clear, but might result from protein degradation. In the case of the aa.101-162 fragment, the low signal detected was likely due to difficulties in transferring such small proteins rather than representative of low expression as this fragment behaved similar to both NS5A and aa. 1-253 (AH-domain I-LCS I) in translation assay.

5.4.3 Residues K108, R112, K139, R157 and R160 are involved in the down-regulation of viral translation by the NS5A domain I aa. 100-161 region

We previously showed that the down-regulation of viral translation by domain I required the poly-U/UC region of the viral 3´-UTR, where domain I has been shown to bind, suggesting that RNA binding by domain I may be involved. Positively charged amino acids arginine and lysine residues are highly favourable in RNA-protein interactions as they are able to interact with the negatively charged phosphate backbone of RNA (557). To identify potential effector or RNA binding sites within the aa. 100-161 region of domain I, four arginine residues at positions 112, 123, 157 and 160 and two lysine residues at positions 108 and 139 were substituted by alanines (**Figure 5.3a**). In co-transfection experiments using the HCV RNA translation reporter, we found that NS5A aa. 100-161 expression plasmids containing the individual amino acid substitutions K108A, R112A, K139A, R157A and R160A impaired the ability of NS5A aa. 100-161 to down-regulate viral translation (**Figure 5.3b**). The individual amino acid substitutions K108A, K139A, R157A and R160A restored viral translation to 65-70% of the vector control which is a significant increase over the inhibition seen with wild-type domain I aa. 100-161. The R112A substitution had the greatest

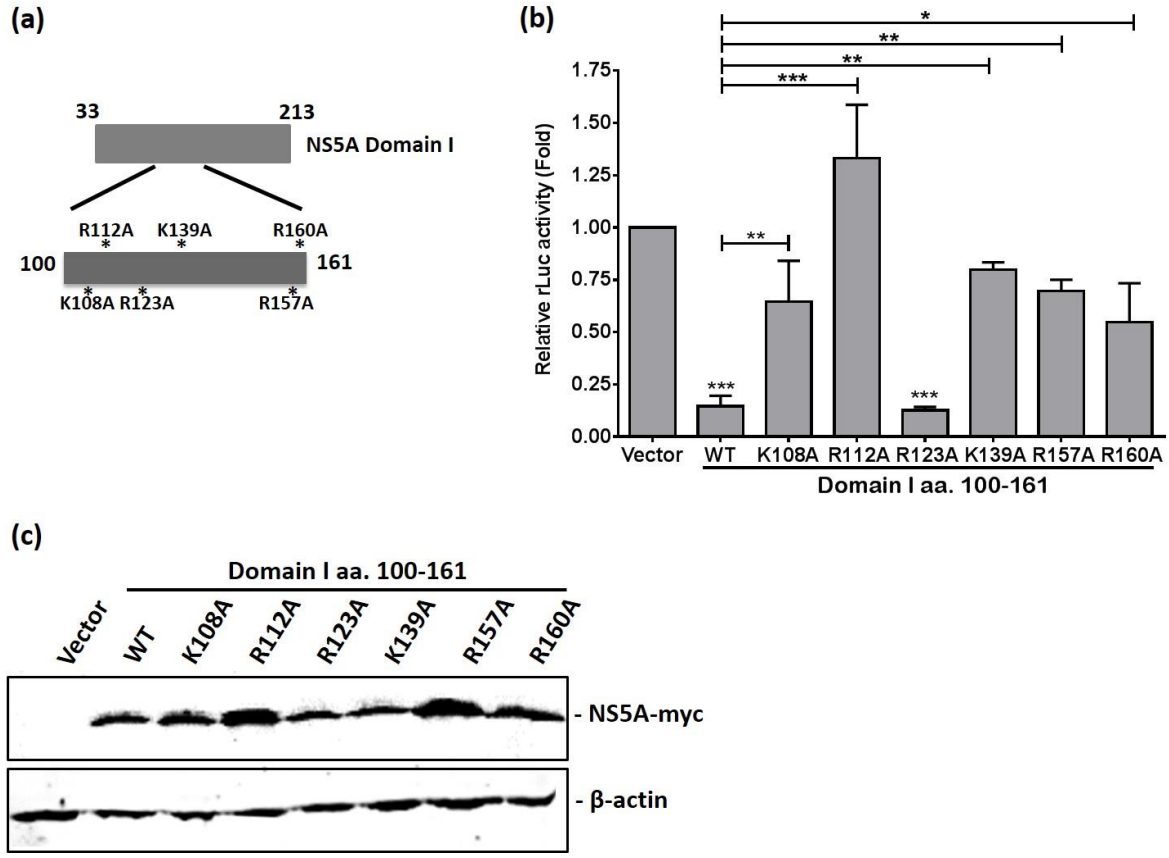

Figure 5.3 - Residues K108, R112, K139, R157 and R160 are involved in the down-regulation of viral translation by NS5A aa. 100-161. **(a)** Diagram of NS5A aa. 100-161 mutations. **(b)** HuH-7 cells were co-transfected with vector, or plasmids expressing myc-tagged NS5A aa. 100-161, wild-type or containing the amino acid substitution indicated, and HCV RNA translation rLuc reporter RNA. Luciferase assay was performed 24 hr after transfection. Statistical differences compared to vector unless otherwise indicated are labelled as * if $p \leq 0.05$, ** if $p \leq 0.01$, or *** if $p \leq 0.001$. **(c)** Expression of the myc-tagged NS5A aa.100-161 proteins in (**b**) was demonstrated by Western blotting using a myc-tag specific antibody. As a loading control, the level of β-actin was determined by a β-actin antibody.

effect on this modulatory function as this mutation completely abolished the down-regulatory effect of the aa. 100-161 region on viral translation. Conversely, the amino acid substitution R123A did not have an effect on the ability of NS5A aa. 100-161 to down-regulate viral translation (**Figure 5.3b**). The expression of the domain I aa.100-161 mutants was confirmed by Western blot (**Figure 5.3c**). There was some variation in the level of expression between the NS5A aa. 100-161 mutants. The reason for the variation was not known. However, it was unlikely to have had an effect on the results obtained in the translation assay. For example, K139A and R157A exhibited different levels of expression, but both demonstrated a significant and similar loss in the ability to down-regulate viral translation compared to Wt. Taken together, these results demonstrated that a number of positively charged residues, particularly R112, play a role in the modulation of viral translation by the aa. 100-161 region of NS5A domain I.

5.4.4 Residues R112 and E148 play a key role in the down-regulation of viral translation by NS5A domain I

In addition to playing a key role in the modulation of viral translation by the NS5A domain I aa.100-161 region, this R112 residue has been directly implicated in the dimerization of domain I. In the Love *et al.* study the authors determined the crystal structure of an NS5A domain I dimer and found that the dimer contact surfaces are formed by residues 92-99, 112-116, 139-143, 146-149 and 160-161 (556). Additionally, R112 was found to form an intermolecular hydrogen bond with glutamic acid 148 (E148) (556). We therefore set out to determine if R112 and E148 are involved in the modulation of viral translation within the context of the NS5A domain I (**Figure 5.4a**). When NS5A domain I R112A and I E148A substitution mutations were used in HCV translation assay, we found that the R112A mutation has a similar inhibitory effect when present within the context of the entire domain I as when expressed within domain I aa.100-161 fragment (**Figures 5.4b and 5.3b**). NS5A domain I E148A mutant was also no longer capable of down-regulating viral translation (**Figure 5.4b**). The expression of the NS5A domain I mutants was confirmed by Western blot (**Figure 5.4c**). These results are indicative of a significant role for both R112 and E148 in the modulatory ability of domain I on viral translation, perhaps through mediating domain I dimerization.

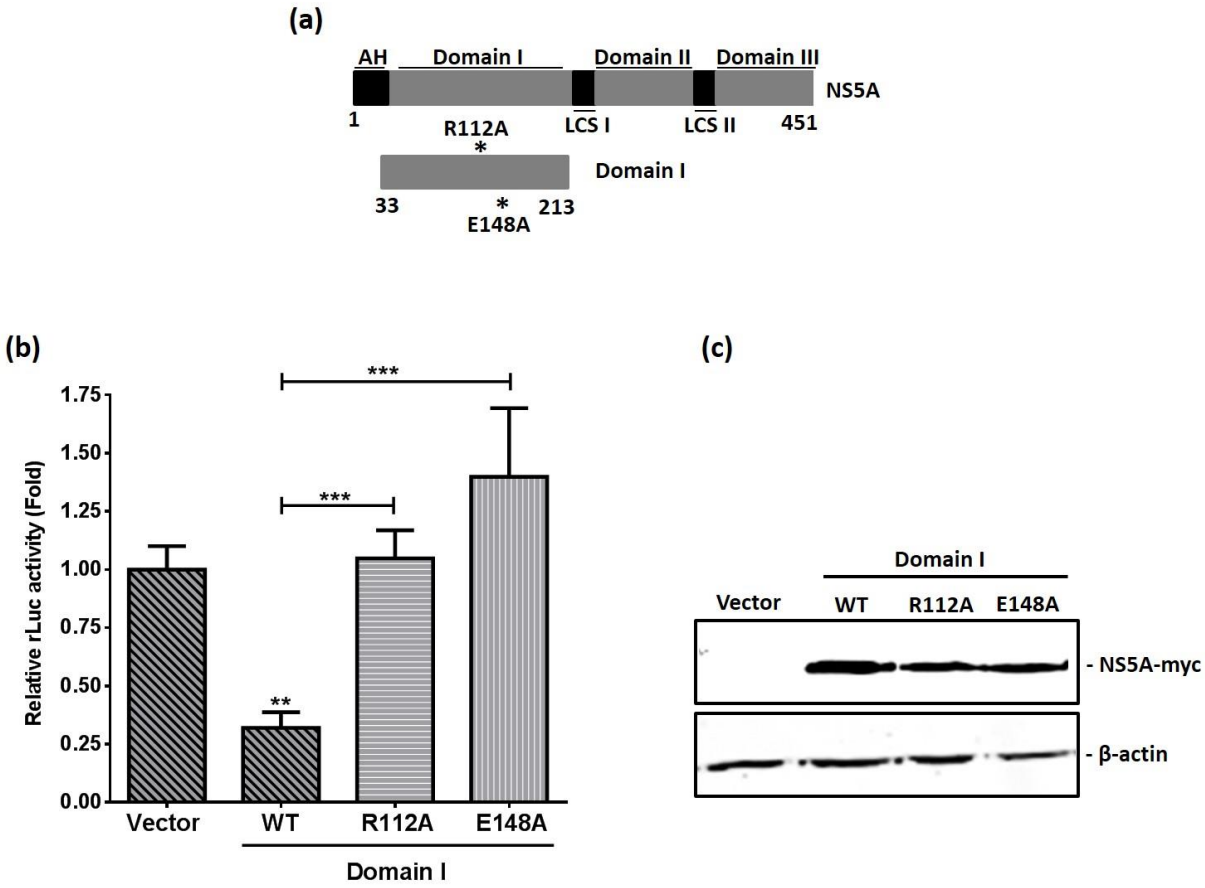

Figure 5.4 - Residues R112 and E148 are involved in the down-regulation of viral translation by NS5A domain I. **(a)** Diagram of NS5A domain I amino acid substitutions used. **(b)** HuH-7 cells were co-transfected with plasmids expressing myc-tagged NS5A domain I, wild-type, R112A, or E148A mutant, and HCV RNA translation rLuc reporter RNA. Luciferase assay was performed 24 hr after transfection. Statistical differences compared to vector unless otherwise indicated are labelled ** if $p \leq 0.01$, *** if $p \leq 0.001$. **(c)** Expression of NS5A domain I proteins in (**b**) was demonstrated by Western blotting using a myc-tag specific antibody. As a loading control, the level of β-actin was determined by a β-actin antibody

5.4.5 Residue C39 is involved in the down-regulation of viral translation by NS5A domain I

To further investigate the effect of dimerization and RNA binding on viral translation modulation by NS5A domain I, we used a C39A mutant, which has been reported to be unable form a dimer or bind RNA (479, 489). When we co-transfected the domain I C39A expression plasmid along with the HCV RNA translation reporter RNA, viral translation was significantly down-regulated compared to vector (**Figure 5.5a**). Interestingly, viral translation after domain I C39A expression was significantly higher than that of wild-type domain I. Expression of the domain I C39A mutant protein was confirmed by Western blot (**Figure 5.5b**). These results suggested that domain I dimerization plays a role in modulating HCV translation.

5.4.6 Mutations R112A and E148A impede dimer formation by domain I

We next investigated the effect of the R112A and E148A mutations on NS5A domain I dimerization in an immunoprecipitation experiment. Cells were co-transfected with Flag-ΔAH NS5A and either ΔAH NS5A-myc or one of the NS5A domain I-myc expression plasmids: domain I, domain I R112A E148A or domain I C39A. Immunoprecipitation was performed on cell lysates with an anti-Flag antibody. If the domain I fragments are able to form dimers with Flag-ΔAH NS5A, they will be co-immunoprecipitated along with Flag-ΔAH NS5A. In this experiment, the C39A mutation acts as a negative control as others have found that this mutation abolishes dimerization (554). The input and Flag-IP samples were analyzed by Western blot using anti-Flag and anti-myc tag antibodies, respectively. As shown in **Figure 5.6**, we found that both myc-tagged ΔAH NS5A and myc-tagged NS5A domain I were co-immunoprecipitated with Flag-tagged ΔAH NS5A. This indicated the formation of NS5A/NS5A and NS5A/NS5A domain I dimers. The R112A E148A double mutation abolished the ability of domain I to interact with the Flag-tagged NS5A because no domain I R112A E148A could be co-immunoprecipitated with Flag-NS5A (**Figure 5.6**). Domain I C39A mutant could not be detected in the immunoprecipitates as expected. These results indicated that the R112A and E148A mutation impedes dimerization by NS5A domain I.

5.4.7 The LCS I region can compensate for E148A, but not R112A, mutation

The LCS I linker region between domains I and II may impact the function of domain I (554, 555). As such we investigated the effect of LCS I on viral translation modulation. As shown in **Figure 5.7a**,

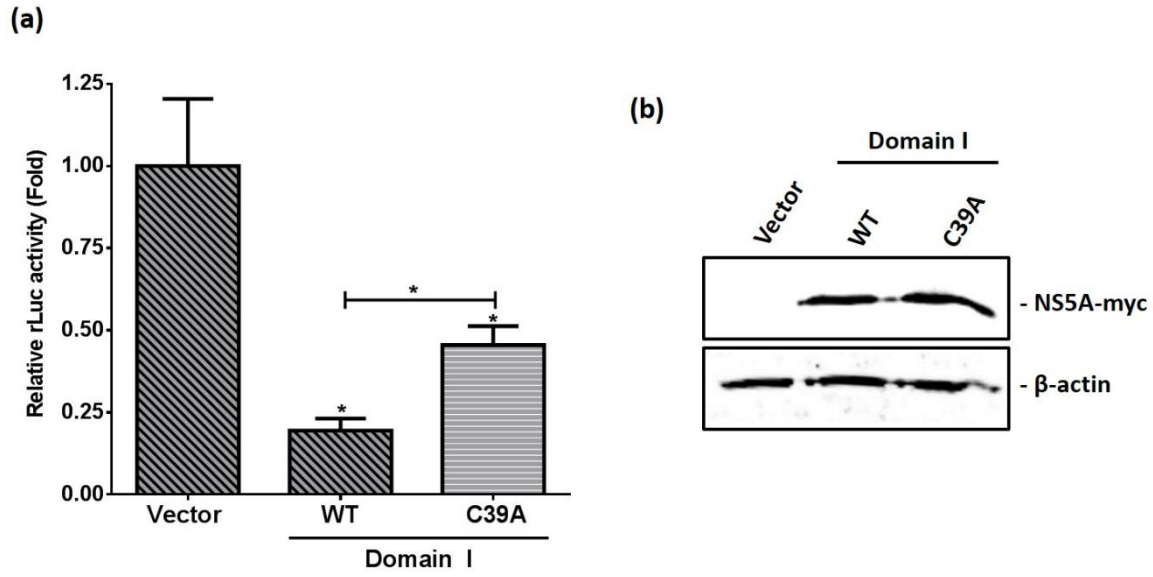

Figure 5.5 - The C39A mutation reduces the ability of NS5A domain I to modulate viral translation. **(a)** HuH-7 cells were co-transfected with vector, plasmids expressing myc-tagged NS5A domain I, wild-type or C39A, and HCV RNA translation rLuc reporter RNA. Luciferase assay was performed 24 hr after transfection. Statistical differences compared to vector unless otherwise indicated are labelled * if $p \leq 0.05$ relative to vector. **(b)** Expression of NS5A domain I proteins in (**a**) was demonstrated by Western blotting using a myc-tag specific antibody. As a loading control, the level of β-actin was determined using a β-actin antibody.

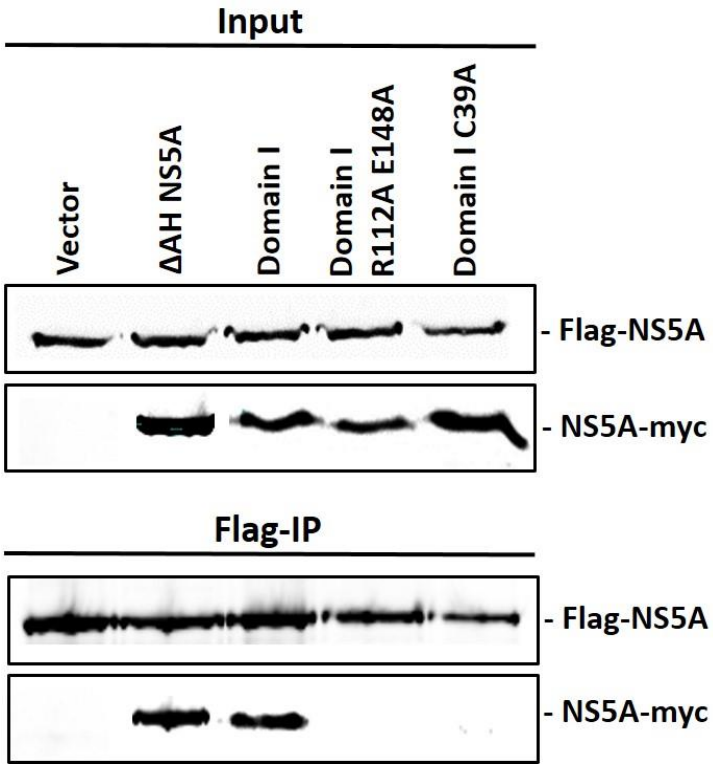

Figure 5.6 - Mutations R112A, E148A, and C39A impede dimer formation by domain I. HuH-7 cells were co-transfected with a Flag-ΔAH NS5A expression plasmid and plasmids expressing myc-ΔAH NS5A, NS5A domain I, NS5A domain I R112A E148A, or NS5A domain I C39A. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody. The presence of myc-tagged NS5A proteins in immunoprecipitates was detected by Western blotting using a myc-tag specific antibody. The input Flag-NS5A protein was demonstrated by Western blotting using a Flag specific antibody. Representative of multiple independent experiments.

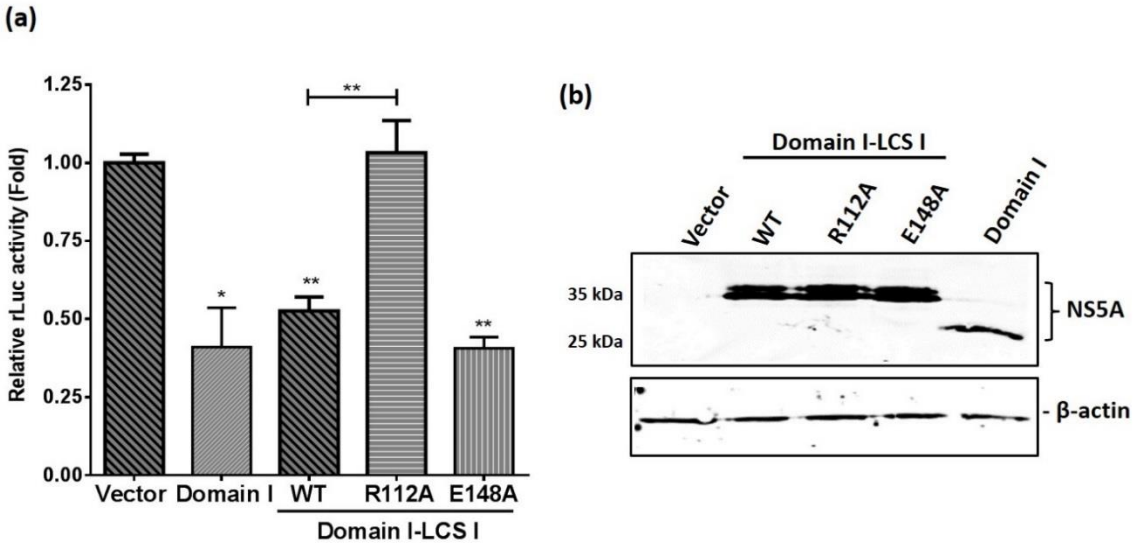

Figure 5.7 - Residue R112 but not E148 is involved in the down-regulation of viral translation by NS5A domain I-LCS I. **(a).** HuH-7 cells were co-transfected with plasmids expressing NS5A domain I, domain I-LCS I, domain I-LCS I R112A or E148A mutants, or vector, and HCV RNA translation rLuc reporter RNA. All NS5A proteins had a myc-tag at the C-termini. At 24 hr after transfection, luciferase assay was performed. Statistical differences compared to vector unless otherwise indicated are labelled * if $p \leq 0.05$, ** if $p \leq 0.01$. **(b).** Expression of the NS5A proteins in (**a**) was demonstrated by Western blot using a myc-tag specific antibody. As a loading control, the level of β-actin was determined using a β-actin antibody.

domain I-LCS I exhibited similar inhibitory effect on viral translation as domain I, suggesting that LCS I is not involved in regulating HCV translation. We also studied the effects of the R112A and E148A mutations on the ability of a domain I-LCS I fragment to modulate viral translation. We found that the R112A mutation negated the ability of the domain I-LCS fragment to down-regulate viral translation to a similar extent as when the LCS I was absent (**Figure 5.7a**). In contrast, the E148A mutation had no effect on the ability of domain I-LCS I to modulate viral translation (**Figure 5.7a**). This E148A mutation abolished this function in a domain I fragment without the LCS-I region (**Figure 5.4b**), suggesting that the LCS I region can compensate for the effect the E148A mutation has on domain I. The expression of the NS5A domain I-LCS I proteins was confirmed by Western blotting (**Figure 5.7b**). The doublets observed in the three domain I-LCS I fragments were most likely due to phosphorylation because a few serine residues in LCS I have been shown to be modified by phosphorylation (361).

5.4.8 The R112A mutation impairs the RNA binding ability of NS5A domain I

We previously showed that the poly-U/UC region is required for viral translation modulation by domain I, suggesting domain I binding to the poly-U/UC region is involved. Mutations of numerous positively charged residues impaired translation modulation by this domain (**Figure 5.3b**). Furthermore, residues implicated in domain I dimerization, which has been linked to RNA binding ability, were also implicated in this modulatory function (**Figure 5.4b**). To investigate whether there is a correlation between translation modulation and RNA binding, we studied RNA binding activity of R112A mutant protein in electrophoretic mobility shift assays (EMSA). Cy5 labelled poly-U/UC RNA was utilized in combination with recombinant ΔAH NS5A domain I or the R112A mutant expressed and purified from *E.coli*. The ΔAH NS5A domain I protein led to an upward shift in labelled RNA at the 6 and 20 μM protein concentrations, indicating the ability of this domain to bind to the poly-U/UC RNA (**Figure 5.8**). However, when the R112A mutant was utilized, this alteration of RNA mobility was not observed (**Figure 5.8**). These results suggested that the R112A mutation impairs the ability of NS5A domain I to interact with the poly-U/UC RNA in the viral 3'UTR. These findings support a mechanism through which domain I down-regulates translation by way of a direct interaction with the viral 3'UTR.

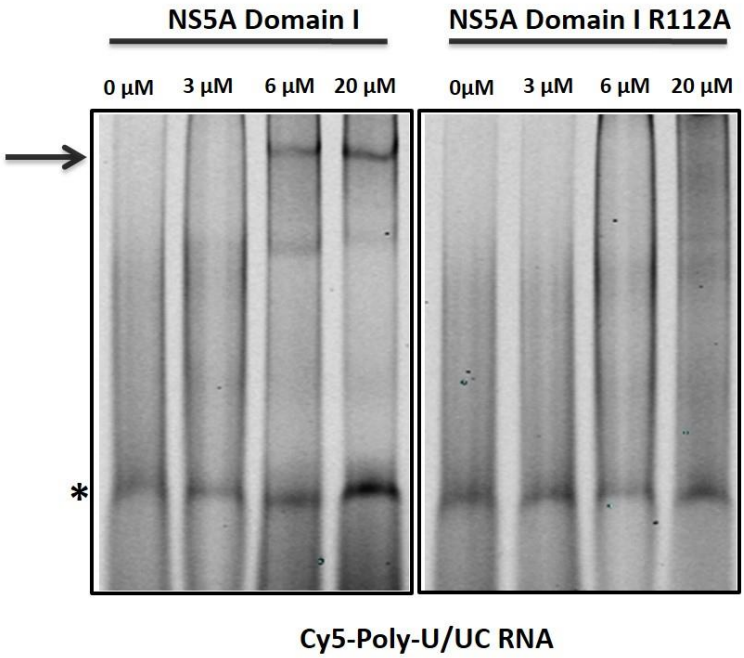

Figure 5.8 - NS5A domain I R112A does not bind to poly-U/UC RNA. The indicated amounts of purified NS5A domain I or NS5A domain I R112A proteins were incubated with 1 pmol of Cy5-poly-U/UC RNA. The binding reactions were loaded into a 8% polyacrylamide gel. The RNA/potein bands were visualized using the Odyssey scanner. Asterix indicates unbound RNA and arrow indicates NS5A/RNA complexes. Representative of multiple independent experiments.

5.4.9 The R112A mutation is lethal for HCV replication

Since NS5A is essential for viral replication and a correlation between NS5A RNA binding and viral replication has been previously identified, we investigated the effect of the R112A mutation on viral replication. To this end, the R112A mutation was introduced into an HCV subgenomic replicon (**Figure 5.9a**). A SgR reporter containing a deletion of the GDD motif within the NS5B polymerase was utilized as a negative control as this deletion renders the SgR replication deficient. As another control, a SgR containing the C39A mutation within NS5A domain I was created as this mutation abolishes the replicative capacity of HCV (554). These SgR reporters were electroporated into HuH-7.5 cells and replicative capacity was determined by luciferase assays at 4, 24, 48, 72 and 96 hr post electroporation. The luciferase activity was normalized against the 4 hr time-point to account for any differences in transfection efficiency. As shown in **Figure 5.9b**, all of the SgR reporters exhibited a similar level of luciferase activity between 24 and 48 hr post-electroporation. A stabilization in the luciferase activity of the wild type HCV SgR was observed from the 48 hr time-point on which was indicative of viral replication. However, the luciferase activity of the R112A mutant continued to decrease at all time-points beyond the 48 hr time-point to a similar extent as the replication deficient C39A and ΔGDD SgR reporters. These results indicated that the R112A mutation renders the SgR replication deficient.

5.5 Discussion

In this study, we further characterized the effect of HCV NS5A domain I on viral translation. Through the use of a series of deletion mutants, we showed that the aa. 100-161 region within this domain was sufficient for down-regulating viral translation (**Figure 5.2**). Furthermore, this function was found to dependent upon a number of positively charged residues within this region (**Figure 5.3**). Of these residues, mutation of the highly conserved R112 had the greatest effect on this down-modulatory function, completely nullifying this activity (**Figure 5.3**). The aa. 100-161 region identified here contained the majority of the domain I dimer contact interfaces identified in both the Love *et al.* dimer and Lambert *et al.* AB dimer (481, 482). The Love *et al.* dimer contains an intramolecular hydrogen bond between R112 and E148 (556). We found that when either R112 or E148 were replaced with alanines within the context of a full domain I fragment, the ability of domain I to down-regulate viral translation was completely lost (**Figure 5.4**). These results suggest that the intramolecular hydrogen bond formed by R112 and E148 is important for down-regulating viral translation.

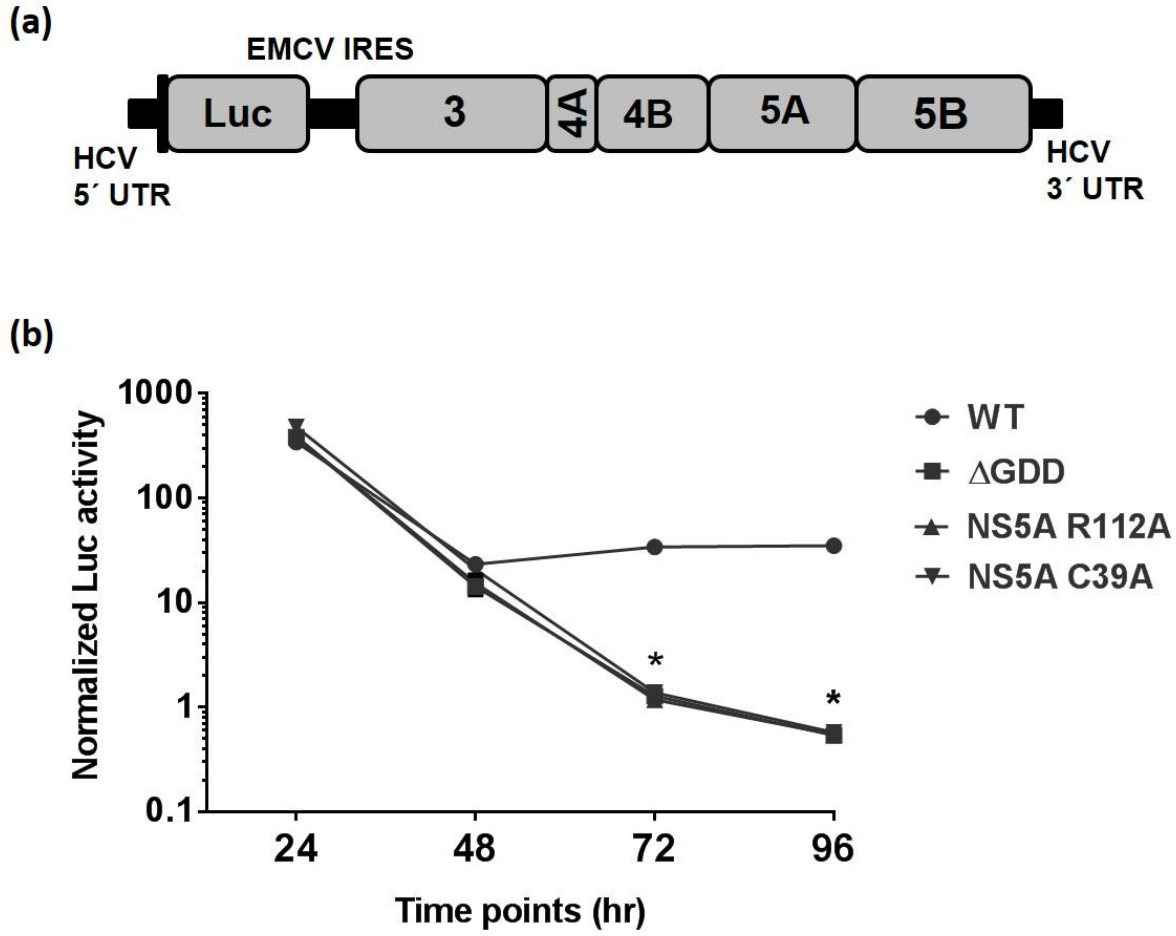

Figure 5.9 - The R112A mutation within NS5A Domain I renders HCV subgenomic replicon replication deficient. **(a).** Diagram of the HCV subgenomic replicon (SgR) with a luciferase reporter. **(b).** HuH-7.5 cells were electroporated with the indicated HCV SgR RNAs. Cells were harvested at the indicated time points and luciferase assay performed. Values were normalized to 4 hr time point and protein concentration. * indicates statistical difference of $p \leq 0.001$ of the three mutant replicons compared to wildtype. Error bars in panel (b) are not visible at this scale.

Based on our findings and this crystal structure, we investigated the effect of the R112A and E148A mutations on the ability of domain I to dimerize. Our results indicated that the R112A and E148A mutations impede the formation of NS5A domain I dimers (**Figure 5.6**). These results suggest that domain I dimerization may have an impact on the ability of this domain to modulate viral translation. Interestingly, another domain I mutant, C39A, which can disrupt domain I dimerization, as shown by the Gallay group (554) and by our own data (**Figure 5.6**), did not completely impede the ability to modulate viral translation by domain I (**Figure 5.5**). C39, along with other three cysteine residues, was proposed to promote NS5A dimerization through disulfide bonds (554). While these results support a role of dimerization in modulating viral translation by NS5A domain I, the differences in the degree of translation down-regulation conferred by C39A and R112A or E148A mutations suggest that the R112A and E148A mutations may interrupt other mechanisms required for viral translation in addition to dimerization.

We next studied the role of LCS I in viral protein modulation by domain I. Our results indicated that LCS I did not have an effect on this function (**Figure 5.7**). However, we obtained interesting data when investigating the R112A and E148A mutations in a domain I fragment containing the LCS I region (**Figure 5.7**). The domain I-LCS I fragment containing the E148A mutation was capable of modulating viral translation to a similar extent as observed for the wild type domain I. The mechanism behind this apparent compensatory function of the LCS I is not clear. The LCS I has been observed by the Cameron group to enhance domain I dimerization (555). Therefore, it is possible that the LCS I enhances domain I dimerization even in the presence of the E148A mutation. Unfortunately, none of the domain I fragments used in crystallization studies completed thus far contained the LCS-I region, so it is unclear how this region could affect the proposed interaction between R112/E148. Alternatively, we have not eliminated the possibility that the R112A and E148A mutations lead to significant structural alterations within domain I. As such, the LCS I region may support the retention of functional structure in a manner that compensates for the effects of E148A mutation. Finally, LCS I is modified by phosphorylation when expressed in mammalian cells (361). It is possible that phosphorylation of LCS I can compensate for the original function of E148 due to its charge which is lost in the E148A mutation.

In an effort to determine the mechanism of the down-regulation of viral translation by domain I, we investigated the effect of the R112A mutation on the ability of domain I to bind to poly-U/UC RNA. Using recombinant domain I and domain I R112A proteins in combination with Cy5-poly-U/UC RNA, we found that the R112A mutation results in a loss of RNA binding ability for domain I (**Figure 5.8**). These findings point to a possible mechanism through which domain I down-regulates viral translation. As the

mechanisms behind the stimulatory effect of the 3′-UTR on viral translation remain inconclusive, it is unclear how NS5A domain I binding within this region interferes with this function. However, this interaction could impede genome circularization, ribosome recycling or the binding of proteins, which are proposed as potential mechanisms to stimulate viral translation by the 3′-UTR (19, 218).

Finally, we found that the R112A mutation was lethal to HCV replication (**Figure 5.9**). This mutation behaved in a similar fashion to the C39A mutation identified by the Gallay group, which identified a link between domain I dimerization, RNA binding and viral replication (554). Therefore our study reaffirms this link but through mutation to R112 rather than mutations within the zinc binding motif in the N-terminal region of domain I.

In summary, we found that the mutation of a single residue within domain I, R112, resulted in the loss of the modulatory function on viral translation exhibited by domain I. Furthermore, this mutation lead to the disruption of domain I dimerization and RNA binding and was lethal to HCV replication. These findings lend support for the previously observed correlation between NS5A dimerization, RNA binding and viral replication (554). NS5A is proving to be an extremely complex protein with a seemingly endless array of functions attributed to it despite the lack of enzymatic activity. Understanding the mechanisms behind such activities will facilitate the development of direct acting antivirals with increasing efficacy.

5.6 Materials and Methods

5.6.1 Plasmid construction and RNA synthesis

The monocistronic HCV RNA translation reporter plasmid T7 HCV 5′-UTR-Core$^{aa1\text{-}16}$-rLuc-NS5B^{5}-3′-UTR of genotype 1b was described previously (chapter 3). This reporter contains T7 promoter, HCV 5′-UTR, sequence encoding the first 16 amino acids of the core protein, an internal *Renilla* luciferase (rLuc) gene, sequence encoding the last five amino acids of the NS5B protein and the 3′-UTR. The 5′-UTR contains the viral IRES which drives the expression of the internal *Renilla* luciferase gene. In other two constructs, the poly-U/UC region in the 3′-UTR or the entire 3′-UTR with the coding sequence for the last five amino acids of the NS5B were cloned downstream of the T7 promoter sequence. HCV subgenomic replicon (SgR) is composed of the HCV 5′-UTR, which directs translation of a luciferase reporter gene, followed by the IRES of the encephalomyocarditis virus (EMCV), which directs translation of the HCV replicase genes NS3-NS5B, and the 3′-UTR (Fig. 8a) (558). The SgR reporter allows for transient replication assay as the amount of luciferase produced can be measured as an indicator of viral replication (559). As a negative control, the GDD sequence within the NS5B viral polymerase was deleted which rendered

replication deficient. Plasmids expressing HCV NS5A with or without the amphipathic α-helix (AH) were reported previously (BH, submitted for publication). Coding sequences for NS5A domain I with or without LCS I were amplified by PCR using HCV-1b N Neo C-5B (540) as template and cloned into pEF/cyto/myc vector (Invitrogen/Life Technologies). NS5A domain I truncations were constructed by a PCR-based approach. Amino acid substitution mutants in the expression plasmid or SgR were generated by site-directed mutagenesis. In additional constructs, a Flag or a myc tag was added to the N- or C-termini of various NS5A expressing constructs, respectively. All plasmids were confirmed by DNA sequencing. HCV RNA translation reporter and SgR RNAs were produced from linearized plasmids by *in vitro* transcription using the MEGAscript T7 *In Vitro* Transcription kit (Ambion).

5.6.2 Cell lines, transfections, HCV transient replication and luciferase assay

Human hepatoma cell lines HuH-7 and HuH-7.5 were kindly provided by Bartenschlager (315) and Rice (543) , respectively. HuH-7 and HuH-7.5 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen/Life Technologies) supplemented with 10% fetal bovine serum and 1% gentamicin and cultured at 37°C and 5% CO_2. In co-transfection experiments, HuH-7 cells in 24-well plates were co-transfected with 1 µg or the indicated amount of expression plasmid and 1 µg of the indicated RNA reporter using Jet-PEI transfection reagent according to the manufacturer's protocol (Polyplus-Transfection). Luciferase assay was performed at 24 hr after transfection. For these luciferase experiments vector control was set to 1 (100%) and error bars represent mean ± SD. In SgR experiments, HuH-7.5 cells were electroporated (270 V, 950 µF, 4 mm cuvette) with the indicated amounts of RNA using an electroporator (Bio-Rad) before they were harvested at the indicated time points. Following incubation, the cells were lysed with Passive Lysis Buffer and luciferase assay was performed according to the manufacturer's instructions (Promega). Luciferase data in these electroporation experiments was normalized to protein concentration which was determined with a BCA protein assay kit (Thermo Scientific). All luciferase experiments are the average of three independent experiments performed in triplicate.

5.6.3 SDS-PAGE and Western blot

SDS-PAGE and Western blots were performed as previously described (560). Briefly, cells were lysed in SDS lysis buffer (1% SDS, 10 mM Tris-HCl, pH 8.0). After SDS-PAGE, proteins were transferred onto nitrocellulose membranes. The membranes were then blocked in 5% skimmed milk in PBS for 1 hr at room temperature before incubation with a primary antibody overnight at 4°C. Blots were then washed with PBS and incubated with the appropriate secondary antibodies (Li-Cor Biosciences) for 1 hr at room temperature. Blots were then washed with PBST (0.1% Tween-20) before a final wash with PBS. Blots were scanned using an Odyssey scanner at the appropriate wavelengths (Li-Cor Biosciences). A monoclonal anti-NS5A antibody was purchased from Virogen and a polyclonal NS5A antibody was kindly provided by Dr. Cameron (561). The myc tag and β-actin antibodies were purchased from Cell Signaling Technology. Western blots shown are representative of multiple independent experiments.

5.6.4 Immunoprecipitation

HuH-7 cells were co-transfected with a Flag-ΔAH NS5A expression plasmid or vector, together with plasmids expressing myc-tagged ΔAH NS5A, domain I, domain I with R112A and E148A mutations, or domain I with C39A mutation. Cells were then incubated at 37°C for 24 hr. Cell lysates were subjected to immunoprecipitation with an anti-Flag antibody (Sigma-Aldrich). The presence of myc-tagged NS5A proteins in the immunoprecipitates was analyzed in Western blotting using a myc-tag specific antibody. Results representative of multiple independent experiments.

5.6.5 Recombinant NS5A domain I protein expression and purification from E. coli

Poly-histidine tagged NS5A domain I proteins, wild-type or R112A mutant, were expressed in *E. coli* with the Expresso T7 SUMO Cloning and Expression system (Lucigen). NS5A domain I proteins were then purified through affinity chromatography under native conditions with the Ni-NTA purification system according to the manufacturer's instructions (Qiagen). Expression and purification of His_6-tagged NS5A domain I proteins were confirmed by both Coomassie blue staining and Western blotting. Purified

proteins were aliquoted and stored at -80˚C. Blue fluorescent protein (BFP) was produced in a similar fashion to be used as a control in RNA binding experiments.

5.6.6 RNA binding electrophoretic mobility shift assays (EMSA) assays

Cy5 labeled poly-U/UC RNA was produced using the MEGAscript T7 *In Vitro* Transcription reagent (Ambion) with Cy5 CTP (GE Healthcare). Prior to binding, the Cy5-poly-U/UC RNA (in 50 mM Tris and 100 mM NaCl) was denatured at 96 °C for 1 min and then placed in boiling water and allowed to cool to room tempearture. The indicated amounts of purified NS5A domain I or NS5A domain I R112A proteins were incubated with 1 pmol of Cy5-poly-U/UC RNA in a binding buffer (20 mM HEPES, 5 mM $MgCl_2$, 5 mM DTT) and 4% glycerol for 5 min at 25 °C. The binding reactions were then loaded into an 8% non-denaturing polyacrylamide gel. The RNA/protein bands were visualized using a Odyssey scanner (Li-Cor Biosciences). Results representative of multiple independent experiments.

5.6.7 MTT assay

See methods described in section 3.6.4

5.6.8 Statistical analysis

All the experiments represent three independent experiments performed in triplicates. The experimental data were analyzed by Student's *t* test. A *p* value of ≤ 0.05 was considered statistically significant. Results reported as Mean ± SD.

5.7 Acknowledgments

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6.0 LINKER BETWEEN CHAPTERS 5 AND 7

Given the success of the strategy followed in the previous chapter at identifying individual residues involved in the modulation of translation by NS5A domain I we chose to extend this to domains II and III. Therefore, we utilized a similar approach as the previous chapter using a combination of deletion and amino acid substitution mutants. Despite being less characterized than domain I residues within these domains have been found to be essential in both replication and assembly.

7.0 MAPPING THE RESIDUES INVOLVED IN THE MODULATION OF VIRAL TRANSLATION BY NS5A DOMAINS II AND III

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Keywords: hepatitis C virus (HCV); HCV NS5A domain III; HCV RNA translation modulation; poly-U/UC sequence

Running title: Modulation of HCV translation by NS5A domains II and III

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7.1 Authors' contribution

All the experiments within this chapter were performed by Brett Hoffman. The Manuscript was written by Brett Hoffman and edited by Qiang Liu.

7.2 Abstract

NS5A is a multifunctional protein that plays a critical role in the HCV lifecycle. Although NS5A has essential roles in viral replication and virus production, its potential role in modulating viral translation has remained questionable. We have previously demonstrated that NS5A down-regulates viral translation through a mechanism requiring the poly-U/UC region of the viral 3´-UTR and that each of NS5As three domains are capable of this function individually. For domain I, we determined that one residue in particular, R112, plays a key role in modulating viral translation and is also involved in the dimerization and RNA binding ability. Additionally, the R112A mutation also proved lethal in an HCV sub-genomic replicon. In this study, we set out to map the regions and amino acids within domain II and domain 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. When these residues are substituted with alanine in full length domain II and domain III fragments they result in a complete loss of this downregulatory affect. Mutation of K312 again leads to a loss of this modulatory effect in a domain II-LCS II-domain III fragment, whereas mutation of residues E446 and S447 in domain III are required for this loss of function.

7.3 Introduction

Hepatitis C virus (HCV), discovered in 1989 as the agent responsible for infectious non-A, non-B hepatitis, currently infects approximately 130-150 million people worldwide, representing approximately 2% of the world's population (1, 2, 562). Up to 75% of those infected develop a chronic infection (31). Many of those infected can remain unaware for decades of their status, as the initial infection tends to be asymptomatic (31). Clinical manifestations of chronic HCV infection include liver steatosis cirrhosis and hepatocellular carcinoma (13, 31). The standard of care for many years consisted of a treatment regimen that included ribavirin and pegylated-interferon, treatment which produced multiple adverse side effects that hindered treatment uptake and adherence (62) . While effective in up to 80% of those infected with

genotype 2 or 3 HCV, this treatment was only effective at best in 50% of those infected with genotype 1, the most common genotype in North America (62). However, tremendous progress has been made in the development of direct acting antivirals (62, 68). Recently, the field has seen the development and regulatory approval of numerous direct acting antivirals such as viral protease and polymerase inhibitors (62, 68). New treatment regimens which combine direct acting antivirals targeting different aspects of the viral lifecycle are effective in up to 90-99% of genotype 1 patients in clinical trials (62, 70, 74). Although major advancements in treating HCV have significantly improved treatment outcome, a vaccine that offers protection against infection has yet to be developed. Additionally, many aspects of the viral lifecycle remain unclear, particularly the role that NS5A, an essential viral protein and attractive drug target, plays in the various aspects of viral infection.

HCV is an enveloped, single stranded positive sense RNA virus and classified within the Flaviviridae family as the only member of genus Hepacivirus (4). The viral RNA contains a single ORF flanked by two highly conserved untranslated regions (UTRs) (15). 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 (19). The 3´-UTR serves as the initiation site for negative stand synthesis during genome replication (19). It is composed of three regions; the variable region, a long poly-U/UC tract and an x-tail region which consists of three stem-loops (19). The 3´-UTR also functions to stimulate viral translation through an unknown mechanism which may involve long range RNA-RNA interactions with the 5´-UTR, binding of cellular proteins or the retention and recycling of ribosomes during subsequent rounds of translation (19, 218). The viral polyprotein is cleaved co- and post translationally by both cellular and viral proteases into at least ten viral proteins (19). This includes three structural proteins (core, E1, E2) and seven non-structural proteins (p7, NS2, NS3, NS4A/B, NS5A and NS5B) (19). Following viral polyprotein processing, genome replication occurs in replication complexes found at an ER-derived membranous web (4). HCV virions are then assembled in close proximity to ER-associated lipid droplets and are exported through the secretory pathway in association with lipoproteins (11).

Numerous cellular and viral proteins have been implicated in the modulation of viral translation but this process remains unclear (15, 19). Particularly unclear is the role that the essential viral protein NS5A may play in this process (19). NS5A, which lacks any enzymatic activity, is a multifunctional phosphoprotein essential for viral replication and assembly, although through unknown mechanisms. NS5A exists within infected cells as basally phosphorylated and hyperphosphorylated forms, with apparent molecular weights of 56 kDa and 58 kDa, respectively. (361). The sites of phosphorylation and kinases responsible remain ambiguous, but cellular kinases PI4KIIIα and CKII have been implicated in

hyper- and basal phosphorylation respectively (453, 478). Phosphorylation has been implicated in regulating various functions of NS5A, possibly though modulating the conformation of NS5A and protein interactions (361, 362). NS5A has been found to interact with numerous viral and host proteins and is an essential component of the viral replication complex. A recent study identified 132 host proteins with which NS5A is proposed to interact (27). NS5A preferentially interacts with proteins central to cell signalling networks, suggesting that NS5A plays a key role in modulating the cellular environment in favour of viral pathogenesis (27). The pathways within the NS5A interactome include cell growth/death, cellular signalling, cell adhesion and transport and the immune response (27).

NS5A is made up of an N-terminal amphipathic helix followed by three domains separated by regions consisting of low complexity sequences (LCS) (480). The amphipathic helix is essential for viral replication and mediates NS5A localization to the replication complex at ER derived membranes (460, 479). The three domains of NS5A have been implicated in numerous functions in the HCV lifecycle and each possess RNA binding ability towards the poly-U/UC tract within the viral 3´-UTR, although with different affinities (363, 364). Domain III binds to this region weakly when compared to domain I and II (363). NS5A has also been observed to bind to sites within the IRES found within the 5´-UTR (364). As NS5A interacts with both ends of the genome, it may play a role in modulating viral translation and the switch from translation to replication which must occur during the lifecycle of a positive sense RNA virus.

Domain I (aa. 33-213) is highly structured, possesses an essential zinc-binding motif and is proposed to mediate NS5A dimerization within infected cells (479). Currently, domain I is the only domain of NS5A which has been crystalized. However, four different domain I structures have been crystalized, all as dimers with differing structures (479, 482, 563). The physiological relevance of these different dimers is unclear. One possibility is that they serve different functions during the viral lifecycle and that their formation is regulated though phosphorylation, protein interactions or by binding the viral RNA (361, 563). Another possibility is that different domain I dimers with different dimer interfaces form an extended multimeric network during infection (482, 563). Based on the selection for resistance mutations, models of NS5A structure and the symmetrical nature of some of the most potent NS5A inhibitors, it is likely that these compounds interact with domain I dimer interfaces (483-486). Furthermore, a correlation between NS5A dimerization, RNA binding and viral replication has been observed, supporting the biological relevance of domain I dimerization (489).

While Domain I has been has been fairly well characterized, much less is known about NS5A domains II and III. Domain II (aa. 254-346), like domain I, is essential for viral replication (418, 454, 490). However, large regions of this domain are dispensable. 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 (418, 454, 490). No crystal structure has been obtained for domain II. It appears to be largely disordered and natively unfolded (491, 492). Despite this disordered state, domain II is capable of adopting different transient conformations which may play a role in modulating domain II function and protein interactions (492, 493). The overall unstructured nature of domain II may provide a larger accessible surface for molecular interactions and the flexibility to bind to many different targets without sacrificing specificity compared to folded proteins (491). Accordingly, domain II has been found to interact with numerous cellular proteins including PKR, PI3K and cyclophilin A and B (458, 494-496). The interaction between domain II and Cyclophilin A (CypA), a protein which possesses peptidyl-prolyl isomerase activity, is essential for viral replication (497, 498) . As such, a number of CypA inhibitors are currently in clinical trials as treatment against HCV infection. The mechanism of action of these inhibitors remains to be clearly defined, but the isomerase activity of CypA is essential for its role in modulating viral replication (498, 499). The resulting cis-trans peptide bond interconversion modulated by this isomerase activity is speculated to cause a structural shift in domain II towards a more extended form (500, 501). This structural alteration is mimicked by CypA inhibitor-resistance mutations which therefore reduce the dependence upon CypA to induce such modifications (500, 501). How this structural shift within NS5A domain II modulates HCV replication remains unclear. It could potentially involve modulating protein interactions and/or the RNA binding ability of domain II (84).

Domain III (aa. 360-451) is similar to domain II in that no crystal structure for this domain has been resolved. It also appears to lack significant secondary structure and to be natively unfolded (479). However, domain III, much like domain II, has been proposed to possess regions of transient secondary structure (505). Domain III exhibits a tendency to form intrinsic α-helices at both its N- and C-terminal regions (505). Such transient structures may play a role in the interaction of NS5A domain III with viral or host factors (505). Domain III appears to be dispensable for viral replication in that deletion of this region is tolerated in viral replicons (419). However, this region may have a role in establishing early replication efficacy in that deletion of this domain leads to delayed replication kinetics in the JFH-1 cell culture system (419). On the other hand, this region plays a critical role in viral assembly (452). The C-terminal region of domain III has been implicated in the localization of NS5A to lipid droplets (LDs) and the efficacy of virus production correlates with the levels of NS5A-core interaction on the surface of LDs (418-420, 452). In particular, a cluster of serine residues within the domain III C-terminal region have been found to play a key role in modulating this interaction and viral assembly (419, 420, 452). Mutation of these residues to alanine impairs NS5A basal phosphorylation and results in the loss of NS5A: Core co-localization on the

surface of LDs, resulting in an inhibition of virion production (419, 452). These mutations also inhibited the interaction of core with viral RNA, supporting a role for NS5A in delivering the viral RNA to the surface of LD's during the assembly process (452). However, these interactions and virus assembly are restored upon mutation of these serines to glutamic acid, which mimics a phosphorylated state (452). These finding suggesting a key role for the phosphorylation in modulating viral assembly (452).

We have previously found that NS5A down-regulates viral translation through a mechanism requiring the poly-U/UC region of the 3´-UTR to which we and others have shown NS5A binds (363, 365). Furthermore, we determined that each of the three NS5A domains is capable of this modulation independently. Upon investigation into domain I, we found that a number of positively charged residues, most notably arginine 122 (R112), play key roles in this effect. Upon mutation of R112 or glutamic acid 148 (E148), with which R112 forms a hydrogen bond within a domain I dimer crystal structure, the ability of domain I to modulate viral translation is abrogated. Furthermore, we found that this R112A mutation appears to impede both the dimerization and RNA binding ability of NS5A domain I. Adding to these results it was found that this R112A mutation was lethal when introduced into a 1b HCV subgenomic replicon (SgR). These results suggested a key role for NS5A domain I R112 in potentially modulating viral translation and also to an essential role in viral replication, perhaps by modulating a switch between the two processes. In this current study we have taken a similar approach to determine the regions and residues of domains II and III of NS5A that are involved in the modulation of viral translation by these domains. We investigated this first through use of a series of deletion mutants to identify effector regions. Amino acid substitutions were then produced within these regions in an effort to map the effect of individual residues. Amino acid substitutions that were found to participate in modulating viral translation were then cloned back into the full domains to determine their importance in the modulation of viral translation by domains II and III. Finally the implicated residues were mutated within a HCV subgenomic replicon to determine if viral replication is affected as well.

We found that K312 and E446 play important roles in the modulation of viral translation by NS5A domains II and III, respectively. When these residues are substituted with alanine in full length domain II and domain III fragments they result in a complete loss of this down-regulatory affect. In a domain II-LCS II-domain III fragment mutation of K312 again leads to a loss of this modulatory effect whereas the concurrent mutation of two residues, E446 and S447, in domain III are required for this affect. However, neither the identified domain II or domain III mutations had a significant effect on the replication of a 1b SgR either alone or in combination.

7.4 Results

7.4.1 Domain II and III downregulate viral translation though a mechanism requiring the poly-U/UC region of the viral 3´-UTR

We first confirmed our previous findings that NS5A domains II and III were capable of modulating viral translation independently through a process requiring the poly-U/UC region within the 3´-UTR. Huh7 cells were co-transfected with a monocistronic RNA HCV reporter and expression plasmids encoding for either domain II or domain III (**Figure 7.1a**). The monocistronic reporter utilized in this study contains the viral 5´-UTR, an internal Renilla Luciferase gene and the viral 3´-UTR. Small portions of the core and NS5B coding sequences are also included to avoid interfering with the secondary structure found within the untranslated regions. A reporter lacking the poly-U/UC region was also utilized to determine the role this region plays. As shown in **Figure 7.1b** the expression of either domain II or domain III independently leads to the down-regulation of translation, relative to vector, of the HCV reporter containing the Wt. 3´-UTR. Translation of the HCV reporter lacking the poly-U/UC region was unaffected by expression of either NS5A domain II or III, confirming the importance of this region in such modulation. These findings are in agreement with our previous results. We found that this is effect is specific to IRES-dependent translation. Domain II or III had little to no effect on the translation of a cap-dependent translation reporter (**Figure 7.1c**). Expression of the domain fragments was confirmed by western blot (**Figure 7.1d**). The migration of domain III was significantly slower than expected from the predicted molecular weight of this fragment. However, this observation is consistent with previous observations and attributed to the abundance of acidic and proline residues (505, 545) . We also determined that the expression of these NS5A domains did not affect cellular viability through use of an MTT assay (**Figure 7.1e**). We have previously demonstrated that both the HCV 5´- and 3´-UTRs are required for modulation by NS5A and that the down-regulation observed is not a result of changes in RNA levels caused NS5A expression.

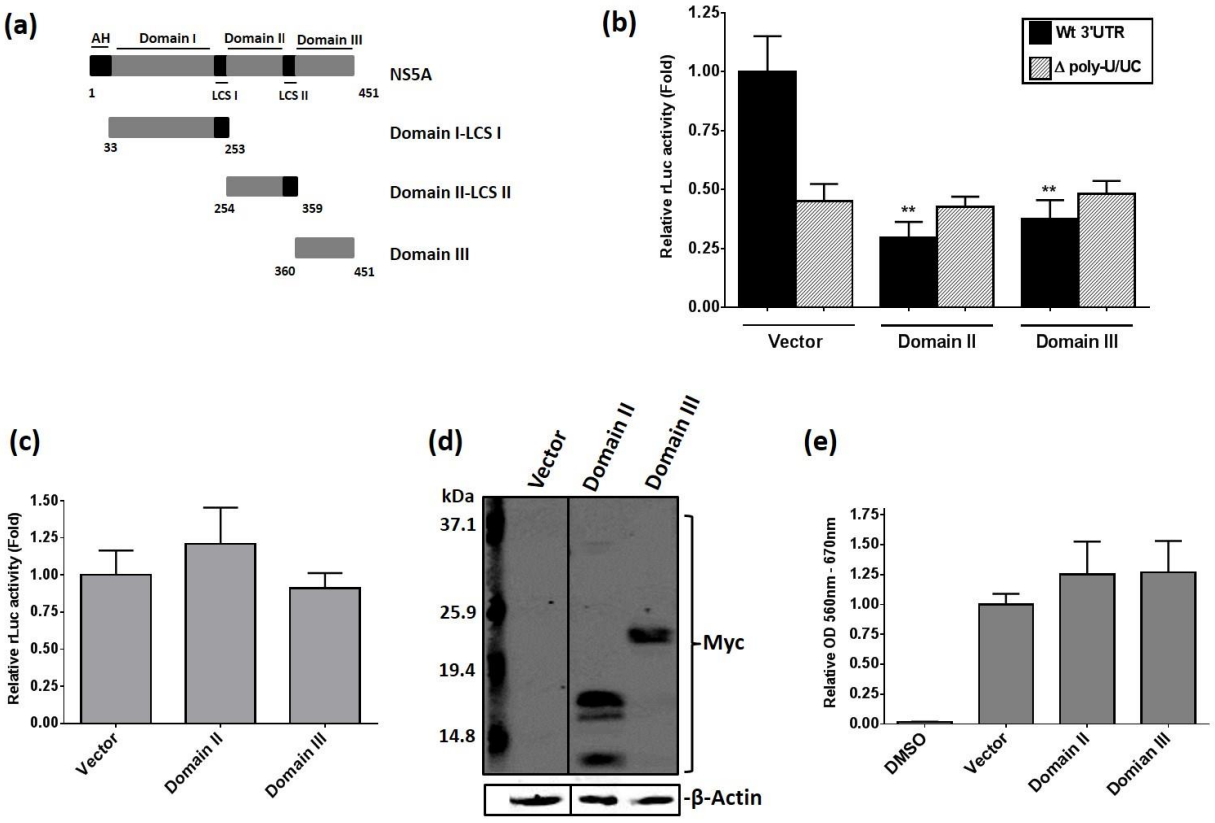

Figure 7.1 - NS5A domains II and III downregulate HCV translation through a mechanism requiring the poly-U/UC region of the 3´-UTR. **(a)** Diagram of NS5A domain composition. Amphipathic helix (AH), individual domains and Low-complexity sequences (LCS) are labelled. Corresponding amino acid residues are numbered according to NS5A protein sequence of HCV 1b. **(b,c)** Huh7 cells were co-transfected with either vector, 1b NS5A domain II or domain III expression plasmid and a (b) monocistronic 1b HCV RNA luciferase reporter with either the full length 3´-UTR or the Δpoly-U/UC 3´-UTR or a (C) capped luciferase RNA reporter. At 24 hr after transfection, luciferase assay was performed. **(d)** Expression of the domain II and domain III fragments was confirmed by western blot using a Myc specific antibody, with β-actin as a loading control. **(e)** Cell viability was determined by MTT assay 24hr. After transfection of either vector, domain II or domain III expression plasmids. DMSO treatment was included as a control. Mean of vector set to 1. Error bars indicate mean $\pm$SD. Statistical differences were indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ relative to vector.

7.4.2 NS5A Domain II region aa. 299-346 is sufficient for the down-regulation of viral translation

To map the region of NS5A domain II required for the translation modulation we constructed an initial set of domain II deletion mutants in which the LCSII region was removed along with deletion of 60aa from the N-terminus in 30aa segments. Using these domain II deletion mutants in combination with the monocistronic HCV RNA reporter described earlier we found that the LCS-II region did not appear to play an essential role in modulation of translation and that the domain II aa. 284-346 fragment lacking first 30 residues of domain II was still capable of modulating viral translation. However upon deletion of 60 residues (aa. 314-346 fragment) from the domain II N-terminus this regulatory effect was lost (**Figure 7.2a**). Expression of the fragments was confirmed by western blot except for the aa. 314-346 fragment which was undetectable despite numerous attempts and varying experimental conditions (**Figure 7.2b**). This was likely due to the difficulties in transferring such small proteins. Despite this issue we moved on to delete a further 15 amino acids from the N-terminus of the aa. 284-346 fragment to produce the domain II aa. 299-346 fragment. We found that this region was still able to down-regulate viral translation of our monocistronic HCV RNA reporter (**Figure 7.2c**). Expression of this fragment was confirmed by western blot (**Figure 7.2d**). It is interesting to note that this aa. 299-346 region contains the aa. 299-315 region previously identified to make contact with poly-U RNA (365). When this region is disrupted, as is the case with the aa. 314-346 deletion mutant, the effect on HCV translation is lost. Additionally, this aa. 299-346 region contains a highly conserved CypA binding site and the interaction between CypA and domain II has been shown to enhance the RNA binding ability of domain II (84, 495, 497). As the poly-U/UC region is required for the effect of domain II on viral translation this suggest that RNA binding by domain II may play a role in this effect.

7.4.3 Residue K312 is involved in the down-regulation of viral translation by NS5A domain II region aa. 299-346

To identify residues within this domain II aa. 299-346 that were potentially involved in the modulation of viral translation by this region a number of positively charged amino acids within this fragment were mutated. These residues are favoured in protein/RNA interactions as they can interact with the negatively charged phosphate backbone of RNA. This aa. 299-346 region contained 4 positively charged amino acids. These residues, arginine 308, lysine 309, lysine 311 and lysine 312 were substituted

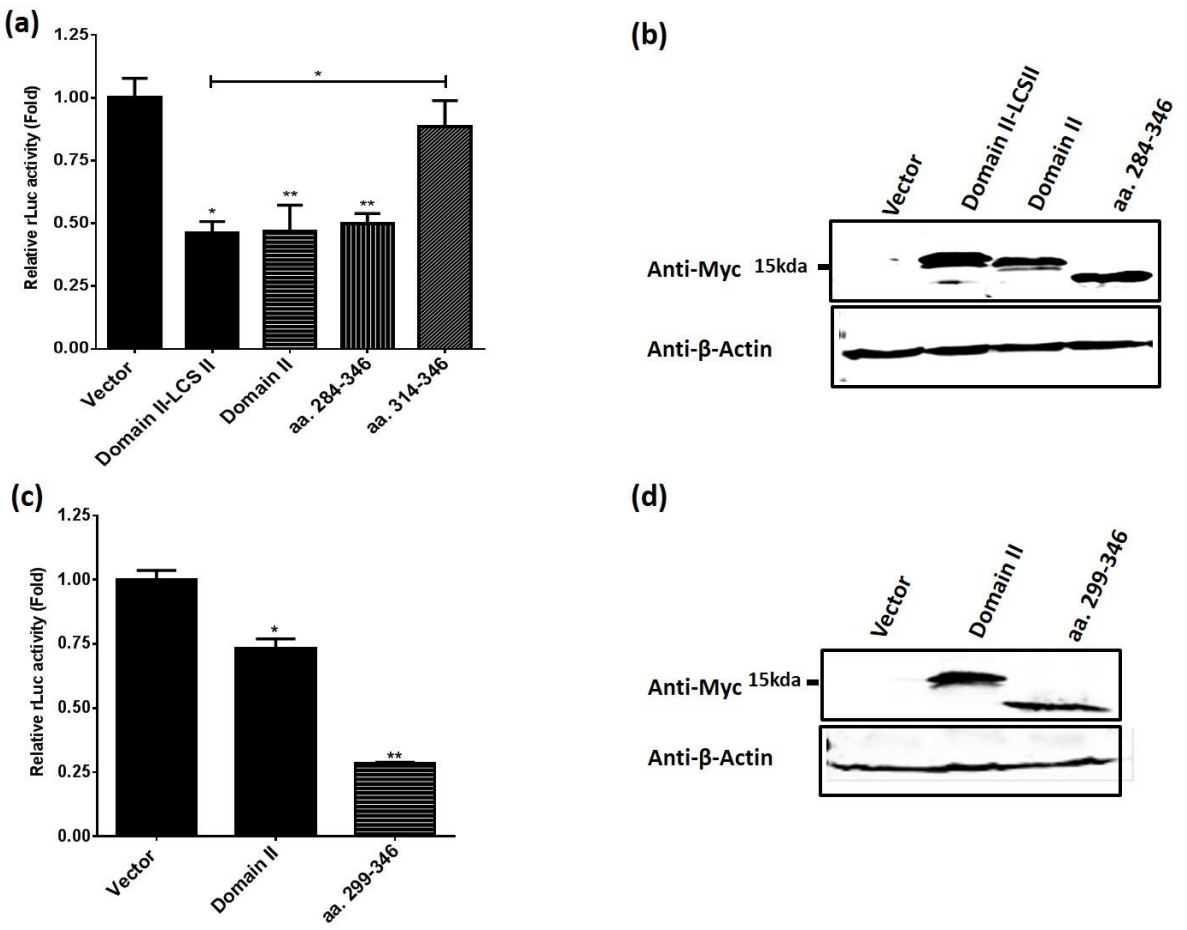

Figure 7.2 - NS5A Domain II region aa. 299-346 is sufficient for the down-regulation of viral translation. **(a, c)** Huh7 cells were co-transfected with either vector, 1b NS5A domain II or the indicated domain II deletion expression plasmid and a monocistronic 1b HCV RNA luciferase reporter. At 24 hr after transfection, luciferase assay was performed. **(b, d)** Expression of the domain II fragments in (a,c) was confirmed by western blot using a Myc specific antibody, with β-actin as a loading control. Approximate location of 15 kDa marker band shown. Mean of vector set to 1. Statistical differences were indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ relative to vector unless otherwise indicated

with alanine individually through site directed mutagenesis. The effect of these amino acid substitutions on the ability domain II aa. 299-346 to modulate viral translation was then investigated using the monocistronic HCV RNA reporter. The domain II aa. 299-346 fragments that contained substitutions of residues arginine 308, lysine 309, and lysine 311 with alanines maintained the ability to modulate viral translation (**Figure 7.3a**). However, mutation of lysine 312 (K312) essentially abolished the ability of this domain II region to modulate viral translation. The fragment which contained substitutions of all four residues behaved in a similar fashion to the K312A mutant. The expression of the domain II aa. 299-346 mutant fragments utilized in this experiment were confirmed by western blot (**Figure 7.3b**). These results point to an essential role of K312 in the ability of aa. 299-346 to modulate viral translation. Furthermore, all of these positively charged residues are found within the 299-315 region that was found to contact poly-U RNA (365). In the same study it was found that when NS5A is bound and cross-linked to poly-U RNA the expected trypsin cleavage is blocked at all four of these residues suggesting they interact directly with the RNA (365). If the modulation of viral translation observed is in fact due in part to RNA binding activity, then these results suggest K312 may play a predominate role in such as interaction.

7.4.4 The K312A mutation negates the ability of NS5A domain II to modulate viral translation but does not affect viral replication.

To confirm the importance of K312 in the modulation of viral translation by NS5A domain II the K312A mutation was introduced into the full length domain II fragment. The ability of this fragment to modulate viral translation was then compared to that of wild-type domain II. Upon co-transfection with a monocistronic HCV RNA reporter we observed that the K312A mutation negates the ability of domain II to modulate viral translation (**Figure 7.4a**). This suggests that this residue is critical for the down-regulatory effect of domain II on HCV translation. Expression of this K312A domain II mutant was confirmed by western blot (**Figure 7.4b**).

To investigate possible additional effects of this mutation in the viral lifecycle, the K312A mutation was cloned into a HCV sub genomic replicon (SgR). These SgR reporters are composed of the HCV 5´-UTR, which directs translation of a *Luciferase reporter* gene, followed by the IRES of the encephalomyocarditis virus, which directs translation of the HCV replicase genes NS3-NS5B, and the 3´-UTR (**Figure 7.4c**). These reporters allow for transient replication assay and the amount of luciferase produced can be measured as an indicator of viral replication. A SgR reporter containing a deletion of the GDD motif within the NS5B polymerase was utilized as a negative control as this deletion renders the SgR replication deficient. Upon

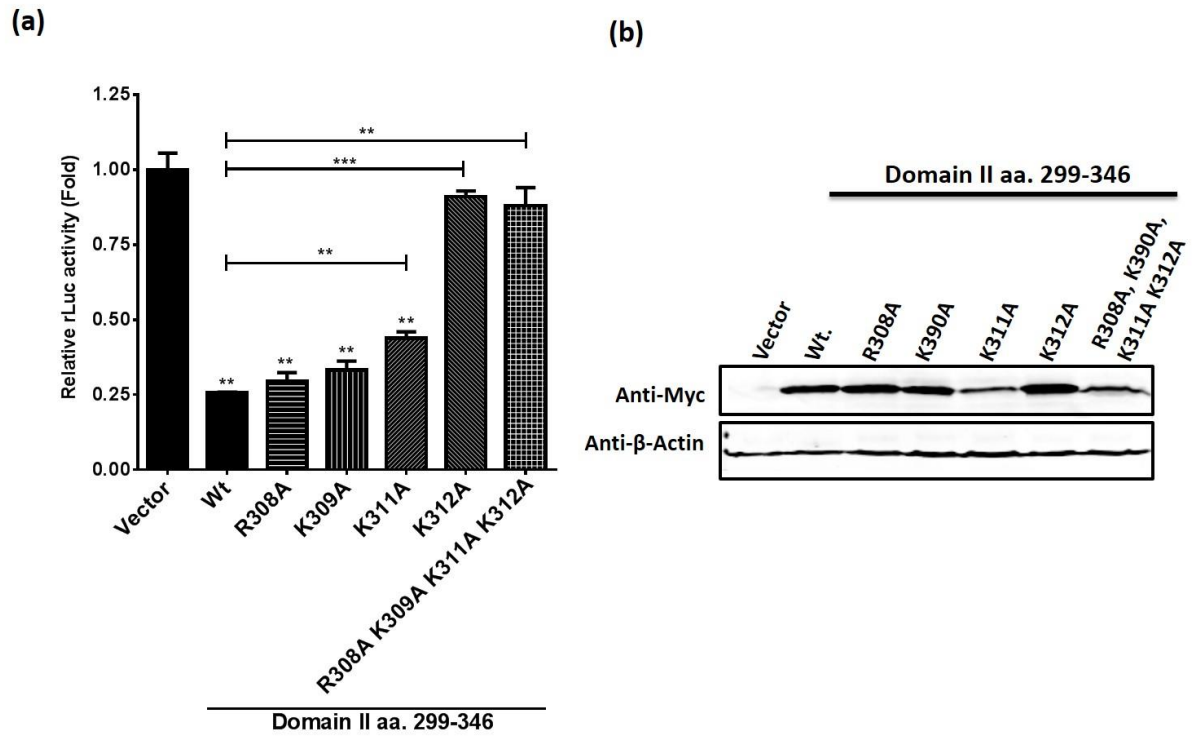

Figure 7.3 - Residue K312 is involved in the down-regulation of viral translation by NS5A domain II region aa. 299-346. **(a)** Huh7 cells were co-transfected with either vector or a domain II aa. 299-346 expression plasmid containing the indicated amino acid substitution and a monocistronic 1b HCV RNA luciferase reporter. At 24 hr after transfection, luciferase assay was performed. **(b)** Expression of the domain II aa. 299-346 fragments in (a) was confirmed by western blot using a Myc specific antibody, with β-actin as a loading control. Mean of vector set to 1. Statistical differences were indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$, *** if $p \leq 0.001$ relative to vector unless otherwise indicated.

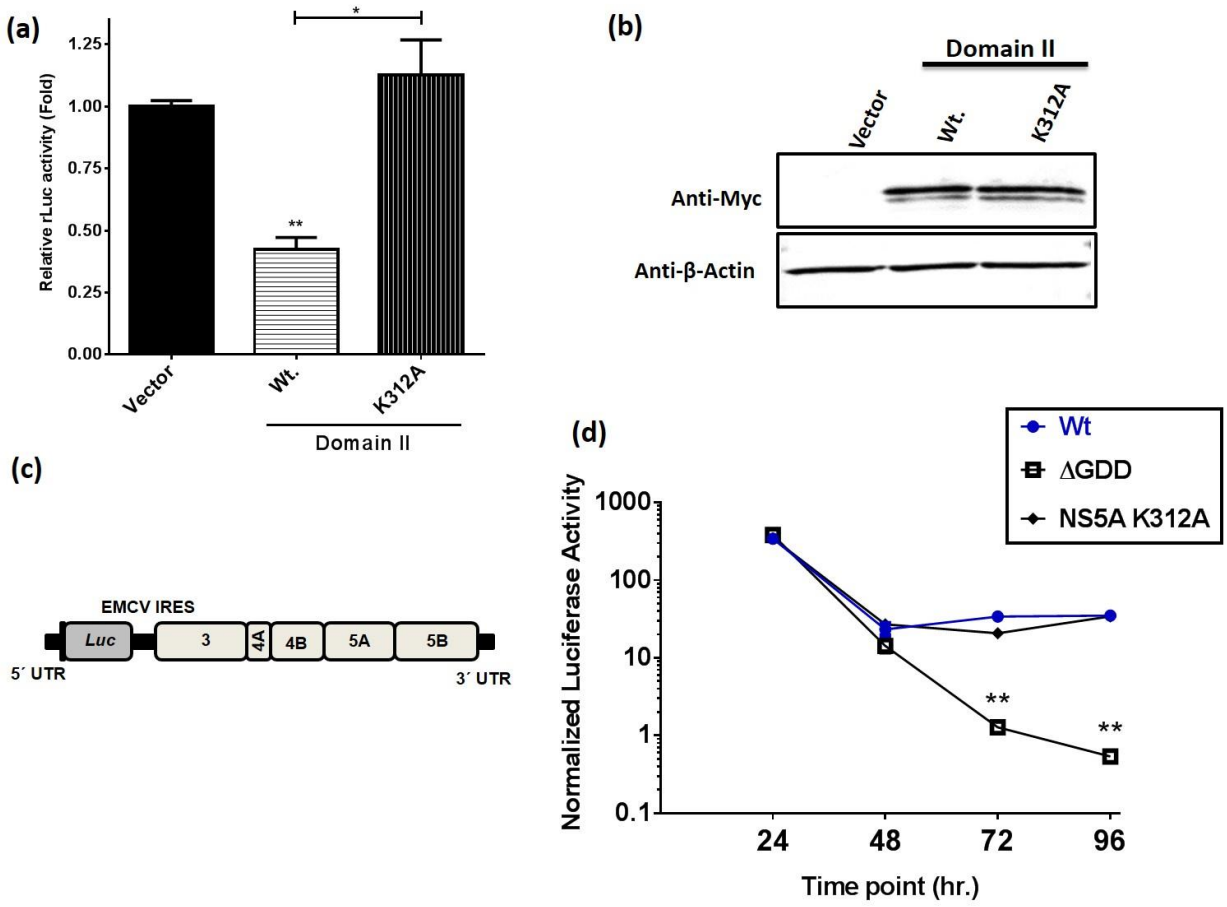

Figure 7.4 - The K312A mutation negates the ability of NS5A domain II to modulate viral translation but does not affect viral replication. **(a)** Huh7 cells were co-transfected with either vector, wt. NS5A domain II or NS5A domain II K312A expression plasmid and a monocistronic 1b HCV RNA luciferase reporter. At 24 hr after transfection, luciferase assay was performed. **(b)** Expression of the domain II fragments in (a) was confirmed by western blot using a Myc specific antibody, with β-actin as a loading control. **(c)** Diagram of the 1b HCV subgenomic replicon (SgR) utilized, see text for details. **(d)** Huh 7.5 cells were electroporated with the indicated 1b HCV SgR and a capped Luciferase mRNA. The ΔGDD mutation within the viral NS5B polymerase renderers the SgR replication deficient. Cells were harvested at the indicated time points and luciferase assay performed. Values were normalized to 4hr time point and protein concentration as determined by BCA assay. Statistical differences were indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ relative to vector/wildtype unless otherwise indicated. Error bars in panel (d) are not visible at this scale.

electroporation into Huh 7.5 cells we found that the K312A mutation within NS5A domain II had no significant effect on viral replication throughout the 96h time course (**Figure 7.4d**). So while K312 appears to play an essential role in the modulation of viral translation by NS5A domain II, possibly by mediating interaction with the poly-U/UC region within the viral 3´-UTR, it does not have an effect on viral replication in the context of full length NS5A within a subgenomic replicon.

7.4.5 Residues G445A, E446A and S447A are involved in the down-regulation of viral translation by NS5A domain III region aa. 420-451

Using the same approach used to identify K312 within domain II, we attempted to identify potential regions and residues involved in the modulation of viral translation by NS5A domain III. An initial set of domain III deletion mutants were produced in which 60 amino acids were removed from the N-terminus of domain III in 30 aa segments. Using these deletion mutants in combination with the monocistronic HCV RNA reporter we found that domain III aa. 420-451 was sufficient for modulating viral translation (**Figure 7.5a**). Oddly, the aa. 390-451 fragment was unable to modulate viral translation, suggesting that the region aa.390-420 may somehow interfere with the function of the aa. 420-451 region. Expression of the domain III deletion mutants was confirmed by Western blot (**Figure 7.5b**). The band observed for the aa. 420-451 deletion mutant was significantly less intense than for domain III and the aa. 390-451 deletion mutant. Although this could be due to lower expression this fragment behaved in a similar fashion to domain III in the translation assay.

Utilizing this domain III aa. 420-451 fragment, we cloned a series of alanine scanning mutants in which we progressively mutated three amino acids at a time within this region to alanine. This approach was taken as this region contained no positively charged amino acids that could potentially be involved in the RNA binding activity of domain III. Domain III was found to bind to the 3´-UTR relatively weakly compared to domains I and II and regions involved in such an interaction remain to be defined (363). The ability of this set of domain III aa. 410-451 alanine scanning mutants to modulate HCV translation was examined in co-transfection experiments with the monocistronic HCV RNA reporter (**Figure 7.5c**). In this experiment one fragment, domain II aa. 420-451 containing alanine substitutions at glycine 445 (G445A), glutamic acid 446 (E446A), serine 447 (S447A), lost the ability to modulate viral translation. Expression of the alanine scanning mutants was confirmed by western blot (**Figure 7.5d**). Next, these amino acid substitutions were cloned into the domain III aa. 420-451 fragment individually to determine the role of each residue in the modulation of viral translation by this region. In a co-transfection experiment the

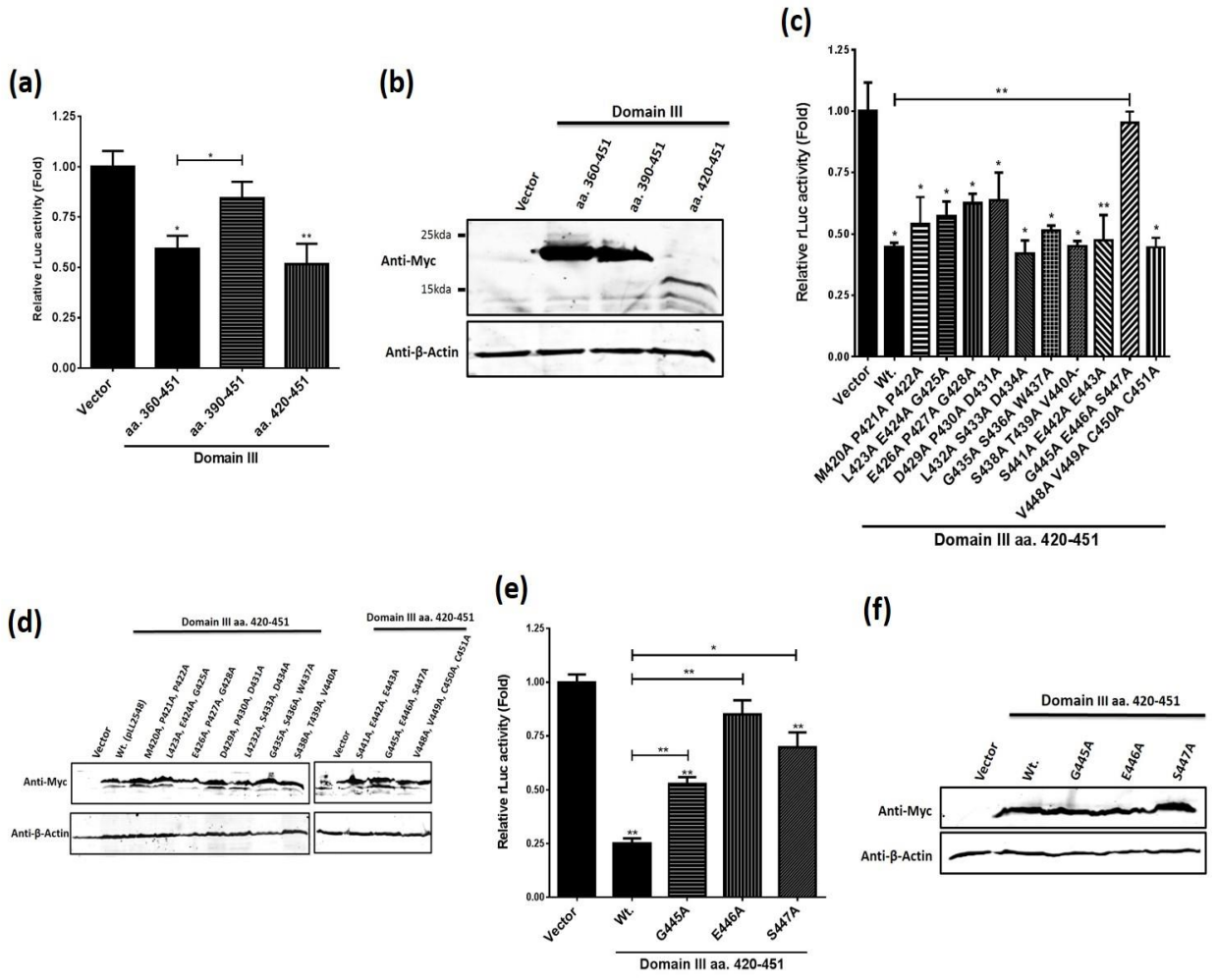

Figure 7.5 - Residues G445A, E446A and S447A play a role in the down-regulation of viral translation by NS5A domain III region aa. 420-451. **(A, C, E)** Huh7 cells were co-transfected with either plasmid vector or the indicated domain III deletion and/or amino acid substitution mutant expression plasmid and a monocistronic 1b HCV RNA luciferase reporter. At 24 hr after transfection, luciferase assay was performed. **(b,d,f)** Expression of the domain III deletion mutant fragments in (a, c, e) was confirmed by western blot using a Myc specific antibody. Mean of vector set to 1. Statistical differences were indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ relative to vector unless otherwise indicated.

E446A mutation was found to have the greatest effect, leading to a loss of a significant down-regulatory effect on viral translation (**Figure 7.5e**). Both the G445A and S447A mutants still lead to a significant decreases in translation levels compared to vector but to a statistically significant lower degree than the wild-type domain III aa 420-451 fragment suggesting this residues may play a smaller role in this effect than E446A. Expression of these domain III aa. 420-451 fragments was confirmed by Western blot (**Figure 7.5f**).

7.4.6 The E446A mutation negates the ability of NS5A domain III to modulate viral translation

To investigate the role of the E446 and S447 residues within the context of domain III, the E446A and S447A mutations were introduced into a full domain III fragment. When these domain IIII mutants were co-transfected with a monocistronic HCV RNA reporter, we found that double domain III E446A S447A mutant leads to a loss of the translational regulatory effect (**Figure 7.6a**). When mutated individually, the E446A mutation also resulted in the loss of this modulatory effect (**Figure 7.6c**). The S447A mutation, on the other hand, appeared to have no effect on this function. (**Figure 7.6c**). These results confirm the importance of the E446 residue in the modulation of translation by domain III. Expression of the domain III mutants was confirmed by western blot (**Figures 7.6b and 7.6d**). The fact that the modulation of translation by domain III is dependent upon E446, a negatively charged amino acid, suggests that this effect may not be the result of domain III binding to the viral RNA. However, due to the ability of this region to adopt transient structure this possibility cannot be completely excluded.

To determine if this E446A mutation had an effect on viral replication, this mutation was cloned into a 1b N SgR, as was done for domain II. The S447A mutation was investigated as well since it was identified as playing a role in the modulation of viral translation within the domain III aa. 420-451 deletion mutant. We did not expect these mutations to have a dramatic effect on viral replication since others have shown that NS5A domain III is largely dispensable for RNA replication. However, deletion of portions of NS5A domain III have been found to lead to a delay in viral replication in the JFH-1 genotype suggesting domain III may play a small role in establishment of the RNA replication at early time-points (419). As expected, within the context of full length NS5A in the SgR, neither the E446A nor S447A mutations alone or in combination had a significant effect on viral replication at any of the time-points (**Figure 7.6e**). How the E446A mutation affects the ability of domain III to modulate viral translation is unclear at this point but potential mechanisms include reduced RNA binding ability, an effect on an interaction between cellular proteins and domain III required for this effect, a shift in potentially required transient structure in this region or a

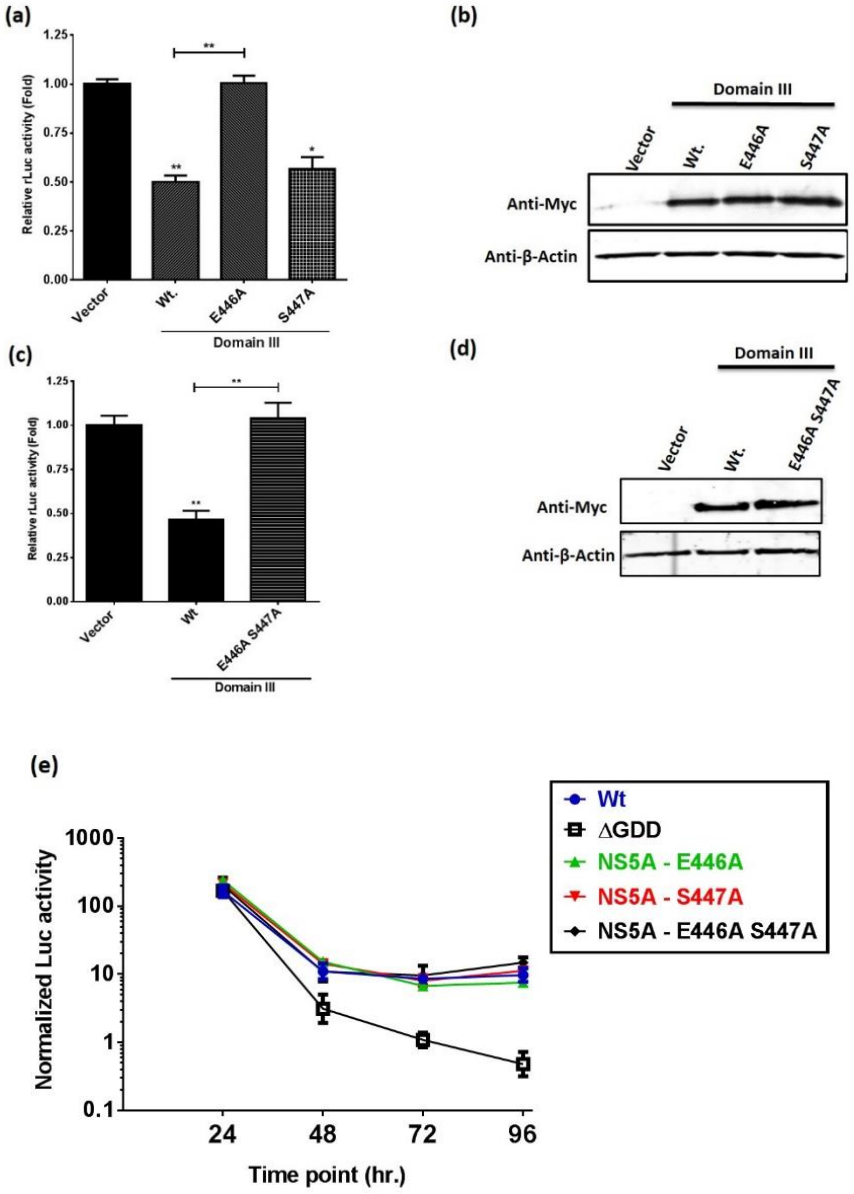

Figure 7.6 - The E446A mutation negates the ability of NS5A domain III to modulate viral translation. **(a, c)** Huh7 cells were co-transfected with either vector, wt. NS5A domain III or the indicated NS5A domain III amino acid substitution expression plasmid and a monocistronic 1b HCV RNA luciferase reporter. At 24 hr after transfection, luciferase assay was performed. **(b, d)** Expression of the domain II fragments in (a, c) were confirmed by western blot using a Myc specific antibody, with β-actin as a loading control. **(e)** Huh 7.5 cells were electroporated with the indicated 1b HCV SgR and a capped Luciferase mRNA. The ΔGDD mutation within the viral NS5B polymerase renderers the SgR replication deficient. Cells were harvested at the indicated time points and luciferase assay performed. Values were normalized to 4hr time point and protein concentration as determined by BCA assay. Mean of vector set to 1. Statistical differences were indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ relative to vector unless otherwise indicated. Some error bars in panel (e) not visible at this scale.

combination of any of these possibilities.

7.4.7 Mutation of K312 alone or E446 and S447 together abrogates the ability of the NS5A domain II-LCS II-Domain III fragment to modulate viral translation

To further investigate residues implicated in the modulation of viral translation by NS5A domains II and III, these mutations were introduced into a domain II-LCS II-domain III fragment. In addition to the K312A and E446A mutations, within domains II and III respectively, we also included S447A, even though it appeared not to play a role in the effect of domain III, given the importance of other nearby serine residues in modulating viral assembly. The effect of these mutations on the ability of this domain II/IIII fragment to modulate viral translation was determined in co-transfection experiments using the monocistronic HCV RNA reporter. As shown in **Figure 7.7a** the K312A mutation within domain II leads to a complete loss of modulatory effect on translation by this domain II-LCS II-domain III fragment whereas the E446A and S447A mutations within domain III do not have an effect. These results suggest that domain II is more dominant in modulation of viral translation by this domain II-LCS II-domain III fragment. The fragment that contains the K312A S447A double mutation also leads to a significant loss of this modulatory effect compared to Wt. but not to the same extent as the K312A mutant. The reasons for this are unclear at this time. It was also observed that the K312A E446A double mutant leads to a significant reduction in the modulatory effect on viral translation compared to Wt. domain II-LCS II-domain III fragment but still displays a slight, but statistically significant, down-regulatory effect (**Figure 7.7c**). The E446A S447A double mutant leads to a complete loss of modulatory effect by this fragment, which is interesting and unexpected as the S447A mutation did not have an effect on the modulation of viral translation by domain III individually. Finally, expression of the triple domain II-LCS II-domain III K312A, E446A, S447A mutant also had no effect on viral translation which is not surprising given the above results. Expression of the domain II-LCS II-domain III fragments was confirmed by western blot (**Figures 7.7b and 7.7d**). As done for domains II and III we investigated a combination of these mutations within both domains on the replication of the 1b SgR. Neither the double, K312A/E446A, nor triple, K312A/E446A/S447A, mutations had an adverse effect on the replicative capacity of the HCV SGR (**Figure 7.7e**). In fact a relatively small but statistically significant enhancement of viral replication, compared to the wild type SgR, was observed for the triple mutant at the 72 hour time-point and for both the double and triple mutant at the 96 hour time-point. The mechanisms behind this and the possible biological relevance are unknown.

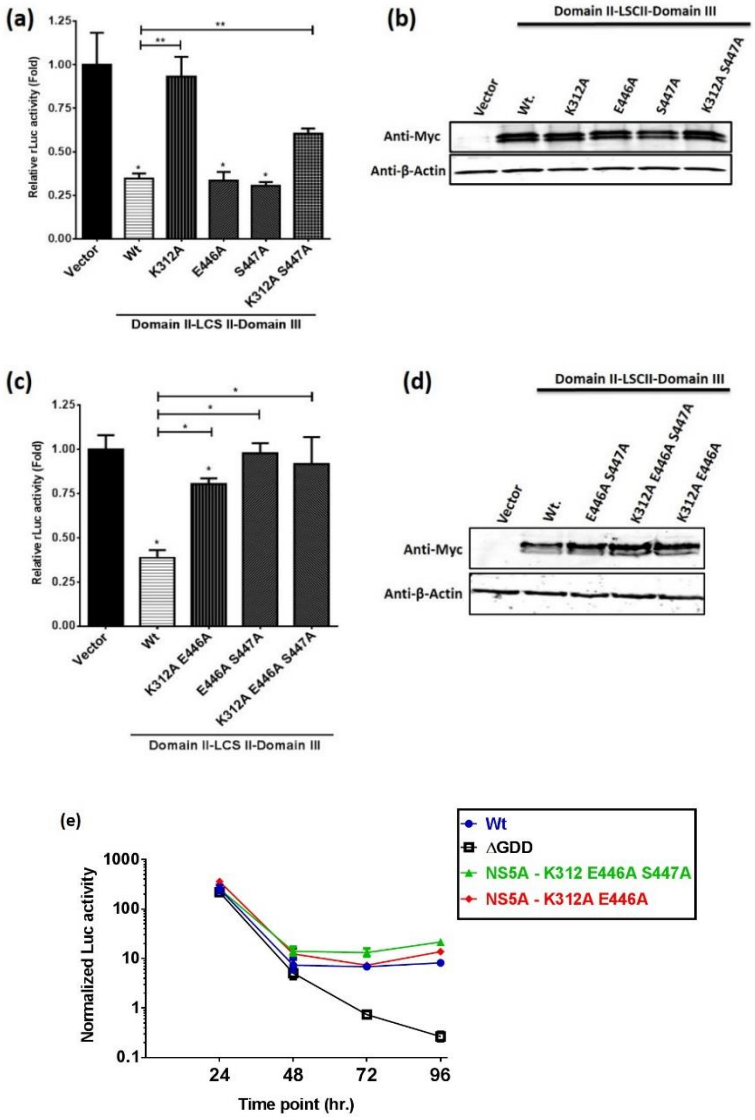

Figure 7.7 - Mutation of K312 alone or E446 and S447 together abrogates the ability of the NS5A domain II-LCS II-Domain III fragment to modulate viral translation. **(a, c)** Huh7 cells were co-transfected with either vector, wt. NS5A domain II-LCS II-domain III or the indicated NS5A domain II-LCS II-domain III amino acid substitution mutant expression plasmid and a monocistronic 1b HCV RNA luciferase reporter. At 24 hr after transfection, luciferase assay was performed. **(b, d)** Expression of the NS5A domain II-LCS II-domain III in (a, c) were confirmed by western blot using a Myc specific antibody, with β-actin as a loading control. **(e)** Huh 7.5 cells were electroporated with the indicated 1b HCV SgR and a capped Luciferase mRNA. The ΔGDD mutation within the viral NS5B polymerase renderers the SgR replication deficient. Cells were harvested at the indicated time points and luciferase assay performed. Values were normalized to 4hr time point and protein concentration as determined by BCA assay. Mean of vector set to 1. Statistical differences were indicated as * if $p \leq 0.05$, ** if $p \leq 0.01$ relative to vector unless otherwise indicated. Error bars in panel (e) are not visible at this scale

7.5 Discussion

We have previously demonstrated that NS5A down-regulates viral translation in a dose-dependent manner through a mechanism requiring the poly-U/UC region within the viral 3´-UTR. We have also demonstrated that NS5A binds to this poly-U/UC region (chapter 3). Furthermore, each of the three NS5A domains retained this modulatory function individually. While this redundancy is somewhat surprising, it is not completely unexpected. Each domain maintains RNA binding capability (363). How NS5A interaction with the 3´-UTR may function to down-regulate translation is unclear at this point as is the mechanism behind 3´-UTR stimulation of IRES mediated translation. Potential mechanisms include binding to viral or host factors involved in circularization of the genome, long range interactions between the IRES and 3´-UTR, or the retention and recycling of ribosomes between the 3´-UTR and IRES (19, 218). It is conceivable that NS5A binding to the poly-U/UC region may interfere with such functions, thereby downregulating viral translation and possibly mediating the switch between translation and replication. In a study focusing the on the mechanism of this down-regulatory effect by domain I, we identified R112 as residue critical in this effect (chapter 5). Substitution of this R112 residue with alanine was found to negate the effect of domain I on viral translation, impair the dimerization of domain I and reduce the RNA binding ability of this domain. Additionally, viral replication was inhibited when this R112A mutation was introduced into a 1b HCV SgR. These findings suggested an essential role for R112 in key functions of domain I in the viral lifecycle and lend credence to the biological relevance of the domain I dimer crystalized by Love *et al.* (482). In this study we sought to extend our initial findings and further investigate the modulation of viral translation by NS5A domains II and III.

Through the use of a set of domain II deletion mutants we first identified domain II aa. 299-346 as sufficient for the modulation of viral translation by domain II. This region contains the aa. 299-315 region that was previously found, along with a second region aa. 267-275 to make contact with poly-U RNA (365). Furthermore, this aa. 299-346 region contains a highly conserved CypA binding site, an interaction essential for viral replication. Binding of CypA to domain II results in structural change within this domain and also stimulate domain II RNA binding (84, 501). As we determined that the poly-U/UC region within the 3´-UTR is required for the modulation of viral translation by domain II, we considered that the mechanism might involve RNA binding. To investigate this possibility positively charged amino acids, which are favorable in protein/RNA interactions, were mutated within this aa. 299-346 region. We observed that the substitution of a lysine residue at position 312 (K312) with alanine negated the ability of this region to modulate viral translation. When this K312A mutation was introduced into a full length

domain II fragment, the ability to modulate viral translation was lost. This finding suggests that K312 participates in some function required for the modulation of translation by domain II. K312 is found within the aa. 299-315 region that makes contact with poly-U RNA (365). RNA cross-linking studies show that the expected trypsin cleavage is blocked at this residue suggesting a direct interaction with the RNA (365). Other residues corresponding to those at which trypsin cleavage is blocked, R308, K309 and K311A were also mutated in this study but had little to no effect on the modulation of translation by this region suggesting that K312 may play a predominant role in potential RNA binding (365).

This K312 residue also corresponds to the very N-terminal residue of the region thought to interact with CypA (495). However, the K312A mutation does not affect viral replication when introduced into the 1b HCV SgR whereas the interaction between NS5A and CypA is essential for viral replication. It is therefore unlikely that the K312A mutation has a negative impact on the interaction of CypA with domain II. CypA binding has been shown to enhance the RNA binding ability of this domain (84). However, the cyclosporine A (CsA) resistance mutation that exhibits CypA independence for viral replication does not exhibit enhanced RNA binding (84). This observation raises the question as to whether the enhanced RNA binding is essential to the role of CypA in modulating viral replication. Therefore this enhanced RNA binding activity of domain II stimulated by CypA may possibly play a role in the modulation of viral translation by domain II and the K312A mutation may interfere with this function. The effect of this K312 mutation on the RNA binding ability of domain II and interaction with CypA and subsequent enhanced binding currently remain unanswered.

In addition to CypA, NS5A has been found to interact with a vast array of host proteins, many involving domain II. Furthermore, domain II has been found to contain two regions, aa. 255-270 and aa. 301-310 (1b N protein numbering), which transiently adopt α-helical structures (493). Such transient structures may play a role in the recognition of binding partners, accordingly, an interaction between the cellular tumour suppressor Bin1 with a region including the aa.301-310 α-helix has been observed (493). The effect of this interaction on the viral lifecycle is unclear but regions affected by Bin1 binding extend significantly outside the boundaries of this transient helix, which is suggestive of changes to the local structure or additional contacts with Bin1 (493). This aa.301-310 transient α-helical structure is found with the domain II aa. 299-346 fragment and is directly N –terminal to the K312A mutation. It is thus plausible that this K312A mutation may affect protein interactions that function to modulate viral translation.

In regards to domain III, which has an essential role in viral assembly, we found that a relatively small region, aa.420-451, was sufficient for its modulation of viral translation. Translation modulation by NS5A domain III requires the poly-U/UC region within the viral 3´-UTR. Domain III has also been observed

to possess RNA binding affinity towards this poly-U/UC region, although relatively weak compared to domain I and II (363). Therefore, we speculated that RNA binding may represent a mechanism for the observed effect (363). However, this aa.420-451 region contained no positively charged RNA residues suggesting an alternative mechanism may be responsible. Through alanine scanning within this aa. 420-451 region we identified two mutations, E446A and S447A which significantly affected its ability to modulate viral translation. A third mutation G445A was also identified, but had a less predominant effect. Upon insertion into a full domain III fragment, the E446A mutation resulted in a complete loss of this domains modulatory function on viral translation whereas the S447A had no observable effect. Although this E446 residue appears to play an essential role in the modulation of viral translation the E446A mutation did not have any effect upon viral replication when inserted into NS5A within the 1b HCV SgR. This result is not surprising. Previous studies have shown that NS5A domain III is dispensable for viral replication (419, 454). The mechanism of the modulatory effect and the role that E446 may play in it remain unclear. The regions of NS5A domain III that are involved in its binding the poly-U/UC region of the viral 3´-UTR remain undefined. Despite the reasons discussed above, this aa. 420-451 may perhaps contains novel RNA binding activity and E446A may play a role in such an interaction.

Domain III, which is globally unfolded, has been found to adopt regions of transient secondary structure. Two regions within domain III, aa. 364-379 and aa.446-449, have been observed to transiently form α-helical structure within Con1 (also genotype 1b) (505). Notably this second helical region is found within the domain III aa.420-451 fragment that is sufficient for modulation of viral translation and contains the E446A residue that is essential for this function. The S447A mutation, which did not affect translation modulation by domain III, is the only residue not conserved between Con 1 and N subtypes within this transient helical region. Transient structures such as these α-helices may be essential for interaction with cellular proteins involved in the HCV lifecycle. CypA, which has an essential interaction with domain II, has also been found to interact with domain III in a region just N-terminal to this transient α-helical structure (505). A CsA resistance mutation, corresponding to V449A (1b N numbering), has been observed in this region (505). CypA has also been found to possess *in vitro* isomerase activity towards this C-terminal region of domain III, resulting in structural modifications with potential functional consequences (505). Therefore, the E446A mutation identified in this study could potentially interfere with the formation or function of this region of transient structure resulting in a disruption of protein interactions that modulate viral translation.

In a final set of experiments, we investigated the effects of the mutations identified within domain II and III in the context of a domain II-LCS II-domain III fragment. This fragment retained the ability to

down-regulate viral translation. The single mutation K312A within domain II resulted in the loss of this modulatory function. On the other hand, the E446A mutation within domain III had no effect on the modulatory ability of this fragment. These results suggest that domain II may play a predominate role in this modulation of translation and can compensate for any loss of function caused by the E446A mutation in domain III. However, the domain II-LCS II-domain III mutant containing the double mutation E446A/S447A within domain III also nullified the effect of this fragment on viral translation. The involvement of S447A mutation was surprising. Whereas this mutation was implicated in this function of domain II aa. 420-451, it did not have a significant effect when cloned into the full domain III. Furthermore, when this S447A mutation was cloned into the domain II-LCS II-domain III fragment in combination with the K312A mutation, the ability of this fragment to modulate viral translation is significantly reduced, but to a lower extent than seen with the K312A mutation alone. The reasons for these conflicting finding are unknown but could include alterations of transient structures or interactions between domains II and III within this fragment that affect either the RNA binding ability of this fragment or protein interactions required for the observed effect on viral translation. A number of other serine residues within C-terminal region of domain III have been implicated in NS5A basal phosphorylation and viral assembly. It would be interesting to examine the phosphorylation status of serine 447 and determine if this plays a role in these observations (419, 420, 452).

At first, it seems unusual that such a wide array of functions and protein interactions have been attributed to NS5A domains II and III. Both of these regions are natively unstructured. However this is a trait shared by numerous cellular proteins often referred to as intrinsically disordered proteins (IDPs), such as p53 and BRAC1 (507, 508). Such IDPs are very abundant within the cell and often function as signalling and regulatory hubs as they exhibit extraordinary binding promiscuity (508). IDPs often adopt transient structured elements that are utilized to carry out functions (508). These characteristics have been also described for domains II and III of NS5A, which are thought to function as complex molecular interaction platforms (362, 493). Structural changes within IDPs can be induced by interactions with nucleic acids, other proteins, membranes, or post-translational modifications such as phosphorylation (508). As discussed, the mutations identified within this study are found within or adjacent to regions of transient structure and thus might result in structural changes or the shifting of isomer populations to favour one confirmation over the other thereby interfering with function.

In this study, we have identified residues, K312 in domain II and E446 in domain III, which are essential for the downregulatory effect of these domains on viral translation. It will be interesting to determine if these mutations affect RNA binding or protein interactions of these domains and if the

alteration of transient functional structure is involved in this effect. Efforts to answer some of these intriguing questions are currently on going in our lab.

7.6 Materials and Methods

7.6.1 Plasmid construction and RNA synthesis

The monocistronic HCV RNA translation reporter plasmid T7 HCV 5´-UTR-Core^{aa1-16}-rLuc-NS5B^{5}-3´-UTR of genotype 1b was described previously (BH *et al.*, submitted for publication). This reporter contains T7 promoter, HCV 5´-UTR, sequence encoding the first 16 amino acids of the Core protein, an internal *Renilla* luciferase (rLuc) gene, sequence encoding the last five amino acids of the NS5B protein and the 3´-UTR. The 5´-UTR contains the viral IRES which drives the expression of the internal *Renilla* luciferase gene. In other two constructs, the poly-U/UC region in the 3´-UTR or the entire 3´-UTR with the coding sequence for the last five amino acids of the NS5B were cloned downstream of the T7 promoter sequence. HCV subgenomic replicon (SgR) is composed of the HCV 5´-UTR, which directs translation of a luciferase reporter gene, followed by the IRES of the encephalomyocarditis virus (EMCV), which directs translation of the HCV replicase genes NS3-NS5B, and the 3´-UTR (Fig. 8a) (558). The SgR reporter allows for transient replication assay as the amount of luciferase produced can be measured as an indicator of viral replication (559). As a negative control, the GDD sequence within the NS5B viral polymerase was deleted which rendered replication deficient. Plasmids expressing HCV NS5A with or without the amphipathic α-helix (AH) were reported previously (chapter 3). Coding sequences for NS5A domain II with or without LCS II and NS5A domain III were amplified by PCR using HCV-1b N Neo C-5B (540) as template and cloned into pEF/cyto/myc vector (Invitrogen/Life Technologies). NS5A domain II and domain III truncations were constructed by a PCR-based approach. Amino acid substitution mutants in the expression plasmid or SgR were generated by site-directed mutagenesis. For all constructs a myc tag was added to the C-termini. All plasmids were confirmed by DNA sequencing. HCV RNA translation reporter and SgR RNAs were produced from linearized plasmids by *in vitro* transcription using the MEGAscript T7 *In Vitro* Transcription kit (Ambion).

7.6.2 Cell lines, transfections, HCV transient replication and luciferase assay

See methods described in section 5.6.2

7.6.3 SDS-PAGE and Western blot

See methods described in section 5.6.3

7.6.4 MTT assay

See methods described in section 3.6.4

7.6.5 Statistical analysis

See methods described in section 5.6.7

7.7 Acknowledgments

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8.0 GENERAL DISCUSSION AND CONCLUSION

8.1 General Discussion

NS5A appears to be involved in nearly every aspect of the viral lifecycle and performs essential functions in both viral replication and assembly. We thus set out to determine how this protein is involved in the regulation of viral translation. We hypothesized that NS5A would modulate viral translation due to its interactions with both the 5´- and 3´-UTRs (363-365). In addition, we aimed at mapping the regions of NS5A responsible for translation modulation in an effort to elucidate the potential mechanism. NS5A is an important antiviral drug target and the further characterization of its roles in the viral lifecycle could facilitate the design of future treatments targeting the multiple functions of NS5A.

In chapter 3, we determined that NS5A down-regulates HCV IRES-mediated translation in a dose-dependent manner through a mechanism that requires the presence of the poly-U/UC region of the 3´-UTR. In addition to our monocistronic HCV RNA reporter, this down-regulatory effect was also observed in the full-length viral genome upon expression of additional NS5A in *trans* and was again dependent upon the presence of the poly-U/UC region. The effect was specific to HCV translation and required the presence of both the HCV 5´- and 3´-UTRs. We and others have shown that NS5A binds to the poly-U/UC region, our results thus suggest that such binding may modulate viral translation. How the interaction between NS5A and the poly-U/UC region could modulate HCV translation remains unclear. The poly-U/UC region is proposed to act as a molecular interaction platform for the recruitment of host and viral proteins that modulate both viral translation and replication (212-215). As the amount of NS5A produced increases after multiple rounds of translation, binding within the poly-U/UC region could potentially interfere with the enhancement of viral translation by the 3´-UTR.

In Classical Swine Fever virus (CSFV), which also belongs to the *Flaviviridae* family but within a different genus (*Pestivirus),* NS5A has also been found to downregulate IRES mediated translation in a dose dependent manner (564, 565). However, this effect was proposed to be dependent upon a potential interaction with the 5´-UTR rather than the 3´-UTR, as we have observed for HCV NS5A (564, 565). Nonetheless, this finding suggests that the down-regulation of viral translation by NS5A may be a conserved mechanism. Despite sharing only approximately 17% amino acid identity, a number of additional functions have been found to be conserved between the CSFV and HCV NS5A proteins. Both perform essential functions within the viral replication complex and during virion assembly (26, 566, 567). A number of serine residues within the C-terminal region of CSFV NS5A play an essential role in viral assembly, very similar to what has been observed for HCV (452, 565). Furthermore, NS5A from both

viruses interacts with numerous host proteins. Both interact with heat shock protein 70 and annexin A2 (425, 568-570). The interaction between NS5A and heat shock protein 70 is proposed to function during viral replication whereas the interaction with annexin A2 is involved in modulating viral assembly in both CSFV and HCV (425, 568-570). Other similarities include that both interact with and stimulate the viral polymerase, both localize to the ER membrane, and both are extensively phosphorylated (366, 571-574).

One question that arises when considering our observations is why a virus would encode a protein that functions to downregulate its own translation? HCV, like all viruses, which poses a positive sense RNA genome, utilizes its genome for both translation and replication (575-577). After successive rounds of viral translation and polyprotein processing, the increasing accumulation of viral proteins involved in viral replication leads to massive rearrangements of intracellular membranes and the formation of the replication complex (576). However, viral RNA templates being actively translated cannot undergo replication due to the opposing direction in which these processes occur (576, 577). Translation begins at the start codon at the 5´- end of the viral genome and the ribosome proceeds down the genome in the 5´- to 3´ direction. Replication is initiated at the 3´ end of the genome and the viral polymerase proceeds down the genome in the 3´to 5´- direction. Therefore, there must be a mechanism in place to switch from translation to replication once the necessary quantity of viral proteins has been produced. Such a transition would also serve prevent the continued accumulation of viral proteins which could potentially be cytotoxic to the infected cell (234). How this switch between stages in the viral lifecycle occurs remains unclear for many viruses. However, for poliovirus one mechanism involved in this switch has been well characterized.

Poliovirus (PV), like HCV, is a single stranded positive sense RNA virus that translates it genome through use of an IRES to produce a polyprotein (576). Although structurally different than that of HCV, the PV 5´-UTR is similarly highly structured and contains six stem loops with stem-loops II to VI comprising the IRES (576). Much like HCV, both canonical and non-canonical cellular translation factors aid in the initiation of translation. One of the cellular factors which plays a key role in this process is the poly(rC) binding protein 2 (PCBP2) (576). PCBP2 binds to stem-loop IV of the poliovirus IRES and forms a ribonucleoprotein complex which stimulates translation, possibly through recruitment of ribosomes (576, 578-580). Furthermore, PCBP2 also binds to stem-loop I of the 5´-UTR from where it can interact with poly(A) binding protein (PABP) which is bound to the PV 3´-poly-A tail (576, 579, 581). This interaction also functions to stimulate viral translation, potentially by circularizing the genome to facilitate ribosome recycling (576, 581). However, the interaction between PCBP2 and stem-loop I of the 5´-UTR is also required for viral replication where it functions in the initiation of negative strand synthesis (576, 580).

PCBP2 contains three hnRNP K homologous (KH) domains, which are common in RNA binding proteins (582). KH3 is necessary for binding to stem-loop IV within the IRES (582). Following PV translation and processing of the viral polyprotein, the viral 3CD proteinase cleaves PCBP2, removing the KH3 domain (576, 580). This cleaved PCBP2 can no longer bind to stem-loop IV of IRES (576, 580, 582). As a result PV translation is inhibited by the loss PCBP2 binding, potentially due to the disruption of ribosome recruitment (576, 580). Furthermore, the viral proteinase has also been found to cleave PABP, disrupting the interaction between the two UTRs and contributing to the inhibition of PV translation and the clearance of ribosomes from the viral genome (581). However, cleaved PCBP2 retains the ability to bind to stem-loop I and perform an essential function in RNA replication (576, 580). It was additionally found that Inhibition of PCBP2 and PABP cleavage impairs viral replication (576, 581). Therefore these findings strongly suggest that the viral protease cleaves cellular ITAFs altering their interaction with the 5′- and 3′-UTRs in order to promote the switch from translation to replication.

For HCV, the molecular mechanisms that may mediate the transition from viral translation to genome replication remain unclear. One study has implicated viral NS3 protease mediated cleavage of La autoantigen in this process (234). Multiple cellular factors which are implicated in both viral translation and replication have been proposed to be involved, including miR-122 and hnRNP L and D (225-228, 234). It is also highly likely that the 3′-UTR of HCV is involved in this process in that it is involved in regulating both translation and replication (577). The mechanism whereby the 3′-UTR modulates viral translation remains unclear but may involve long-range RNA-RNA interactions between the 5′- and 3′-UTRs, the binding of cellular factors which function to enhance translation and/or ribosome recycling (19, 218). Conceivably, the binding of NS5A within the poly-U/UC region could disrupt the stimulation of viral translation by the 3′-UTR by interfering with these mechanisms. Therefore the switch between viral translation and replication could result from the combined efforts of multiple viral factors such as NS3 functioning at the IRES to remove La autoantigen while NS5A functions at the 3′-UTR. This process would be similar to the situation in PV where the viral 3CD proteinase cleaves translation enhancing factors at both end of the viral genome.

The cellular factors shown to bind within the poly-U/UC and stimulate viral translation include HuR, LSm1-7 and IGF2BP1 (216, 217, 231). Interestingly, LSm1-7 and IGF2BP1 also bind within the 5′-UTR to IRES stem-loops II and IV, respectively (216, 227). These proteins also interact with other factors such as hnRNP D and La autoantigen, which bind within the 5′-UTR and function to stimulate viral translation (15). IGFBP2 is also capable of self-interaction (231). This interaction network suggests that these factors are involved in an interaction between the 5′- and 3′-UTRs that functions to stimulate viral translation

possibly through recruitment of additional translation factors or the recycling of ribosomes. It is therefore possible that as concentrations of NS5A increase after successive rounds of translation, factors favouring viral translation are displaced from the poly-U/UC region by NS5A. Preliminary results obtained in our lab, which are not presented in this thesis, are consistent with such a model. We observed that the expression of IGF2BP1 stimulates viral translation, dependent on presence of the poly-U/UC region. When NS5A and IGF2BP1 are co-expressed, however, the down-regulatory effect of NS5A dominants. Experiments such as these will be continued in our lab in an effort to investigate this potential mechanism.

NS5A has also been found to interact with a number of host proteins that bind to the viral genome and function to stimulate viral replication, including the FUSE binding protein (FBP), NF90, PCBP2 and the dual functioning hnRNP L (227, 382, 583). The mechanisms behind the stimulation of viral replication by these factors remains to be determined. FBP was found to interact with NS5A and bind to the poly-U/UC region (382). Overexpression of FBP results in stimulation of replication and inhibition of viral translation suggesting a role in the switch between the two (382). PCBP2 binds to both the stem-loop I in the 5´-UTR, similar to its role in PV replication, and the poly-U/UC region within the 3´-UTR (227, 583). It is also involved in stimulating viral replication (227, 583). PCBP2 is implicated in modulating viral translation. It can interact within domain IV of the IRES but its role in modulating viral translation remains unclear (15, 583). Stem-loop I within the 5´-UTR is essential for viral replication, through unknown functions, and NS5A is also speculated to interact with this stem-loop (364). Both hnRNP L and NF 90 bind both the 3´-UTR and within the 5´-UTR upstream of the IRES (227). NS5A also interacts with the NS5B polymerase and stimulates its polymerase activity (366, 573, 584). Furthermore, this interaction has been implicated in recruitment of NS5B to the viral 3´-UTR in *in vitro* replication experiments (366, 573, 584). Therefore, NS5A interacts with a set of proteins which function to promote viral replication and bind to both the 5´- and 3´-UTRs and could potentially be involved in the circularization of the genome in a fashion that favours replication similar to other positive sense RNA viruses (585, 586).

Given the number of host proteins that bind to the poly-U/UC region and participate in either viral translation or replication, one function of NS5A binding to the poly-U/UC region may be to coordinate these interactions. As the concentration of NS5A increases, it may function to displace a set of cellular factors which function in stimulating viral translation and recruit a different set of host proteins involved in modulating viral replication. To investigate this potential mechanism, a number of different experimental approaches could be followed, for example, RNA binding competitions and RNA pull-down experiments similar to those performed by Li *et al.* and Nag *et al.* in the presence and absence of NS5A (227, 587). Additionally, iCLIP (individual-nucleotide resolution UV crosslinking and immunoprecipitation)

could be utilized to investigate how different levels of NS5A expression affect the binding patterns of host proteins which function to enhance viral translation, similar to the approach used by Flynn *et al.* to study the binding of PCBP2 and HCV RNA (588). The results from experiments such as these could provide an insight into the mechanism of the modulation of viral translation by NS5A and how it affects the RNA binding activity of cellular proteins.

Additionally, the effect NS5A on ribosome recycling, one potential mechanism of translational enhancement by the 3′-UTR, should also be investigated in future studies. The effect of NS5A on the ability of the 3′-UTR to bind the 40S ribosome could be accomplished using an RNA binding competition assay. In a study by Bai *et al.,* a system in which luciferase activity was measured in situ over the course of an *in vitro* translation reaction was utilized to demonstrate that the 3′-UTR does not affect the rate of a single round translation but does function to enhance subsequent rounds of translation (218). *In vitro* produced and purified NS5A could be added to this system to determine the effect on ribosome recycling. Furthermore, the effect of NS5A on the formation of polysomes in in vitro translation reactions could be investigated as ribosome recycling by the 3′-UTR has been shown to significantly enhance polysome formation (218).

The 3′-UTR has also been found to influence the folding of the IRES through direct RNA-RNA interactions and likewise the 5′-UTR influences the structure of the 3′-UTR (197, 198). Such modulation may regulate viral translation and replication and may perhaps play a role in the transition between the two processes (197, 198). However, this structural modification has only been shown in the absence of proteins. It would be interesting to evaluate the effect of NS5A's interaction with the poly-U/UC region on such interactions. This could be accomplished by utilizing SHAPE (selective 2'-hydroxyl acylation analyzed by primer extension) assays in the presence of NS5A.

Having established that NS5A functions to downregulate viral translation we then set out to figure out which region(s) were responsible for this effect. We found that each of the three NS5A domains were able to carry out this effect individually, again requiring the poly-U/UC region. Although surprising, this result was not completely unexpected as each domain has been shown to possess RNA binding activity towards the poly-U/UC region (363). Based on these results, we aimed to map the regions within each of the three NS5A domains responsible for this modulatory effect using a series of deletion mutants and amino acid substitutions.

In chapter 4 we focused on NS5A domain I that performs multiple essential functions in viral replication and is proposed to be the main driver of NS5As RNA binding activity. Domain I also mediates the dimerization of NS5A which is thought to be involved in both RNA binding activity and viral replication.

However, the functions of a domain I dimer remain unclear due to the isolation of 4 different crystal structures (479, 481, 482). It is possible that all of the dimer structures are physiologically relevant and perform different functions within the viral lifecycle. Alternatively, NS5A may form a large multimeric complex. In this chapter we determined that the domain I region aa. 101-161 was sufficient for the down-regulation of viral translation by NS5A domain I. Interestingly, this region contained the majority of the contact interfaces of the domain I dimers identified by both Lambert *et al.* and Love *et al.* A number of positively charged amino acids within this aa. 101-161 region were implicated in the modulatory effect. The mutation of K108, K139, R157 or R160, to alanine partially disrupted the ability of domain I to modulate viral translation. However, mutation of R112 to alanine led to a complete loss of this regulatory function. This R112 residue is within the contact interface of the Love *et al.* dimer. Moreover, it is proposed to form an intermolecular hydrogen bond with E148, suggesting it plays a key role in the structure of this dimer. Upon mutation of this E148 residue to alanine, we again observed a complete loss of the ability of domain I to modulate viral translation, suggesting the importance of the hydrogen bond between the two residues in dimer formation. In further support of this model, we found that a domain I fragment containing both R112A/E148A mutations fails to dimerize. Given the proposed role of NS5A dimerization in mediating NS5A domain I RNA binding, we tested the effect of this R112A mutation on the ability of NS5A domain I to bind to poly-U/UC RNA. We observed that this mutation impaired the ability of domain to interact with the RNA. Furthermore, when this R112A mutation was inserted into an HCV replicon, a loss of replicative capacity was observed. Therefore, these results indicated that the R112 residue is critical for the downregulatory effect of NS5A domain I on viral translation and performs an essential function in viral replication. It also suggests that dimerization and RNA-binding activity of domain I is involved in modulating these effects. Our results also suggest a biological relevance of the domain I dimer observed in the Love *et al.* study. These results were also similar to those obtained by Lim *et al.* who identified a correlation between domain I dimerization, RNA-binding activity and viral replication through mutation of a number of cysteine residues in the N-terminal zinc binding motif (489).

It is somewhat surprising that R112, which appears to play a prominent role in the Love *et al.* dimer, was found to play a key role the RNA binding ability of domain I. The Love *et al.* dimer model lacks the large positively charged groove proposed to act as an RNA binding site found in the Tellinghuisen *et al.* dimer (479, 482). The highly conserved contact interface of Love *et al.* dimer, including R112, is fully exposed in Tellinghuisen *et al.* dimer and may function as a site of molecular interaction when in this conformation (482). Alternatively, the R112A mutation identified in this study may affect the transition between different dimer conformations during the viral lifecycle or the formation of larger multimeric

NS5A complexes that conceivably consist of NS5A dimers in different conformations via interactions with alternating exposed surfaces (481, 482, 488). However, a number of results obtained in this chapter questioned the role of dimerization in the ability of domain I to modulate viral translation. Firstly, we found that through the addition of the LCS-I region to domain I, the effect of the E148A mutation on translation modulation could be compensated for. This suggests that the LCS-I can either make up for structural defects caused by E148A mutation or perhaps phosphorylation, which occurs extensively within the LCS-I, produces negatively charged residues that can interact with R112A. Although not expressed *in cis*, which is thought to be required for full hyperphosphorylation, our results suggest that our domain I-LCS-I fragment undergoes phosphorylation due to presence of a doublet on Western blot. Studies by the Cameron group have suggested that this LCS-I region contributes to domain I dimerization. To clarify the role of the LCS-I, immunoprecipitation experiments utilizing the domain I R112A/E148A-LCS I fragment could be performed. In addition, phosphatase treatment could be added to determine the role that phosphorylation within the LCS-I region may play in this effect.

Secondly, we found that a domain I C39A mutant partially retains the ability to modulate viral translation. We and others have found that this domain I mutant also impairs viral dimerization and it has been found to inhibit RNA binding by domain I and is lethal to viral replication in the study by Lim *et al.* (489). These findings suggest that NS5A domain I dimerization may play a role in the observed effect of domain I on viral translation but not be absolutely required for this effect. Perhaps the domain I monomer retains low efficiency binding to the poly-U/UC region, binding which is dependent upon R112. Alternatively, It remains possible that domain I fragments containing the C39A mutation are still able to dimerize to a degree necessary for the modulation of viral translation but to a weaker extent that is undetectable by the assays used. The presence of the viral reporter in the translation studies could stimulate this activity as U-rich RNA has been found to enhance dimerization (365). An inefficient ability to dimerize could explain the reduced modulatory ability of this C39A mutant relative to Wt. domain I. An approach which could be utilized to clarify the role of domain I dimerization and RNA binding in the modulation of viral translation would be through the use of symmetrical NS5A inhibitors such as DCV. DCV is thought to target the NS5A dimer and has been observed to impair the ability of domain I to bind to U-rich RNA (487). This is proposed to be the result of trapping the dimer in a conformation that does not bind RNA (487). Assessing the ability of domain I to modulate viral translation in the presence of DCV could provide insights into the importance of dimerization and RNA binding in the mechanism of modulation.

The importance of this R112 residue in multiple functions of NS5A involved in both translation and replication suggests that it mediates functions conserved among the different HCV genotypes. This model is supported by the conservation of this residue across all six circulating HCV genotypes (**Figure 8.1**). The conservation of this residue and its essential roles in the viral lifecycle suggest that R112 could be targeted in the development of novel direct acting antivirals. The identification of new drug targets is important in attempts to increase treatment efficacy and to combine with existing antivirals in order to decrease the potential of resistance.

In chapter 5, we shifted our focus onto NS5A domains II and III and used an approach similar to that utilized for domain I in an effort to map the regions and residues that mediate the ability of these domains to modulate viral translation. For domain II, we determined that the aa. 299-346 region is sufficient for the down-regulation of viral translation. Through the mutation of positively charged amino acids within this region, we were able to identify K312 as the main mediator of this effect. As mutation of this residue lead to a complete loss of the modulation of viral translation by domain II. However, mutation of this residue within a HCV replicon had no observable effect on viral replication indicating that the function mediated by this residue is limited to viral translation.

The mechanism behind the modulation of viral translation remains unclear. However, the requirement for the poly-U/UC region and the disruption that mutation of a positively charged residue produces leads us to speculate that RNA binding may be a potential mechanism. In support of this model, the K312 residue has previously been observed to contact poly-U RNA, resulting in a block of trypsin mediated cleavage in RNA binding studies (365). This aa. 299-346 region also contains the CypA binding site (495). K312 is located just N-terminal to this site, within a region whose structure is altered by the isomerase activity of CypA (495). The binding of CypA to domain II enhances the RNA binding activity of this domain towards poly-U RNA (84). This enhanced binding, however, is not thought to mediate the essential function of CypA in viral replication (84). Mutations which facilitate CypA independent replication do not demonstrate this enhanced binding (84). Therefore, it is possible that this CypA binding alters the structure of the region containing the K312 residue resulting in enhanced RNA binding and the down-regulation of viral translation. This possibility could be investigated through mutation of the CypA binding site to determine if this interaction has an effect on the ability of domain II to modulate viral translation.

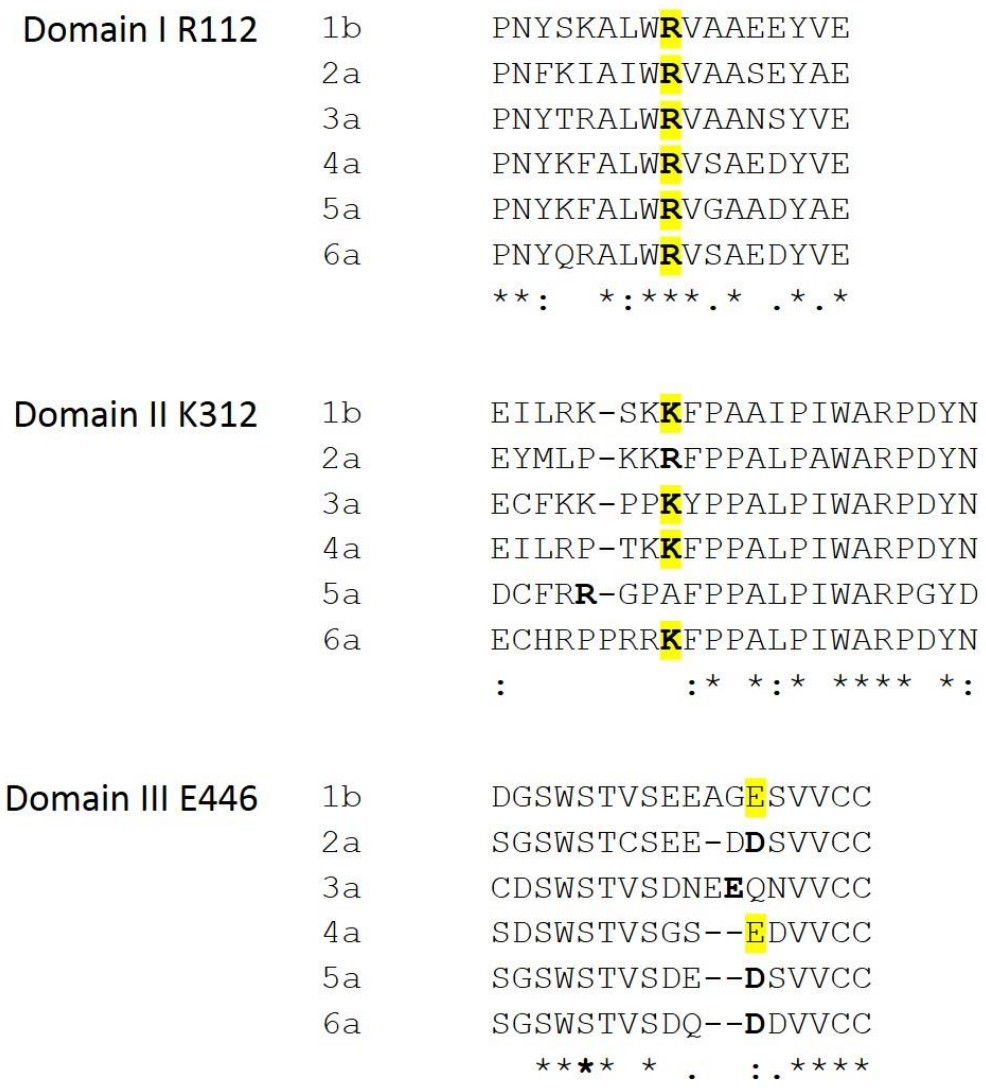

Figure 8.1 – Sequence alignment of residue implicated in the modulation of viral translation by NS5A domains I, II and III. Alignment performed using Clustal Omega software. Sequences of prototypical HCV strains retrieved from HCV database (http://hcv.lanl.gov/content/index). Conserved residues are bold and highlighted whereas residue with similar characteristics to the implicated residues are in bold.

In addition to RNA binding, the interaction between domain II and host proteins may be involved in the modulation of viral translation observed. The domain II aa. 299-346 region sufficient for modulating viral translation has been found to contain a region (aa. 301-310) capable of transiently adopting α-helical structures and bind host proteins such as Bin1 (493). Therefore the K312 mutation proximal to this region could potentially alter the structure of this region, interfering with interactions with host proteins. The effects of this mutation on the structure within this region could be investigated though the use of NMR analysis and the binding of cellular proteins could be investigated through a pull-down assay. NS5A has also been found to act as a transcriptional activator and domain II plays an essential role in this function. Domain II contains an acidic region that is responsible for the majority of the trans-activation activity (502-504). The K312A mutation which negated domain II effect on viral translation is located just N-terminal to this region. Perhaps through alterations of transient domain structure, this K312A mutation may affect the transcriptional activation properties of this region. Even though this fragment lacks an NLS, NS5A lacking an NLS are still transported into the nucleus, although to a lower extent, likely due to passive migration (504). As the transcriptional activation properties of NS5A have been observed to correlate with viral replication, it is possible that the up-regulation of factors that favour viral replication play a role in the switch between translation and replication and could result in the down-regulation of viral translation observed (504). However, this possibility remains to be investigated.

In terms of sequence conservation this K312 residue is conserved across genotypes 1, 3, 4 and 6 (**Figure 8.1**). However, in genotype 2 this residue corresponds to an arginine residue that like lysine is positively charged, suggesting it could function similar to K312. In genotype 5, whereas K312 corresponds to a neutral alanine residue, there are two arginine residues at positions 308 and 309 that could function in a similar manner.

Within domain III, we identified the aa. 420-451 region as sufficient for the down-regulatory effect on viral translation. We subsequently found that mutation of E446 to alanine resulted in a loss of the modulatory ability of domain III on viral translation. Similar to K312 identified within domain II, this domain III mutation was found not to affect viral replication. How this domain modulates viral translation and the role of E148 remains unclear. Even though this residue is negatively charged, it is possible that it is involved in the RNA binding activity of domain III through function in a novel RNA binding fold. The effect of the E148 mutation on the RNA binding ability of domain III remains to be investigated. However, given the small size of the aa. 420-451 region and the relatively weak binding affinity domain III possess towards the poly-U/UC region it is possible that this region mediates other functions. Similar to the K312 mutation in domain II, this E446 residue may play a role in transient structure adopted by domain III. E446

falls within the aa. 446-449 region of domain III found to transiently form an α-helical structure which may be important for the interaction with host proteins (505). Interestingly, CypA has been found to bind within this C-terminal region and a CsA resistance mutation is observed at position V449A (505). The function of CypA binding within domain III is unknown (505). It would be interesting to determine if CypA binding enhances the RNA binding activity of this region in a fashion similar as domain II and if this is involved in the modulation of viral translation by domain III. Furthermore, given the essential role domain III plays in viral assembly, the effect of the E446A mutation on this processes should also be determined.

In regards to the sequence conservation of this E446 residue, it is only conserved between genotypes 1 and 4 (**Figure 8.1**). However, there is an aspartic acid residue, which is very similar to glutamic acid in its negative charge, at this position in genotypes 2, 5 and 6. Genotype 3 possesses a glutamic acid one position away at position 445. Therefore, it appears that a negatively charged acid in this region of NS5A domain III performs a conserved function in the viral lifecycle.

To further investigate the effects of the R112A, K312A and E446A mutations on the viral lifecycle the cellular localization and motility of NS5A containing these mutations should be assessed. Within infected cells, NS5A localizes to the ER associated membranous web in addition to the surface of lipid droplets where it performs functions essential to genome replication or viral assembly, respectively (511). Furthermore, two populations of NS5A-positive foci, likely representing distinct replication complex populations, are observed in infected cells (589, 590). One population is composed of large structures demonstrating restricted mobility whereas the second population is characterized by small, fast-moving structures (589, 590). These distinct NS5A containing replication complex populations may have distinct functional roles during the viral lifecycle, however, this remains to be determined.

From the results obtained in this work, we have determined that NS5A functions to downregulate viral translation in addition to its already demonstrated essential roles in viral replication and assembly. Furthermore, we have mapped this effect to individual residues within each domain of NS5A and proposed potential mechanisms. It is clear that NS5A is capable of carrying out a remarkable number of pro-viral functions within the cell, especially for a protein that lacks enzymatic activity. The continued characterization of NS5A and the processes that modulate its functions may aid in the development of future treatments against this complex and enigmatic protein.

8.2 Conclusions

- NS5A down-regulates viral translation in a dose dependent manner though a mechanism requiring the poly-U/UC region within the viral 3´-UTR
- Each domain of NS5A is capable of modulating viral translation independently, also through a mechanism requiring the poly-U/UC region within the viral 3´-UTR
- Residue R112 is essential for the effect of NS5A domain I on viral translation
- Mutation of R112 and E148 impairs NS5A domain I dimerization
- The R112 residue is involved in RNA binding by NS5A domain I as the R112A mutation impairs this activity
- The R112A inhibits viral replication when inserted into an HCV subgenomic replicon
- The K312 residues is essential for the down-regulation of viral translation by NS5A domain II but has no effect on replication of an HCV subgenomic replicon
- Mutation of E446 negates the translation modulatory activity of NS5A domain III but has no effect on replication of an HCV subgenomic replicon
- Mutation of K312 within domain II negates the ability of an NS5A domain II-LCS II-domain III fragment to modulate viral translation while mutation of two residues, E446/S447, is required within domain III for this effect
- No combination of these mutations within domains II and III has a significant effect on replication of an HCV subgenomic replicon

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