Interaction of hepatitis C virus polymerase with host cell proteins

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Interaction of Hepatitis C Virus Polymerase with Host Cell Proteins

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Thesis Presented for

the Degree of Doctor of Philosophy in the School of Biological and Biomedical Sciences at the University of Durham

Infectious Diseases Group

School of Biological and Biomedical Sciences

University of Durham

Durham

July 2009

DECLARATION

I declare that this thesis is my own work that has been composed by myself and does not include any work forming a part of a thesis presented for a degree in this or any other University.

Ahmed Attia Ali Mohamed

July 2009

ACKNOWLEDGEMENTS

I would like to express my grateful thanks to Paul Yeo for giving me the opportunity to work in his lab on this interesting subject. I greatly appreciate his valuable supervision, support, kind encouragement, unfailing advice and sincere guidance to complete this thesis.

My deep appreciation to Arvind Patel (MRC Virology Unit, Institute of Virology, Glasgow) for his generous help, valuable efforts, encouragement throughout the course of this work and providing essential reagents required for the work in thesis and also for giving me the chance to do the HCV replicon work within his department.

I am deeply grateful to Paul Denny for his generosity and support especially during the last year of my work that facilitated the completion of my thesis.

I wish to thank Diane Hart and Simon Padbury for their technical assistance and friendship throughout the course of PhD.

I would like to dedicate my thesis to my parents as the work would not have been possible unless their continuous support and encouragement. Many thanks for my wife and my daughter for supporting me and making my life happier in UK during the course of my study.

I would like to thank the Egyptian Government and the National Research Centre in Egypt for supporting me, funding the project and giving me the chance to study in UK.

ABSTRACT

Hepatitis C virus (HCV) interacts with host cell proteins to modify cellular pathways creating a favourable environment that facilitates its replication and persistence. The purpose of the work presented in this thesis was to identify cellular proteins that can interact with NS5B, the virus's RNA-dependent RNA polymerase, that may contribute to the virus's biology.

A number of cellular proteins were found to interact with NS5B using the yeast two-hybrid system. These proteins were involved in cellular pathways such as interferon signalling, lipid transport and metabolism, protein trafficking, cell proliferation and apoptosis. Of these, phospholipid scramblase 1 (PLSCR1) and zinc finger protein 143 (ZNF143) were selected for further investigation. The interactions were confirmed *in vitro,* and, for PLSCR1, the region that interacted with NS5B was determined to be within the amino-terminal region of the protein (61-137 a.a.). NS5B interacted with PLSCR1 and ZNF143 via a single interacting region localized in its N-terminus (1-153 a.a.).

Expression of PLSCR1 or ZNF143 enhanced the ability of interferon to stimulate transcription from an interferon-stimulated response element (ISRE) reporter construct. Co-expression with NS5B was found to down-regulate this activity. Expression of a number of interferon-stimulated genes was investigated in the presence of NS5B, PLSCR1 or ZNF143 but no significant effect was observed. Overexpression of PLSCR1 had no effect on HCV sub-genomic replicon replication, while reduction of its expression by short hairpin RNA (shRNA) enhanced replication. Overexpression of ZNF143 was found to have a suppressive effect on replication but downregulating its expression did not enhance replication.

In addition to using the yeast two-hybrid system to identify NS5B-interacting proteins, an *in vitro* pulldown assay coupled with mass spectrometry identified α- and β-tubulin associated with NS5B *in vitro* and *in vivo.* Subsequently this association was demonstrated to be an indirect interaction

but the intermediatory partner was not identified. The domain that mediated the association with α- and β-tubulin was determined to be within the N-terminus of NS5B (1-153 a.a.). Nocodazole, an inhibitor of tubulin polymerization, had a marked effect on the association of α-tubulin with NS5B displacing it from the complex but had no effect on β-tubulin's association. Utilizing an HCV sub-genomic replicon, nocodazole was shown to have a significant inhibitory effect on replication.

Taken together the data presented in this thesis showed that NS5B had a multitude of potential interactions with a variety of cellular proteins. The biological significance of some of these interactions on the cellular response to IFN and replicon replication was investigated. This work has generated a number of novel observations on the interaction between the virus and the cell that warrant future investigation.

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ABBREVIATIONS

a.a.	Amino Acid
AD	Activation domain
Ade	Adenine
AH	Amphipathic helix
Amp	Ampicillin
APS	Ammonium persulfate
BAX	Bcl-2–associated X protein
Bcl2	B-cell lymphoma 2
BD	Binding domain
BSA	Bovine serum albumin
BVDV	Bovine viral diarrhoea virus
C	Core
CCLR	Cell culture lysis reagent
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CKII	Casein kinase II
CSFV	Classical swine fever virus
CTL	Cytotoxic T lymphocytes
DENV	Dengue virus
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
E	Envelope
E. coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
ER	Endoplasmic reticulum
eIF	Eukaryotic translation initiation factor
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FBS	Foetal bovine serum
GDP	Guanosine diphosphate

Grb2	Growth factor receptor-bound protein 2
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HAV	Hepatitis A virus
HAX1	HS1 (Haematopoietic Lineage Cell-Specific Protein 1)-Associated Protein X-1
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HEK293T	Human Embryonic Kidney (HEK) 293T Cells
His	Histidine
hnRNPL	Heterogeneous nuclear ribonucleoprotein L
hPLIC1	Human homolog 1 of protein linking intergrin-associated protein and cytoskeleton
HRP	Horse radish peroxidase
Huh7	Human Hepatoma Cell line
hVAP	Human vesicle-associated membrane protein-associated protein A
HVR	Hypervariable region
IFN	Interferon
IPS-1	Interferon promoter stimulator-1 (IPS-1) adaptor protein
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRES	Internal ribosomal entry site
IRF	IFN-regulatory factor
ISRE	IFN-stimulated response element
ISGF3	IFN-stimulated gene factor 3
JAK	Janus kinase
JEV	Japanese encephalitis virus
JFH1	Japanese fulminant hepatitis
Kan	Kanamycin
kb	Kilo bases
kDa	Kilo daltons
L	Liter
LD	Lipid droplet
LDL	Low density lipoprotein

Leu	Leucine
luc	Luciferase
M	Molar
MAPK	Mitogen-activated protein kinase
MS	Mass spectrometry
NANBH	Non-A, non-B hepatitis
NF-kB	Nuclear factor kappa-B
Ni	Nickel
NLS	Nuclear localization signal
NS	Non-structural
OD	Optical density
O/N	Overnight
ORF	Open reading frame
OSBP8	Oxysterol Binding Protein 8
p	Significance
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKR	Interferon-inducible protein kinase
PLSCR1	Phospholipid scramblase
PRK2	Protein kinase C-related kinase 2
PTB	Polypyrimidine tract-binding
RdRp	RNA-dependent RNA-polymerase
RIG-1	Retinoic acid-inducible gene
RNA	Ribonucleic acid
RNAi	RNA interference
RT	Room temperature
RT-PCR	Reverse transcriptase- Polymerase chain reaction
RTN3	Reticulon 3
SD	Minimal Synthetic Dropout
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SFRS10	Splicing Factor, Arginine/Serine-Rich 10
SFV	Semliki forest virus

SH3	Src-homology 3
shRNA	Short-hairpin RNA
SOS	Son of sevenless
SP	Signal peptidase
SPP	Signal peptide peptidase
SR-BI	Scavenger receptor class B type I
STAT	Signal transducers and activator of transcription
SVR	Sustained virologic response
TBEV	Tick-borne encephalitis virus
TBS	Tris buffer saline
TIMM50	Translocase of Inner Mitochondrial Membrane 50
TJ	Tight junction
TLR	Toll-like receptor
TMD	Transmembrane domain
TNF	Tumour necrosis factor
TRIF	Toll-like receptor 3 adaptor protein
Trp	Tryptophan
UTR	Untranslated region
UV	Ultraviolet
VERO	African Green Monkey Epithelial Kidney Cells
VLDL	Very low density lipoprotein
VLPs	Virus like particles
VSV	Vesicular stomatitis virus
WB	Western blot
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus
Y2H	Yeast two-hybrid
ZNF143	Zinc finger protein

CHAPTER 1

Introduction

1.1 Introduction

Hepatitis C virus (HCV) is one of the main infectious causes of hepatitis, a liver injury associated with an influx of acute or chronic inflammatory cells into the liver (Crawford, 1997). It is a major health problem worldwide with approximately 130-180 million people infected with the virus (WHO, 1999; WHO, 2004). Most of cases (60-80 %) go from an acute to a chronic stage and patients can develop long-term complications such as liver cirrhosis and hepatocellular carcinoma (HCC) (Bukh *et al.*, 1993) such that 27 % of cirrhosis and 25 % of HCC cases in the world occur in HCV infected individuals (Perz and Alter, 2006).

In some countries, such as Egypt, the virus has infected more than 20 % of the population (Frank *et al.*, 2000). Most of HCC cases (64 %) in Egypt are attributable to HCV infection and as the incidence of HCV infection is increasing, HCC cases increase which places a significant burden on the Egyptian economy (Hassan *et al.*, 2001).

1.2 HCV Infection Prevalence

According to the most recent World Health Organization (WHO) estimate, 2-3 % of the world's population (approximately 130-180 millions) has been infected with HCV (WHO, 1999; WHO, 2004). As shown in Table 1.1 and Fig. 1.1, HCV infection is endemic worldwide and the distribution of the infection varies broadly among geographic areas. Africa and Asia accommodate most of the countries with the highest reported infection prevalence rates (more than 2.9 %). Industrialized nations like North America, northern and western Europe, and Australia have a lower infection prevalence (less than 1 %) (Shepard *et al.*, 2005).

Table 1.1: Reported HCV infection prevalence in the world.

WHO region	Total Population (Millions)	HCV prevalence Rate %	Infected Population (Millions)
Africa	602	5.3	31.9
Americas	785	1.7	13.1
Eastern Mediterranean	466	4.6	21.3
Europe	858	1.03	8.9
South-East Asia	1500	2.15	32.2
Western Pacific	1600	3.9	62.2
Total	5811	3.1	169.7

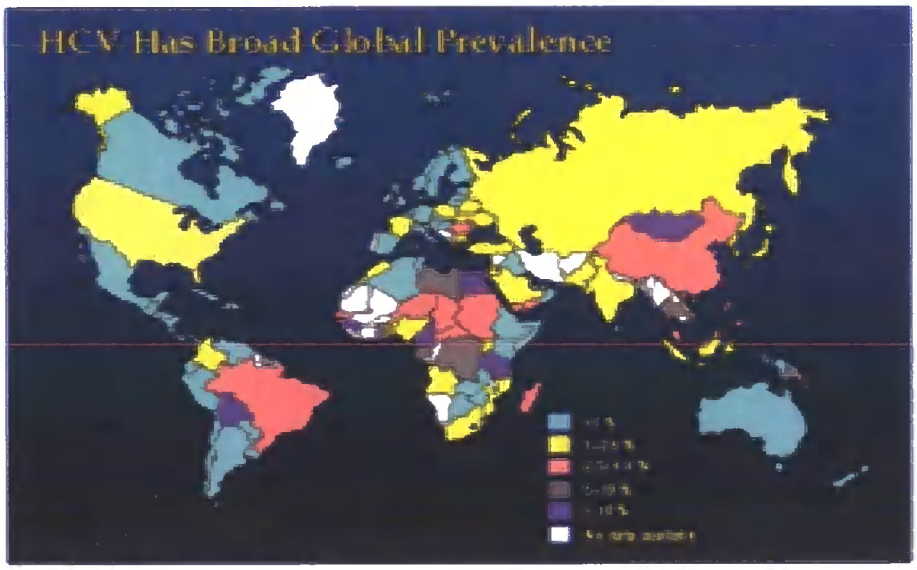

Fig. 1.1: HCV prevalence worldwide (WHO, 2004). HCV has a broad global prevalence. In some countries, such as Egypt and Mongolia, the virus has infected more than 20 % of the population.

In Europe, the United Kingdom and Scandinavia have the lowest reported prevalence rates of between 0.01 and 0.1 % (Shepard *et al.*, 2005). In Central Europe, the HCV prevalence ranges from 0.2 % in the Netherlands to 1.2 % in France. HCV prevalence ranges from 2.5 to 3.5 % in Southern Europe (Spain, Italy, Greece, and Southern France) (Esteban *et al.*, 2008).

In the United States, an HCV prevalence survey covering the period from 1999 to 2002 found that HCV infection was present in approximately 1.6 % of

the population, meaning that about 4.1 million people were infected with HCV at that time, of which 3.2 million individuals would have had a chronic infection. In Asia, it was reported that HCV prevalence ranges from 1.5-2.3 % in Japan, 1.6 % in Malaysia, 1.85 % in India, 3.2 % in China (with high rates in some poor areas such as Mongolia where it is about 31 %) and 3.2-5.6 % in Thailand (Shepard *et al.*, 2005; Sy and Jamal, 2006).

In Africa, Egypt has the highest reported prevalence rate in the world (15-20 %). Here it has been shown that the parenteral antischistosomal therapy (PAT) campaign from 1961 to 1986 by the Egyptian Ministry of Health played the major role in the spread of HCV throughout Egypt as it used contaminated glass syringes for injection of PAT (Frank *et al.*, 2000).

1.3 Disease and Risk Factors

Exposure to HCV can result in two stages of infection; an acute phase and a chronic phase. The acute phase is mild or asymptomatic in most of cases, while the chronic stage develops in 80 % of patients and can result in serious consequences such as liver cirrhosis (20 %) and HCC (5 %). Many factors can affect the rate of progression to cirrhosis and HCC such as alcohol abuse, age at time of infection, viral titre, viral genotype and co-infection with other viruses such as hepatitis B virus (HBV) and human immunodeficiency virus (HIV) (WHO, 1999).

The main routes of HCV transmission are through blood transfusion, use of blood products from unscreened donors, use of contaminated or inadequately sterilized medical equipments, and the intravenous drug abuse (the major route of HCV transmission in the UK). The minor routes of infection are from mother to baby, sexual transmission is possible but uncommon, tattooing, ear piercing, body piercing or acupuncture with unsterile equipment and sharing razors or toothbrushes contaminated with blood (Sy and Jamal, 2006; WHO, 1999).

1.4 History of HCV

Two different types of viral hepatitis causing two distinct liver diseases were identified during the World War II called infectious hepatitis and homologous serum hepatitis. The two liver diseases were later termed *hepatitis A* and *Hepatitis B* caused by hepatitis A virus (HAV) and hepatitis B virus (HBV), respectively (Major *et al.*, 2001).

After the discovery of hepatitis B surface antigen (HBsAg) in 1965 (Blumberg *et al.*, 1965) and its association with hepatitis B (Blumberg *et al.*, 1968; Okochi and Murakami, 1968; Stoliarova and Burlev, 1973), accurate and sensitive assays were developed to identify hepatitis B virus (Lander *et al.*, 1971).

A novel infection was documented in 1975 when it was noted that many cases of post-transfusion associated hepatitis were due to an unidentified infectious agent. This was neither due to HBV nor HAV and was clinically and epidemiologically distinct from these two infections, leading to be termed a non-A, non-B hepatitis (NANBH) (Feinstone *et al.*, 1975; Mosley *et al.*, 1977). The etiological agent causing NANBH was shown to be inactivated by chloroform, suggesting that it was an enveloped virus (Feinstone *et al.*, 1983). The approximate size of the virus was determined by filtration to be 30-60 nm in diameter (He *et al.*, 1987).

In 1989, a random lambda phage cDNA library was constructed from nucleic acid extracted from the plasma of a NANBH infected chimpanzee. This cDNA expression library was screened with serum from an NANBH patient as a source of NANBH antibodies. A complementary DNA clone, termed 5-1-1, was isolated which encoded an antigen specifically associated with NANBH and was shown to be derived from a viral RNA molecule of at least 10,000 nucleotides (Choo *et al.*, 1989). The 5-1-1 clone antigen became the basis for the first serologic assay for NANBH antibodies and the virus was subsequently described as hepatitis C virus (HCV). HCV was found to be associated with most cases of NANBH throughout the world (Choo *et al.*, 1990; Kuo *et al.*, 1989).

1.5 HCV Taxonomy

HCV is the single member of the *Hepacivirus* genus in the *Flaviviridae* family. The *Flaviviridae* (from the Latin word flavus, yellow) is a large family of related human and animal viruses with a positive sense single-stranded RNA genome. It includes with the *Hepacivirus* (from Greek word *hepar, hepatos*, liver) two other genera, *Flavivirus,* and *Pestivirus* (from the Latin pestis, plague) and a group of unclassified viruses (GB) (Fig. 1.2) (Lindenbach and Rice, 2001).

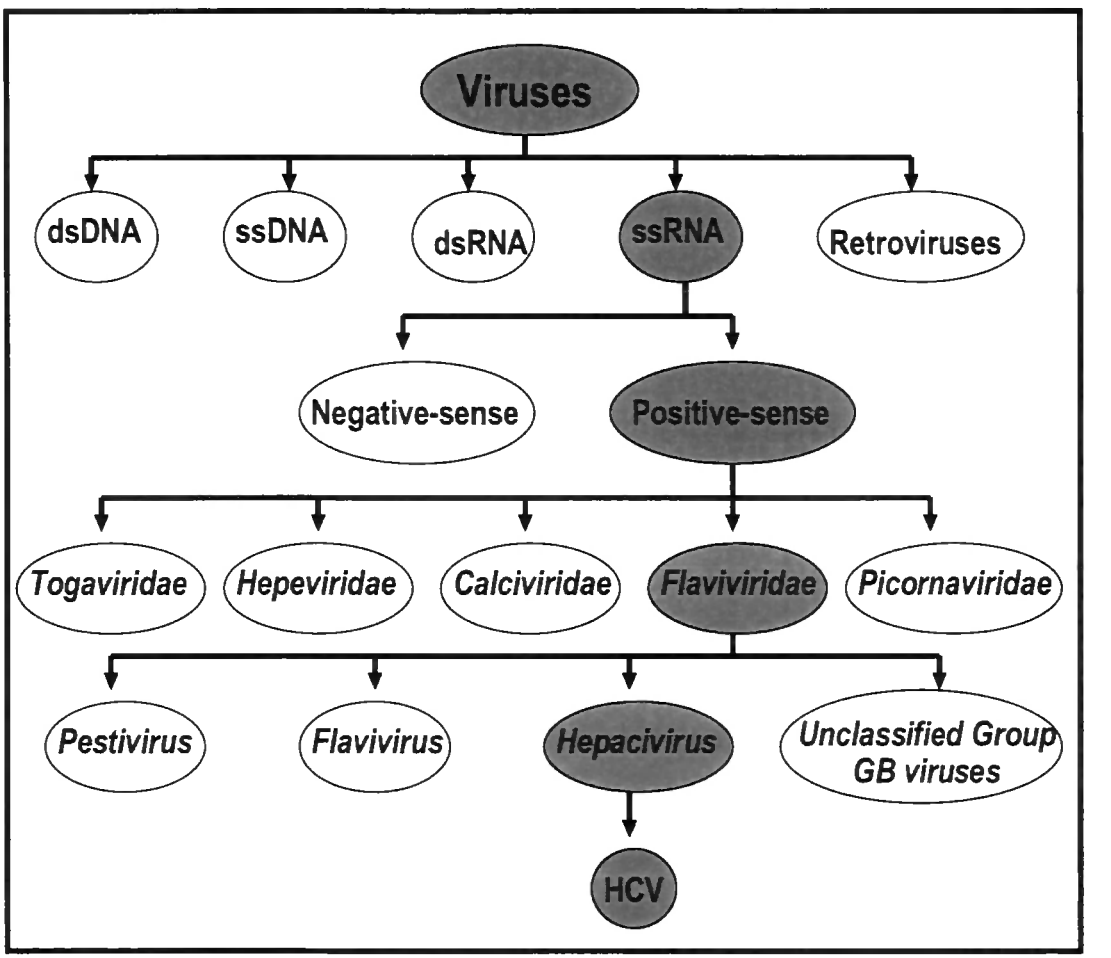

Fig. 1.2: HCV Taxonomy. HCV is ssRNA virus of positive sense. It belongs to *Hepacivirus* in *Flaviviridae* family (Lindenbach and Rice, 2001).

The *Flavivirus* genus is represented by yellow fever virus (YFV), dengue viruses (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV). The *Pestivirus genus* has animal pathogenic viruses such as bovine viral diarrhoea virus (BVDV), and classical swine fever virus (CSFV). The degree of relatedness between viruses within the *Flaviviridae* family is shown in Fig. 1.3 where HCV exhibits more similarity toward the GB viruses, an intermediate degree of relatedness with pestiviruses, and more distant relatedness to flavivirues. Sequence similarities in part of non-structural 3 protein (NS3) region of HCV and GBV-A and GBV-B are 47% and 55%, respectively, while the similarity in the same region between GBV-A and GBV-B is 43.5% (Simmonds, 1996). Although viruses of *Flaviviridae* belonging to different genera have different biological properties, the three genera share common features such as virion morphology, genome organization, protein sequence similarities and replication strategy (Miller and Purcell, 1990; Murphy *et al.*, 1995).

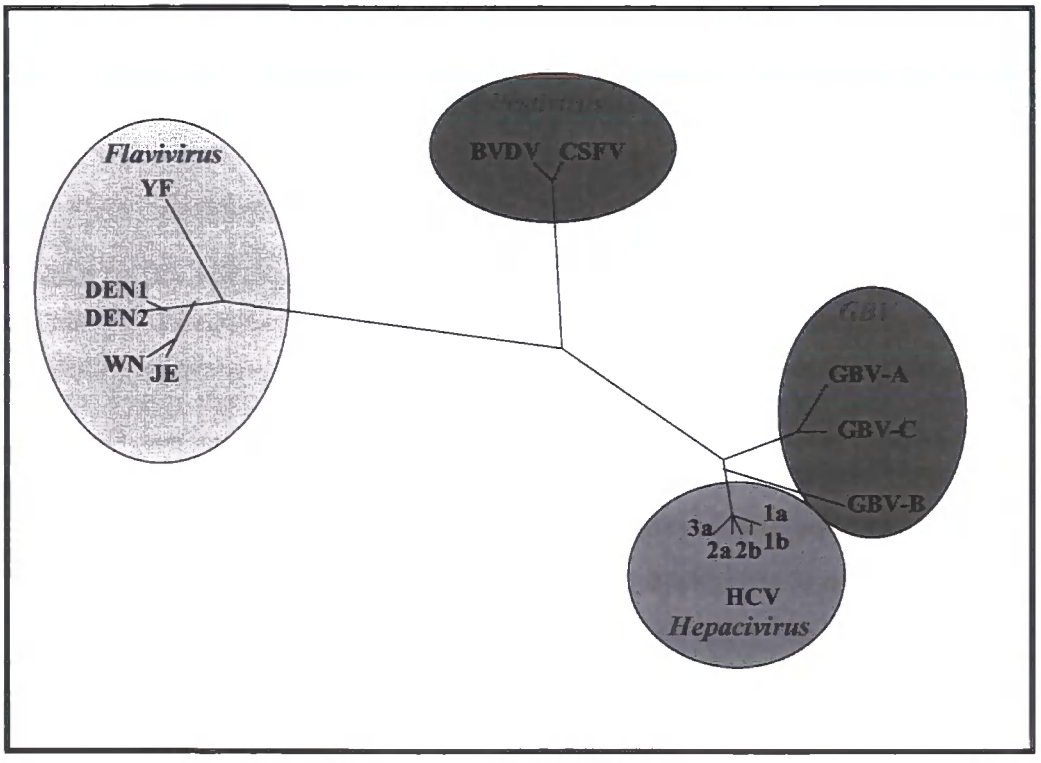

Fig. 1.3: Phylogenetic tree of *Flaviviridae* family members. It based on parsimony analysis of NS3 helicase regions. *Flaviviridae* contains *Hepacivirus, Flavivirus, Pestivirus* and the unclassified viruses (GB). HCV is more similar to GBV than other *Flaviviridae* genera (Lindenbach and Rice, 2001).

1.6 HCV Genotypes

Based on phylogenetic analysis of nucleotide sequences derived from subgenomic regions such as core/envelope 1 (E1) and non-structural 5B (NS5B), as well as complete genome sequences, HCV has been classified into six major genotypes (1 to 6), with a number of closely related subtypes (a, b, c, etc) within each genotype. The genotypes are relatively closely related with between 67 % to 69 % sequence similarities over the entire genome compared with 75 % to 80 % between subtypes (Fig. 1.4) (Simmonds *et al.*, 2005; Simmonds *et al.*, 1993).

Different methods have been used to determine HCV isolate genotype such as restriction fragment length polymorphism (RFLP) in the 5` untranslated region (5` UTR) and NS5 coding region (Davidson *et al.*, 1995), direct sequencing of an amplified segment of the genome like 5` UTR, core or NS5B (Okamoto, 1995), and by hybridization of an amplified segment of DNA to genotype specific probes from the 5` UTR region (Stuyver *et al.*, 1993).

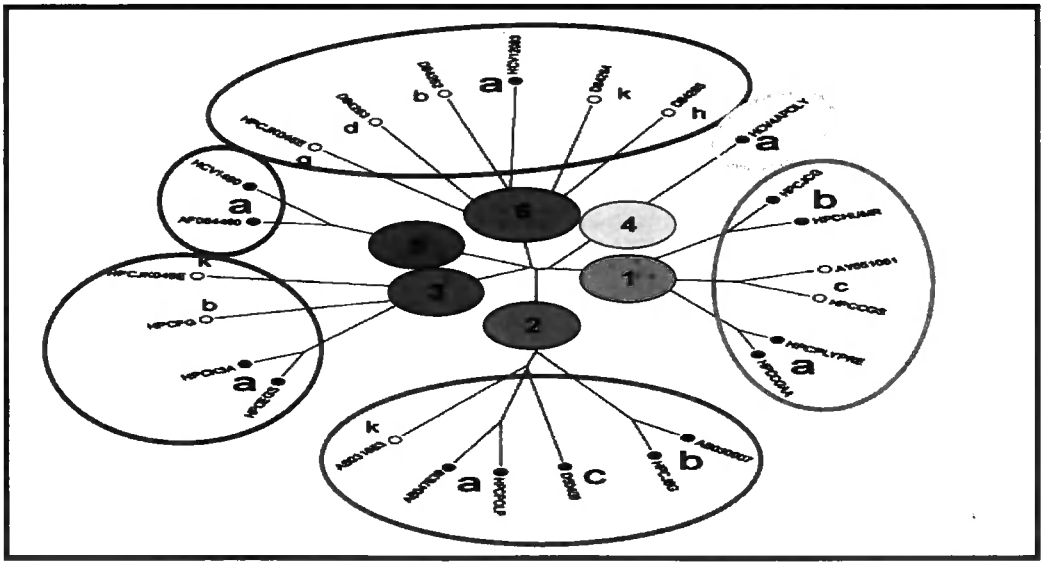

Fig. 1.4: Evolutionary tree of complete ORF sequences for HCV genotypes and subtypes (Simmonds *et al.*, 2005). HCV has 6 major genotypes denoted 1 to 6. Each genotype has many subtypes.

Genotypes 1a, 2a, and 2b have a global distribution, while genotype 4 is mainly concentrated in Africa and the Middle East. The distribution of genotypes worldwide is shown in Figure 1.5 and summarized in Table 1.2 (Simmonds, 1996; Simmonds *et al.*, 2005; Webster *et al.*, 2000).

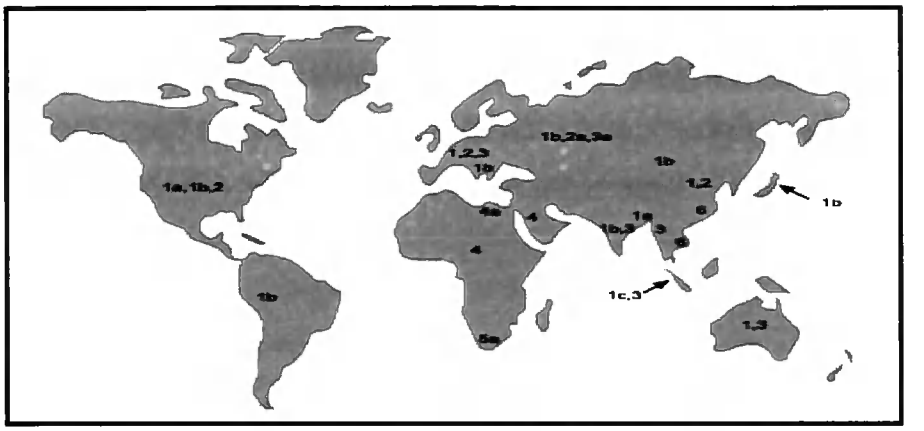

Fig. 1.5: Distribution of HCV genotypes and subtypes worldwide (Webster *et al.*, 2000). Genotypes 1a, 2a, and 2b have a global distribution, while genotype 4 is mainly concentrated in Africa and the Middle East.

Table 1.2: Distribution of HCV genotypes and subtypes worldwide

Country	Main genotypes
USA and Canada	1a, 1b, 2a, 2b, 3a
South America	1a, 1b, 2, 3a
Northern Europe	1a, 1b, 2b, 3a
Western Europe	1a, 1b, 2a, 2b, 3a
Southern Europe	1b, 2c
Eastern Europe	1b
Asia	
South and Eastern Asia	3, 6
Turkey	1b
Middle East	4
China	1b, 2a, 2b
Africa	
Egypt	4a
Central Africa	1, 4
Western Africa	2
South Africa	1, 2, 3, 5a
Northern central Africa	4
Pacific	
Australia	1a, 1b, 2a, 2b, 3a
Taiwan	1b, 2a, 2b
Japan	1a, 2a, 2b
Hong Kong	6a, 1b, 2a, 2b
Thailand	1b, 2, 3, 6
Malaysia	1b, 2, 3
Vietnam	1b, 2, 6

Different genotypes are found to respond differently to the current interferon treatment. In general, people infected with genotypes 1 and 4 respond poorly to interferon intervention compared with the other genotypes (el-Zayadi *et al.*, 1999; Enomoto *et al.*, 2007; Hino *et al.*, 1994; Jiao and Wang, 2005; Kamal *et al.*, 2007; Orito, 2001; Soriano *et al.*, 2005; Yuen and Lai, 2006; Zein *et al.*, 1996). Therefore determining the HCV genotype before starting treatment is important as it allows the duration of treatment and the dose given to be planned in advance.

The optimal duration of treatment for HCV using interferon-α 2b plus ribavirin is 48 weeks or longer for genotype 1b (Drusano and Preston, 2004; Yu *et al.*, 2006). However, for genotype 2 and 3 the duration of treatment required to develop a sustained virologic response (SVR) in 80-85 % of patients is only 24 weeks (Dalgard *et al.*, 2008). Treatment of genotype 4 for 36 weeks is considered sufficient (Kamal *et al.*, 2007), while treatment of genotype 6 for 48 weeks is recommended (Nguyen *et al.*, 2008). Genotype 5 infection is relatively easy to treat with response rates similar to those seen for genotypes 2 and 3 (Nguyen and Keeffe, 2005).

1.7 Virion Structure

The HCV particle is composed of a single-stranded positive-sense RNA molecule surrounded by a capsid made up of many identical subunits of virus core (C) protein which together form the nucleocapsid. This is surrounded by an envelope made up of a lipid bilayer derived from host membranes in which two virus glycoproteins (E1 and E2) are inserted and project about 6 nm from the surface of the virion particle (Fig. 1.6) (Kaito *et al.*, 2006).

The virion size is 55-65 nm in diameter, it is spherical and its envelope is derived from the cell membranes as a result of budding from the endoplasmic reticulum (ER) (Feinstone *et al.*, 1983; He *et al.*, 1987; Ishida *et al.*, 2001; Kaito *et al.*, 2006; Kaito *et al.*, 1994; Li *et al.*, 1995; Watanabe *et al.*, 1995).

Free HCV particles as detected in HCV infected patient plasma were determined to have densities of about 1.14-1.16 g/cm^3 using sucrose density gradient centrifugation (Kaito *et al.*, 1994). It was shown using immunoelectron microscopy that the HCV inner nucleocapsid is 33-40 nm in diameter and has an icosahedral shape, and it was subsequently shown to have a buoyant density of 1.22-1.25 g/cm^3 (Ishida *et al.*, 2001).

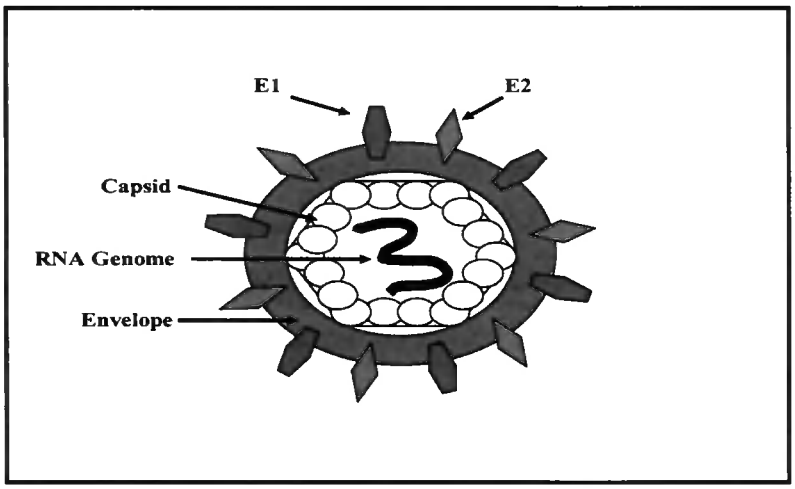

Fig. 1.6: HCV virion particle. The HCV particle is composed of a single-stranded positive-sense RNA molecule surrounded by a capsid which together form the nucleocapsid. This is surrounded by an envelope made up of lipid bilayer in which two virus glycoproteins (E1 and E2) are inserted (Lindenbach and Rice, 2001).

1.8 HCV Genome Organization

HCV genome is a single stranded, positive-sense RNA molecule of 9.6 kb (Choo *et al.*, 1991). It encodes a single long open reading frame (ORF) flanked by two untranslated regions (UTR) at the 5` and 3` ends. The ORF encodes a single polyprotein of approximately 3030 amino acids which is processed co- and post-translationally by various cellular and viral proteases into structural proteins (core, E1, and E2) localized at the N-terminus and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) localized at the C-terminus (Fig. 1.7) (Bartenschlager *et al.*, 1995; Grakoui *et al.*, 1993b). HCV genome organization is similar to that of flaviviruses and pestiviruses (Choo *et al.*, 1991).

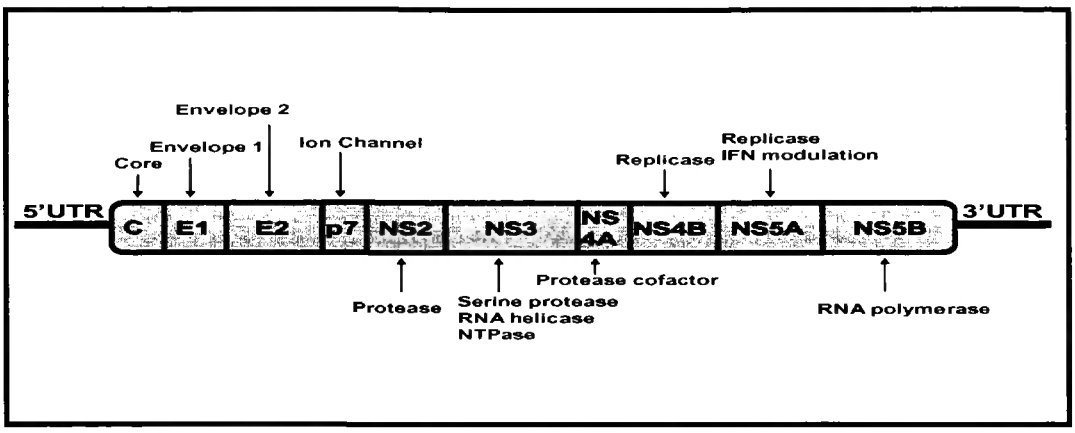

Fig. 1.7: HCV Genome Organization. It encodes a single ORF flanked by two 5` and 3` UTRs. The ORF encodes a single polyprotein which is processed co- and post-translationally by various cellular and viral proteases into ten proteins core, E1, E2 p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. The main functions of each protein are indicated (Bartenschlager *et al.*, 1995).

1.9 5` Untranslated Region (5` UTR)

HCV 5` UTR is a highly conserved RNA region of 341 nucleotides (Han *et al.*, 1991). The region has a high degree of secondary and tertiary structure with a number of stem-loop domains (Fig. 1.8) (Brown *et al.*, 1992; Honda *et al.*, 1999). It is characterized by the presence of an internal ribosomal entry site (IRES) of about 300 nucleotides that directs translation of the viral ORF in a cap-independent process (Brown *et al.*, 1992; Fukushi *et al.*, 1994; Reynolds *et al.*, 1996; Wang *et al.*, 1993).

The IRES is highly conserved between all HCV genotypes (Laporte *et al.*, 2000). Its secondary and tertiary structure was predicted from a comparative sequence analysis with other related pestiviruses such as BVDV and CSFV (Brown *et al.*, 1992). It consists of four structural domains, I to IV, in addition to a pseudoknot that forms a bridge between domains III and IV (Kieft *et al.*, 2002; Lukavsky *et al.*, 2003).

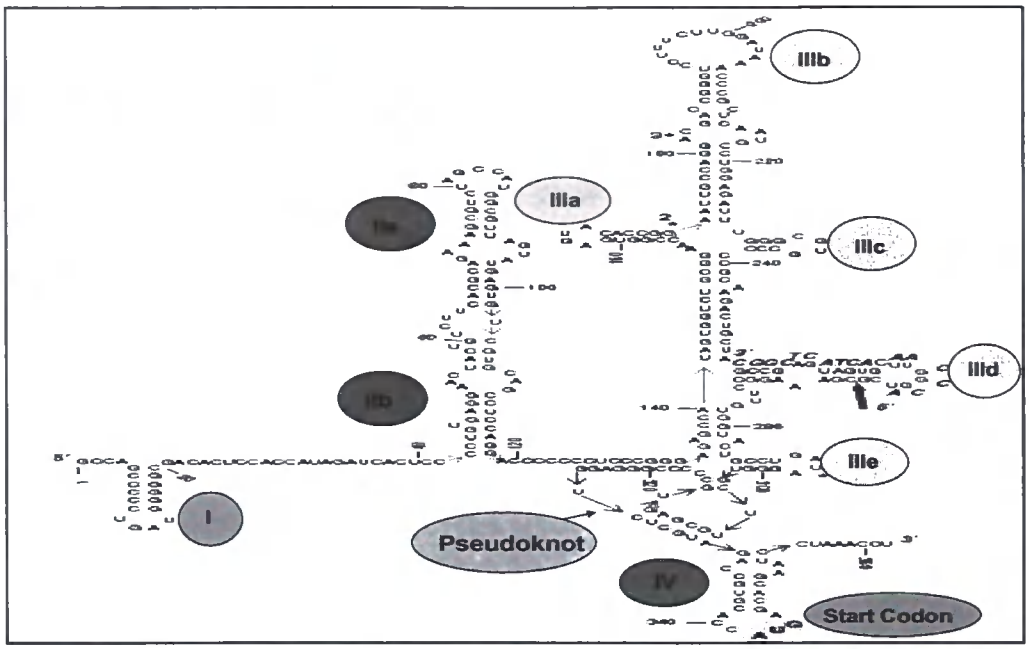

Fig. 1.8: Secondary structure of HCV 5` UTR (Honda *et al.*, 1999). It consists of four structural domains, I to IV and a pseudoknot forming a bridge between domains III and IV. Domain II has two stem loops while domain III has 5 stem-loops.

Domain I is a short hairpin not required for the translational activity of the IRES but it may have a regulatory role in translation. Domains II, III, and IV interact with the ribosomal 40S subunit and are essential for IRES activity (Honda *et al.*, 1996; Rijnbrand *et al.*, 2001). Domain II is highly conserved between the *Flaviviridae* family members suggesting an important role for this domain in protein translation (Honda *et al.*, 1999). Recently, Domain II was found to have a functional role in promoting eIF5-induced GTP hydrolysis and eIF2/GDP release from the initiation complex during the assembly of the 80S initiation complex (Locker *et al.*, 2007).

Domain III consists of 5 stem-loops denoted a, b, c, d, and e. It mediates the initial recruitment of translational initiation complexes. It can also interact with the 40S ribosome subunit and drives the formation of the IRES RNA-40S-eIF3 ternary complex. Domain IV may unfold to position the start codon in a suitable position for pairing with the initiator $tRNA^{met}$. The pseudoknot, between

domains III and IV, is likely to help in positioning domain IV and the start codon for translation initiation (Kieft *et al.*, 2001). IRES domains II, III, and IV work synergistically to tightly, and precisely position the initiation complex onto the HCV ORF start codon (Boehringer *et al.*, 2005; Kieft *et al.*, 2001).

IRES elements were first reported in the 5` UTR region of the poliovirus RNA genome (a picornavirus). It was observed that eukaryotic ribosomes could bind internally to the 5` UTR of poliovirus RNA and mediate cap-independent translation from the viral RNA. This was confirmed by showing that the poliovirus IRES mediated translation when inserted as an intercistronic spacer in a bicistronic mRNA reporter construct (Pelletier and Sonenberg, 1988). The same finding was also reported for the 5` UTR region of encephalomyocarditis virus RNA using a bicistronic reporter construct and *in vitro* translation assays (Jang *et al.*, 1988). An IRES also resides in the 5` non-coding region of the human immunoglobulin heavy-chain binding protein (BiP) mRNA, a stress response protein. BiP can be translated in poliovirus-infected cells when the cap-dependent translation of host cell mRNAs is inhibited indicating that the IRES translational initiation mechanism is also used by eukaryotic mRNAs and not only by viruses (Macejak and Sarnow, 1991).

The cap-dependent mechanism for protein translation of cellular mRNAs can be summarized in the following way: the 40S ribosome subunit associates with eIF3 followed by binding of the ternary complex (eIF2-GTP-Met-tRNA) to the 40S subunit. mRNA is activated by binding to cap-binding cellular factors such as eIF4E, eIF4G, eIF4A, and eIF4B to form the cap-binding complex in an ATP-dependent process facilitated by the presence of 7-methyl guanosine cap structure at the 5` end of cellular mRNAs (Fig. 1.9). This complex binds to the 40S subunit to form the 48S complex. The 48S complex starts ATP-dependent scanning of the mRNA for the AUG start codon and then the 60S subunit joins the 48S complex forming the 80S initiation complex which starts translation (Merrick, 2004; Semler and Waterman, 2008).

In cap-independent translation mediated by IRES, the IRES elements form secondary and tertiary structure on the RNA upstream to the AUG start codon. The IRES thus serves as a nucleus for the formation of the initiation complex by direct binding to the 40S subunit (Otto and Puglisi, 2004). Direct IRES-40S ribosome binding induces a conformational change in the 40S subunit to orchestrate the assembly of the translational preinitiation complex (Spahn *et al.*, 2001). This binding positions the ribosome on the initiation codon to start the translation by a prokaryotic-like mechanism without the need for eukaryotic initiation factors, such as eIF4E, eIF4A, eIF4B, and eIF4G (Fig. 1.9) (Hellen and Pestova, 1999; Otto and Puglisi, 2004; Pestova *et al.*, 1998; Sizova *et al.*, 1998).

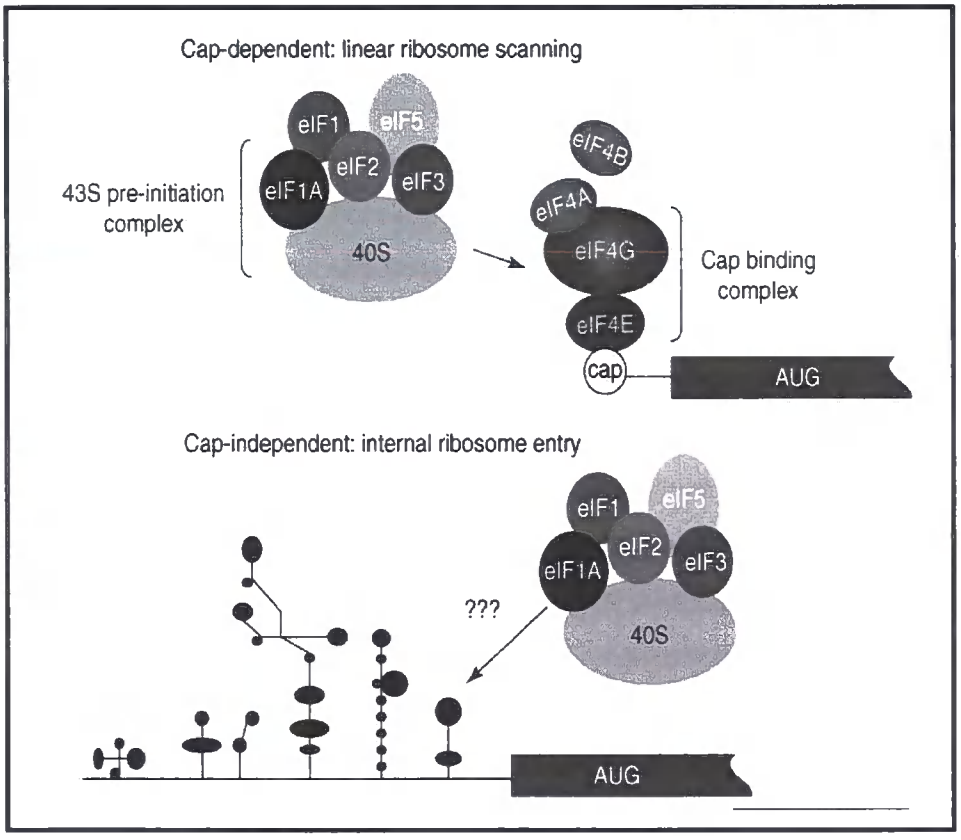

Fig. 1.9: Cap-dependent and Cap-independent (IRES) translational models (Semler and Waterman, 2008). Cap-dependent translation requires the presence of eukaryotic initiation factors such as eIF4E, eIF4A, eIF4B, and eIF4G while in the Cap-independent translation the IRES can mediate the direct binding with 40S subunit without the need for these factors.

Many viral and cellular proteins have been shown to bind the 5` UTR and play an important role in regulating IRES-mediated translation. HCV core protein can affect IRES-mediated translation by competitively binding to the stem loop III-d domain preventing its interaction with the 40S subunit (Shimoike *et al.*, 2006; Shimoike *et al.*, 1999), while NS5A was found to downregulate IRES activity by an unknown mechanism (Kalliampakou *et al.*, 2005).

La protein, RNA polymerase III transcription initiation and termination factor, was found to enhance IRES-mediated translation by binding to 5` UTR (Ali and Siddiqui, 1997). Transient expression of La protein completely restored the inhibitory action of IFN on IRES-mediated translation (Ali and Siddiqui, 1997; Shimazaki *et al.*, 2002). Nuclear ribonucleoprotein L (hnRNP L) was shown to interact with the 3` end of the IRES and this binding was correlated with the translational efficiency (Hahm *et al.*, 1998), while binding of eukaryotic translation initiation factor eIF3 to the HCV IRES enhanced the efficiency and accuracy of binding of the IRES to the 40S subunit resulting in better positioning of the start codon (Buratti *et al.*, 1998; Sizova *et al.*, 1998). The polypyrimidine tract-binding (PTB) protein was shown to interact with the 5` UTR and was required for IRES-mediated translation (Ali and Siddiqui, 1995) but others have reported no effect for PTB on IRES-driven translation (Brocard *et al.*, 2007; Nishimura *et al.*, 2008).

1.10 3` Untranslated Region (3` UTR)

The HCV genome terminates with a 3` untranslated region, 3` UTR. It consists of three elements, a short region that has a variable sequence among the genotypes which is followed by a polyU/UC stretch, and finally ends with a highly conserved region of 98 bases termed the core element or the 3` X region which forms three stem-loop structures (3` SL I, II, and III) (Fig. 1.10) (Kolykhalov *et al.*, 1996).

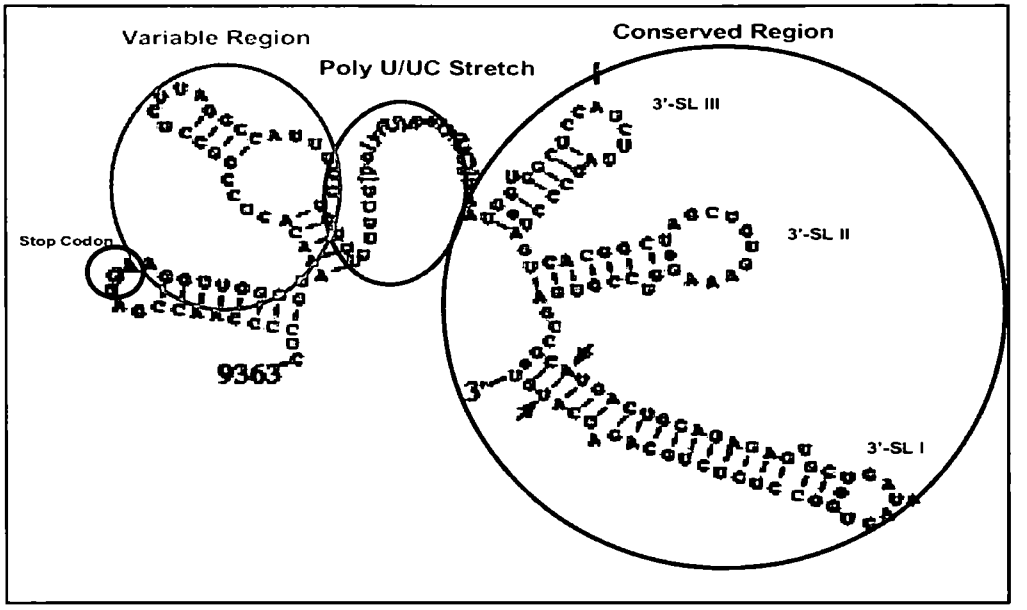

**Fig. 1.10: The secondary structure of HCV 3` UTR region.** It consists of three regions; a short variable region followed by a polyU/UC stretch, and finally ends with a highly conserved region of 98 bases, the 3` X region, which forms three stem-loop structures (Kolykhalov *et al.*, 1996).

The poly U/UC-repeat region varies in length and sequence amongst the different genotypes, whilst the 3` X region is highly conserved between genotypes and forms a distinct secondary structure. This region can interact with viral and cellular proteins such as the viral RNA-dependent RNA polymerase (RdRp) and PTB suggesting an important role for this region in HCV genome replication (Ito and Lai, 1997; Tanaka *et al.*, 1995; Tanaka *et al.*, 1996; Tsuchihara *et al.*, 1997; Yamada *et al.*, 1996). Like the 5` UTR, the poly U tract was shown to interact with La protein which was shown to enhance IRES-mediated translation (Spangberg *et al.*, 1999). The Poly U/UC and 3` X regions, but not the variable region, were subsequently shown to be critical for *in vivo* HCV infectivity (Yanagi *et al.*, 1999).

The 3` X region was shown to enhance translation mediated by the HCV IRES which may suggest a role for this region in the switch between translation and replication (Ito *et al.*, 1998; Song *et al.*, 2006). It was also possible that the role of this region in the enhancement of IRES activity is by enabling ribosome recycling for successive rounds of translation (Bradrick *et al.*, 2006).

1.11 Virus-Encoded Proteins

HCV genome encodes a single polyprotein (approximately 3030 amino acids) which is processed co- and post translationally by various cellular and viral proteases into 3 structural proteins, core (C), two envelope (E1, E2) proteins, and 7 non-structural proteins, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 1.11). In the following section, HCV protein structures and functions will be discussed in more detail.

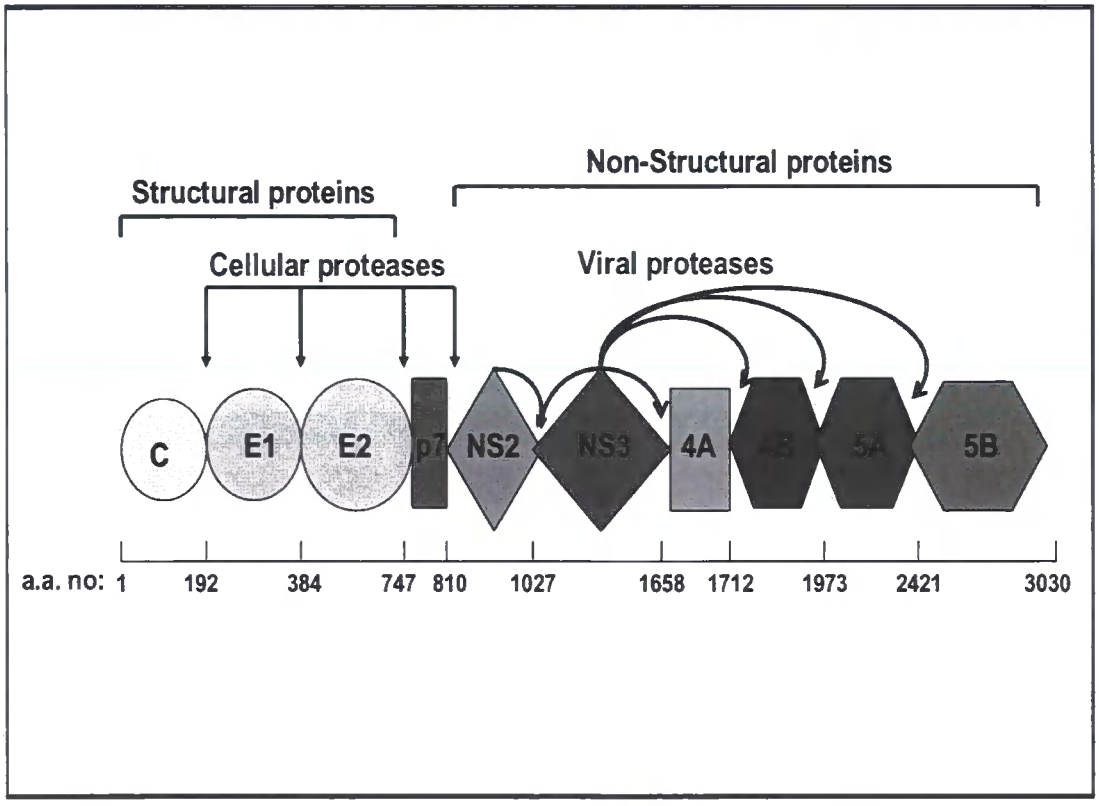

Fig. 1.11: HCV polyprotein processing. The virus polyprotein (approximately 3030 amino acids) is processed co- and post translationally by various cellular and viral proteases into 3 structural proteins, core (C), two envelope (E1, E2) proteins, and 7 non-structural proteins, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B. Amino acids at the start and end of each protein are indicated (Bartenschlager *et al.*, 1995).

1.11.1 Core Protein

The first cleavage product of the HCV polyprotein by cellular proteases is the basic capsid or core protein with a molecular weight of 23 kDa. The mature form of core is a C-terminaly truncated form of 21 kDa (Fig. 1.12). It is the main structural component of the viral capsid (Yasui *et al.*, 1998). The protein's sequence is highly conserved between HCV genotypes (Bukh *et al.*, 1994; Hijikata *et al.*, 1991). The core protein is synthesized in the cytoplasm and binds to the endoplasmic reticulum (ER) via its hydrophobic C-terminal region (Santolini *et al.*, 1994).

Based on the hydrophobic profile of core amino acid content, the protein consists of three domains. A hydrophillic domain 1 (amino acids 1-122) is localized at the N-terminus. Domain 2 (amino acids 123-174) is a hydrophobic region that mediates the attachment of core to lipid droplets after the signal peptide peptidase (SPP) cleavage protecting it from degradation upon the exposure to the cytoplasm (McLauchlan *et al.*, 2002). Domain 3 (amino acids 175-191) at the C-terminus is a highly hydrophobic region that acts as a signal sequence and membrane anchorage domain (Ma *et al.*, 2007; McLauchlan, 2000).

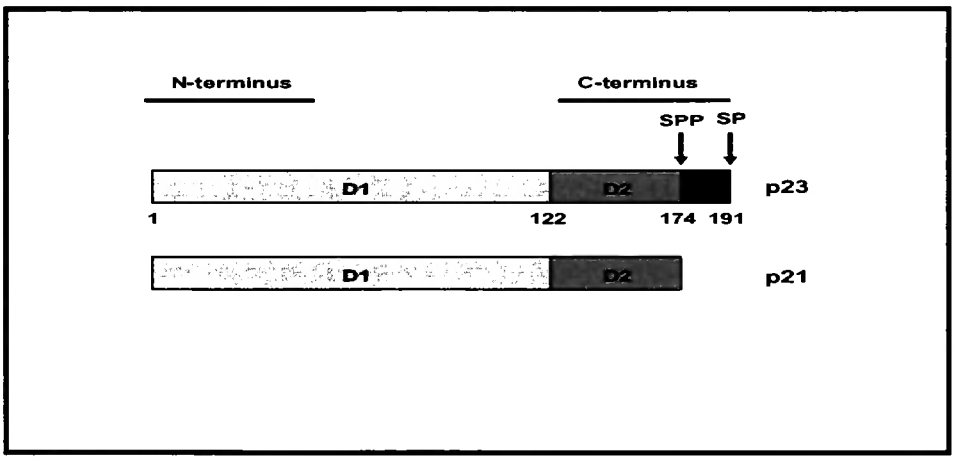

Fig. 1.12: HCV core products and their hydrophobic domains. Two forms of core protein (p23 and p21) are illustrated. Complete release and maturation of core requires cleavage of the polyprotein by a signal peptidase (SP) and signal peptide peptidase (SPP). Amino acid numbers at the borders of each domain are indicated (McLauchlan *et al.*, 2000).

Complete release and maturation of core protein requires cleavage of the polyprotein by a signal peptidase (SP) and another cleavage event of the transmembrane core signal peptide by an intramembrane protease, the signal peptide peptidase (SPP), (Fig. 1.13) (Hussy *et al.*, 1996; Ma *et al.*, 2007). This second cleavage is important for releasing core from the ER membrane permitting its targeting to lipid droplets, an intracellular organelle of neutral lipid surrounded by phospholipid monolayer bounded by proteinaceous coat, (McLauchlan *et al.*, 2002). It is also required for virus production by facilitating association of core with the viral genome, thereby creating suitable sites for capsid assembly (Targett-Adams *et al.*, 2008).

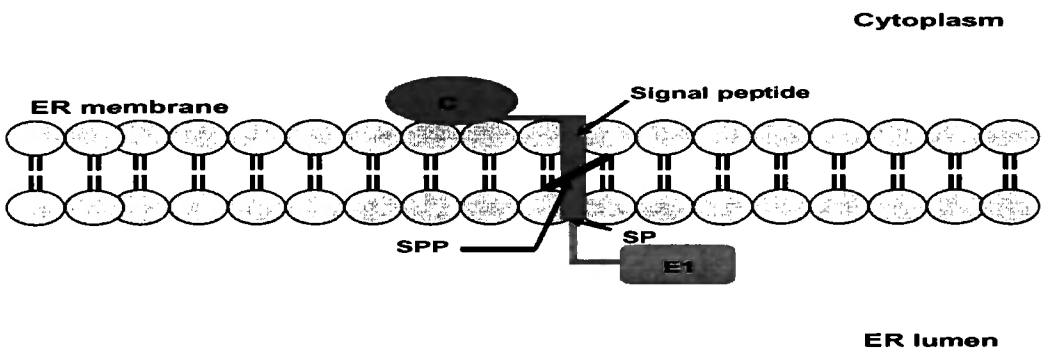

Fig. 1.13: Core processing by signal and signal peptide peptidases. Core is localized to ER via its hydrophobic C-terminal region. SP and SPP cleavages are required for complete release and maturation of core protein (Ma *et al.*, 2007).

Core possesses an RNA-binding activity in the N-terminus that is involved in nucleocapsid formation (Hwang *et al.*, 1995; Shimoike *et al.*, 1999). Core can modulate the expression of HCV ORF and a role has been suggested for it in the switch between viral polyprotein synthesis and genome encapsidation (Reynolds *et al.*, 1995; Shimoike *et al.*, 1999). Core interacts with itself in a homotypic reaction within the amino-terminal hydrophilic region starting the first stage of viral assembly (Matsumoto *et al.*, 1996). The C-terminal region can

interact directly with the E1 envelope protein which may be important in virus morphogenesis (Lo *et al.*, 1996; Nakai *et al.*, 2006). Core also interacts with NS5A and this interaction is thought to be important for the association of core with the viral genome and nucleocapsid assembly (Masaki *et al.*, 2008).

Core can also interact with the cytoplasmic domain of the lymphotoxin-beta receptor (LTβR), a member of the tumor necrosis factor receptor family. LTβR is involved in a number of cellular functions such as NF-kB activation, immunoregulatory responses, proliferation, differentiation, immune organ development, and apoptotic signaling. Interaction of core with LTβR may regulate these functions allowing HCV persistence and establishing a chronic infection (Matsumoto *et al.*, 1997; You *et al.*, 1999). Furthermore, core can induce apoptosis through induction of ER stress and calcium depletion (Benali-Furet *et al.*, 2005) or by the interaction with 14-3-3epsilon protein, a member in a family of conserved regulatory molecules expressed in all eukaryotic cells. This interaction results in dissociation of Bax/14-3-3epsilon complex releasing Bax that activates the mitochondrial apoptotic pathway (Lee *et al.*, 2007; Ray *et al.*, 1996).

Core can interact with a number of cellular proteins that regulate lipid metabolism such as the retinoid receptor α (RxRα), a transcriptional regulator for cellular lipid synthesis (Yamaguchi *et al.*, 2005). This may be directly linked to the development of liver steatosis, an accumulation of lipids in cell (Moriya *et al.*, 1997; Roingeard and Hourioux, 2008). It was also demonstrated that core can modulate microsomal triglyceride transfer protein activity which is responsible for very-low-density lipoprotein (VLDL) assembly leading to an accumulation of triglycerides in cells and ultimately induction of liver steatosis in mice (Perlemuter *et al.*, 2002). An *in vitro* study, showed that core protein from genotype 3a patients with liver steatosis caused an accumulation of high levels of triglyceride in Huh7 cells when compared to other genotypes (Abid *et al.*, 2005). This difference between genotypes was attributable to the presence of a phenylalanine residue at position 164 in domain 2 specific for genotype 3a where this residue has a higher affinity for lipids (Hourioux *et al.*, 2007).

An immunomodulating function has been suggested for core as it can interact with STAT1 preventing its IFN-induced phosphorylation and activation. This inhibits STAT1's nuclear translocation and disrupts the IFN-stimulated gene transcription (Bode *et al.*, 2003; de Lucas *et al.*, 2005; Kawamura *et al.*, 2006; Lin *et al.*, 2006). Core can also suppress the host immune response by dysregulating T and B lymphocytes function through the interaction with a complement receptor, gC1qR (Yao *et al.*, 2008). It has been shown that core can reduce interferon regulatory factor 1 (IRF-1) expression at the transcriptional level (Ciccaglione *et al.*, 2007).

Using computer-based sequence analysis, an alternate open reading frame (ARF) was identified encoding a predicted 16 kDa highly basic protein (F protein or ARFP). The alternative ORF overlaps the core-encoding region in the +1 reading frame relative to the main polyprotein ORF (Xu *et al.*, 2001). F protein ORF is present in all HCV genotypes but with different masses. F protein expression was observed during HCV infection and specific antibodies for this form were detected in HCV infected patients (Troesch *et al.*, 2005; Varaklioti *et al.*, 2002; Walewski *et al.*, 2001).

Two mechanisms have been suggested for production of F protein, the first is by a ribosomal frameshift translation mechanism and the second is by an internal translation initiation mechanism. Efficient translation initiation of the core+1 (F protein) ORF is mediated by internal initiation codon(s) within the core+1-coding sequence, located at the internal methionine codons 85/87 or at codon 26 (GUG or GCG in the viral variants) (Fig. 1.14) (Baril and Brakier-Gingras, 2005; Vassilaki *et al.*, 2008; Vassilaki and Mavromara, 2003). F protein was shown to have a cytoplasmic distribution with partial endoplasmic reticulum localization in interphase cells, whereas in dividing cells it localizes to the microtubules of the mitotic spindle (Vassilaki *et al.*, 2007).

F protein expression is suppressed by core which may suggest a link between F protein expression and the level of viral replication (Wolf *et al.*, 2008). F protein does not have the common functional properties with core

protein such as modulation of c-myc and p53 promoter activities and the biological function of F protein has been not determined yet (Basu *et al.*, 2004).

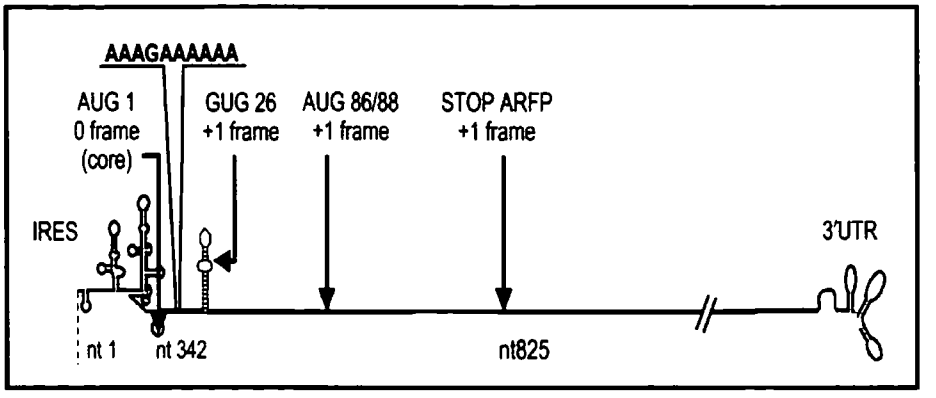

Fig. 1.14: HCV genome sequence indicating alternate reading frame protein (ARFP) translation start site (Wolf *et al.*, 2008). ARFP or F protein translation can start at codons 26 or 86 due to the ribosome slippage at 8-11 amino acids of core sequence.

1.11.2 Envelope Proteins

The envelope region of the HCV ORF encodes two envelope proteins that are processed co-translationally by host cellular proteases into two glycoproteins, E1 (31 kDa) and E2 (70 kDa) (Hijikata *et al.*, 1991). Both E1 and E2 consist of a large N-terminal ectodomain and a C-terminal transmembrane domain. E1 and E2 ectodomains are retained predominantly in the ER lumen as a result of the intramembrane signal peptide of core at E1 N-terminus and a retention signal sequence present in E1 and E2 C-terminal regions (Fig. 1.15) (Choukhi *et al.*, 1998; Cocquerel *et al.*, 1998; Dubuisson *et al.*, 1994; Duvet *et al.*, 1998; Martire *et al.*, 2001; Saadoun *et al.*, 2004; Santolini *et al.*, 1994). E1 and E2 ectodomains are heavily modified by N-linked glycosylation that is required for the proper folding of proteins in the ER (Slater-Handshy *et al.*, 2004).

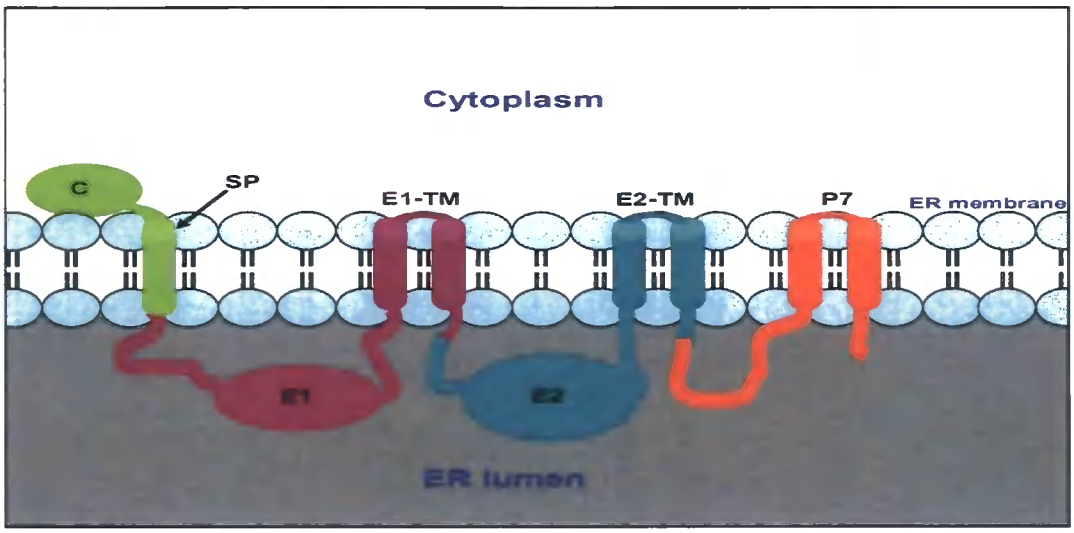

Fig. 1.15: Intracellular localization of core, E1, E2 and p7 and their transmembrane domains. E1 and E2 ectodomains are localized in ER lumen. E1 and E2 have a transmembrane (TM) domain at their C-termini. The figure is modified from (Roingeard and Hourioux, 2008).

Three different classes (I, II, and III) of fusion proteins have been identified in viruses. Class I is represented by orthomyxo-, paramyxo-, retro-, and filoviruses. Their fusion proteins mature by proteolytic cleavage of the nascent protein producing a membrane-anchored domain with a fusion peptide at or near the amino-terminus. Class I fusion proteins form spiky projections at the surface of the virion and undergo irreversible conformational changes to form a hairpin post-fusion structure of three-stranded coiled coils of α-helices (Teissier and Pecheur, 2007).

Class II fusion proteins are represented by the *Flaviviridae* (Tick-borne encephalitis virus (TBEV) and HCV) and *Togaviridae* (Semliki forest virus (SFV)). They are synthesized as a complex of two proteins lying flat on the surface of the virion arranged as β-sheets with three distinct domains (DI, DII, and DIII) forming a dimer of head-to-tail orientation. Class II fusion peptides are internal and located at the tip of domain II buried at the dimer interface. A change in pH causes irreversible conformational changes that expose the

fusion peptide to the cellular target membrane facilitating the interaction of the fusion peptide with the lipid bilayer (Fig. 1.16) (Teissier and Pecheur, 2007).

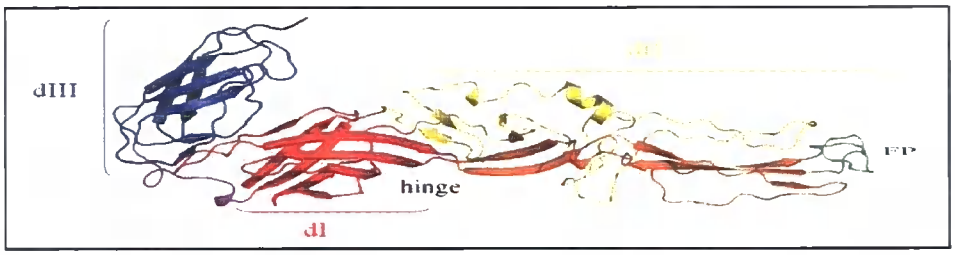

Fig. 1.16: Class II fusion protein of SFV. Class II fusion protein consists of three domains (dI, dII and dIII). The fusion peptide (FP) is present in domain II (Teissier and Pecheur, 2007).

Class III as represented by *Rhabdoviridae* (Vesicular stomatitis virus, VSV) and *Herpesviridae* (Herpes simplex virus, HSV1) shares some common features with class I such as three-stranded coiled-coils of α-helices and also some features of class II like a long three-stranded β-sheets. Class III fusion proteins are characterized by a reversible conformational change exposing the fusion peptide upon exposure to low pH (Fig. 1.17) (Teissier and Pecheur, 2007).

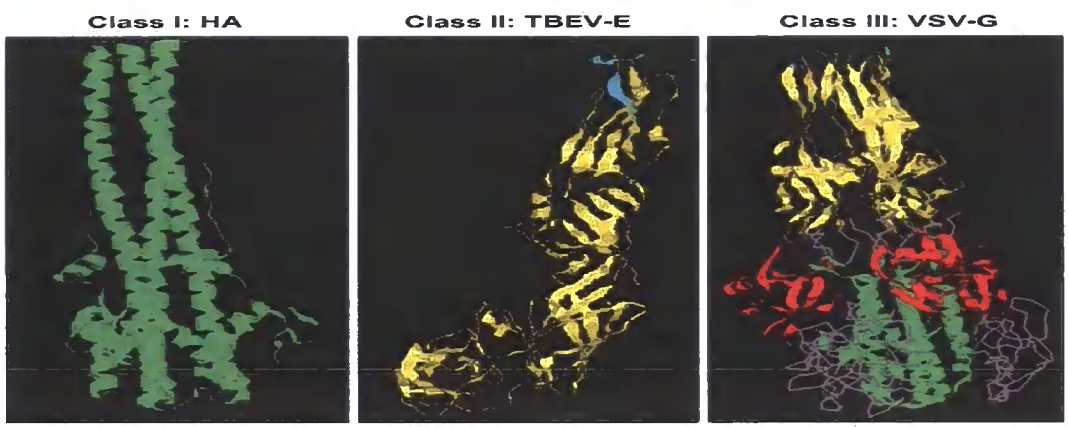

Fig. 1.17: 3D-structures of a prototype of the three classes of fusion proteins. Class I, trimer of the influenza hemagglutinin (HA) at low pH. Class I is three-stranded coiled coils of α-helices while class II arranged as β-sheets. Class III has both, three-stranded coiled-coils of α-helices and three-stranded β-sheets (Teissier and Pecheur, 2007).

Envelope protein E of TBEV was used as a template for building 3D-structure models for HCV E1 glycoprotein and the results suggested that E1 belongs to class II fusion proteins (Fig. 1.18) (Garry and Dash, 2003; Yagnik *et al.*, 2000).

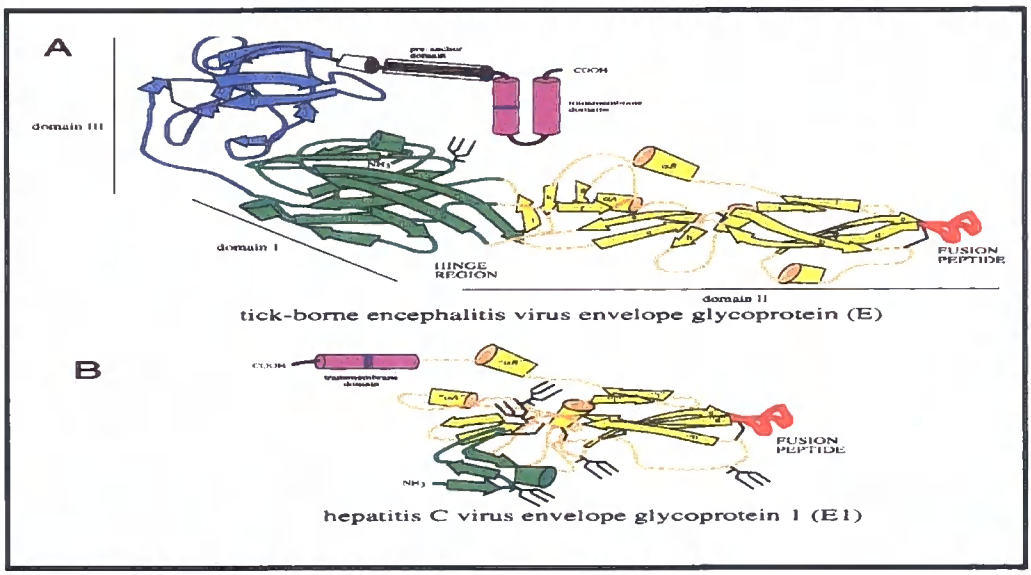

Fig. 1.18: 3D structure of TBEV-E and HCV-E1 proteins. Proteomics computational analyses were used to predict the structure of HCV E1 protein using TBEV-E protein structure as a template (Garry and Dash, 2003).

E1 and E2 associate slowly to form two types of stable complexes, a disulfide-linked form representing misfolded aggregates and a non-covalent E1E2 heterodimer complex which represents the native form of the HCV virion glycoproteins that constitute the virion envelope (Brazzoli *et al.*, 2005; Deleersnyder *et al.*, 1997; Grakoui *et al.*, 1993b). Productive folding of E2 is assisted by E1, while the folding of E1 is affected not only by E2 but also by core and may be other viral proteins (Brazzoli *et al.*, 2005; Merola *et al.*, 2001).

E1 contains a highly conserved hydrophobic domain at the C-terminus that is similar to the fusion peptides in other *Flavivirus* proteins suggesting that E1 has a role in fusing viral and cellular membranes (Garry and Dash, 2003). E1 and E2 transmembrane regions strongly partition into phospholipid membranes,

bind and interact with negatively-charged phospholipids inducing membrane structural changes leading to viral and cellular membrane fusion (Perez-Berna *et al.*, 2008a; Perez-Berna *et al.*, 2008b).

An unglycosylated form of E2 (38 kDa) has been observed and, in contrast to the glycosylated form, is localized mainly in the cytoplasm. This form can interact with the interferon-inducible protein kinase, PKR, in the cytosol suggesting that E2 has a role in interferon resistance (Pavio *et al.*, 2002). E2 has a region with sequence homology to phosphorylation sites in PKR and the translation initiation factor eIF2α termed E2-PKR-eIF2alpha phosphorylation homology domain (PePHD). Via this domain, E2 inhibits phosphorylation of eIF2α by PKR negating its inhibitory effect on protein synthesis (Taylor *et al.*, 1999; Taylor *et al.*, 2001).

E2 has three hypervariable regions, HVR1, HVR2, and HVR3 located within its N-terminal region (Fig. 1.19) (Troesch *et al.*, 2006; Weiner *et al.*, 1991). The diversity inside these regions is a result of the accumulation of mutations during viral replication due to the high error rate of the RNA-dependent RNA polymerase (RdRp) (Pileri *et al.*, 1998). This variability is the basis of immune escape that contributes to viral persistence (Farci *et al.*, 2000; Kato *et al.*, 1992; Kato *et al.*, 1994). Heterogeneity within HVR regions generates multiple forms of closely related but distinct species (quasi-species) of HCV within the same individual and these quasi-species are associated with interferon treatment failure in some cases (Enomoto *et al.*, 1994; Morishima *et al.*, 2006).

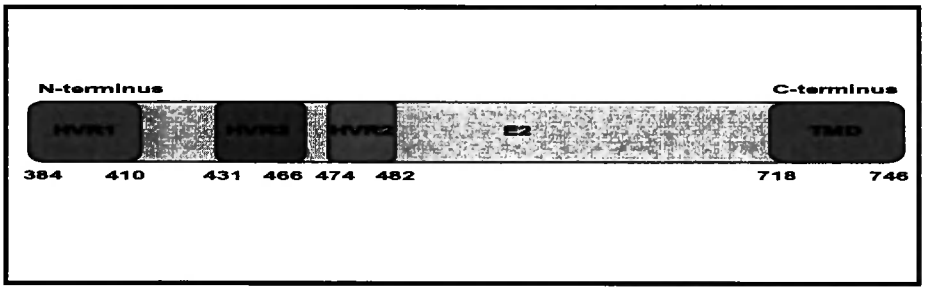

Fig. 1.19: HCV E2 glycoprotein main domains. E2 has three hypervariable regions, HVR1, HVR2, and HVR3 located within its N-terminal region. TMD is localized at the C-terminus (Troesch *et al.*, 2006).

E2 is responsible for HCV attachment to the target cell by binding to potential cell receptors. A number of molecules have been implicated as receptors for HCV: CD81 (a member of the tetraspanin family cell surface membrane proteins) which expressed on various cell types including hepatocytes (Pileri *et al.*, 1998), the low density lipoprotein (LDL) receptor (Thomssen *et al.*, 1992), the very low density lipoprotein (VLDL) receptor (Agnello *et al.*, 1999; Monazahian *et al.*, 1999; Wunschmann *et al.*, 2006) and the scavenger receptor class B type I (SR-BI) (Grove *et al.*, 2007; Scarselli *et al.*, 2002; Zeisel *et al.*, 2007). Co-expression of CD81 and SR-BI receptors in non-hepatic cells does not confer susceptibility to HCV entry suggesting that an additional hepatocyte-specific co-factor(s) is required for HCV entry (Bartosch *et al.*, 2003).

Recently, claudin-1 (CLDN1), a tight junction (TJ) protein that is highly expressed in the liver, was identified as an essential co-receptor for HCV entry. Expression of CLDN1 in non-hepatic 293T cells which naturally express CD81 and SR-BI did confer susceptibility to HCV infection (Evans *et al.*, 2007), although CLDN1 expression failed to make other non-hepatic cells (HeLa and NIH3T3 cells) susceptible to HCV infection; this may be due to the intracellular distribution of CLDN1 where it was highly enriched at sites of cell-cell contact in permissive cell lines but resided predominantly in intracellular vesicles in non-permissive cells (Yang *et al.*, 2008). Cell entry via SR-BI and CD81 was enhanced by the presence of HDL (Voisset *et al.*, 2005; Voisset *et al.*, 2006).

Thus it is clear that many cellular receptors and co-receptors are involved in HCV attachment and entry. The initial viral attachment to the cell membrane may involve glycosaminoglycans and LDL followed by interaction with three entry factors: SR-BI, CD81, and CLDN1. After that, the virus enters the cells by endocytosis followed by fusion of viral and cellular membranes (Fig. 1.20) (Helle and Dubuisson, 2008). Expression of EWI-2wint, a partner of CD81, in hepatocytes inhibits the interaction of HCV envelope glycoproteins with CD81 blocking the HCV entry suggesting that a lack of EWI-2wint expression in hepatic cells is a contributing factor to the hepatic tropism of HCV (Rocha-Perugini *et al.*, 2008).

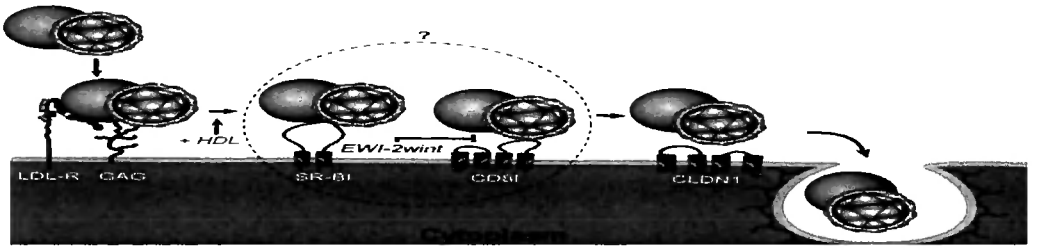

Fig. 1.20: A model for HCV entry. The viral attachment to the cell membrane may involve glycosaminoglycans and LDL followed by interaction with three entry factors: SR-BI, CD81, and CLDN1. After that, the virus enters the cells by endocytosis (Helle and Dubuisson, 2008).

1.11.3 p7 Protein

p7, a small (33 amino acids) hydrophobic protein, is localized within the endoplasmic reticulum. It has two putative transmembrane domains (TMDs) connected by a cytoplasmic loop (Fig. 1.14) (Carrere-Kremer *et al.*, 2002). It is released from the nascent polyprotein by cellular signal peptidases that reside in the ER (Mizushima *et al.*, 1994).

In vivo and *in vitro* cross-linking studies in HepG2 cells have indicated that p7 oligomerizes as a hexamer to form a calcium ion channel that is blocked by the antiviral drug, amantadine (Griffin *et al.*, 2003). This activity is similar to that of viroporins, a class of virally encoded proteins that homo-polymerize to form ion channels, suggesting a role for p7 in the flow of ions from the ER into the cytoplasm (Gonzalez and Carrasco, 2003). A genotype 1a clone with a mutagenized p7 is not infectious when introduced into chimpanzees suggesting that p7 is essential for infectivity (Sakai *et al.*, 2003). A role for p7 in virus assembly and release has been suggested as a mutation in p7 suppresses virus release and impairs production of infectious particles (Jones *et al.*, 2007; Steinmann *et al.*, 2007).

1.11.4 NS2 Protein

NS2 (23 kDa) is cleaved from the polyprotein by a cellular signal peptidase at the p7/NS2 boundary and by a viral protease at the NS2/NS3 junction (Santolini *et al.*, 1995). Based on the hydrophobicity profile and glycosylation studies, NS2 topology was predicted to be a transmembrane protein with four transmembrane domains with both termini localized to the ER lumen (Fig. 1.21) (Yamaga and Ou, 2002). The protease responsible for the cleavage at the NS2/NS3 junction is a cysteine protease composed of the C-terminus of NS2 and the N-terminus of NS3 (Fig. 1.22). It is cis-active and requires zinc ions for its activity (Hijikata *et al.*, 1993; Lorenz *et al.*, 2006; Tedbury and Harris, 2007).

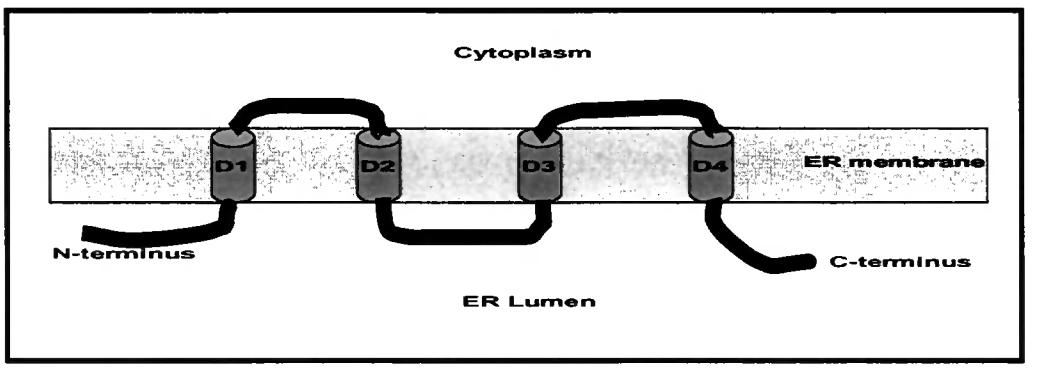

Fig. 1.21: Predicted NS2 topology. NS2 has four transmembrane domains with both termini localized in the ER lumen (Yamaga and Ou, 2002).

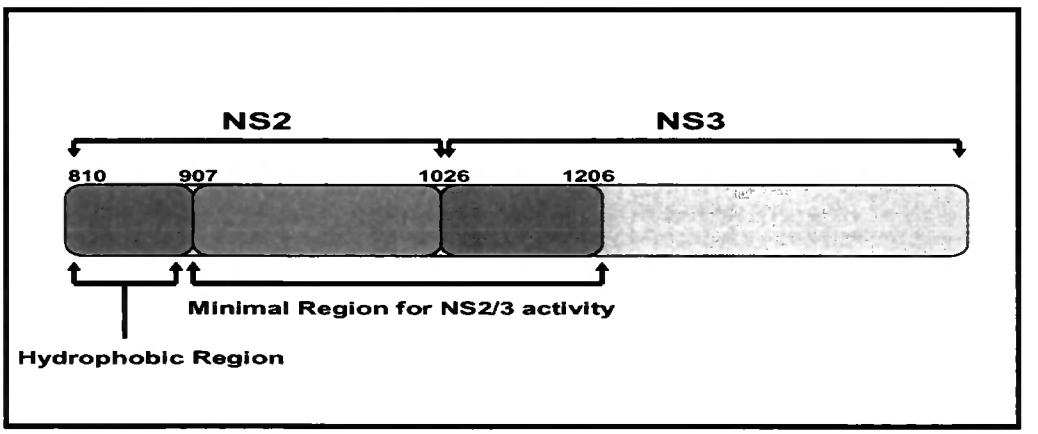

Fig. 1.22: NS2/3 protease structure. A schematic diagram showing main domains of NS2 including the minimal region required for NS2/3 activity (Welbourn and Pause, 2007).

Although NS2 is not essential for viral replication, as subgenomic replicons can replicate without NS2 (Lohmann *et al.*, 1999), NS2 was found to form homodimers and interact with the other non-structural proteins suggesting that NS2 may indirectly participate in replication by stabilization of the replication complex or by recruiting cellular factors that enhance replication (Dimitrova *et al.*, 2003). NS2/3 protease activity is essential for viral replication *in vivo* as a HCV genome lacking NS2/3 activity failed to establish an infection when introduced into chimpanzees (Kolykhalov *et al.*, 2000).

NS2/3 processing is required for accumulation of sufficient NS3 protein for HCV RNA replication as fusion of NS2 with NS3 induces rapid degradation of NS3 which could be a mechanism for regulation of viral replication (Welbourn *et al.*, 2005; Welbourn and Pause, 2007). NS2 protein is also required for NS5A phosphorylation (Liu *et al.*, 1999). NS2 mutant genome impaired the intracellular accumulation of virus particles and failed to produce an infectious virus (Jones *et al.*, 2007).

NS2 was shown to interact with the pro-apoptotic CIDE-B (cell death-inducing DNA fragmentation factor (DFF45)-like effector) protein inhibiting its apoptotic effect on cytochrome c release from the mitochondria (Erdtmann *et al.*, 2003). NS2 can inhibit the expression of a number of cytokines such as IFN (IFN-α, IFN-β and IFN-lambda1/IL-29) and chemokines (CCL5, CXCL8 and CXCL10) suggesting an important role for NS2 in negating the host antiviral response that may lead to viral persistence (Kaukinen *et al.*, 2006).

1.11.5 NS3 protein

NS3 (70 kDa) has a serine protease activity that is responsible for cleavage events at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B junctions and with the NS2 cysteine protease for the NS2/NS3 boundary cleavage (De Francesco and Steinkuhler, 2000; Grakoui *et al.*, 1993a; Lorenz *et al.*, 2006). The cleavage at NS3/NS4A junction occurs in *cis* but the cleavage at the other junctions occurs in *trans* (D'Souza *et al.*, 1994; Lin *et al.*, 1994). NS3

serine protease activity requires NS4A as a cofactor to form a complex essential for efficient cleavage at the NS3/NS4A, NS4A/NS4B, and NS4B/NS5A junctions. NS4A enhances, but is not essential for, NS3 mediated cleavage at the NS5A/NS5B junction (Failla *et al.*, 1994; Lin *et al.*, 1994; Lohmann *et al.*, 1996; Tanji *et al.*, 1995). The NS3 protease catalytic site is formed by a catalytic triad of His57, Asp81, and Ser139 (Fig. 1.23). These three catalytic residues are highly conserved in all identified serine proteases in the flaviviruses and pestiviruses (Grakoui *et al.*, 1993a).

NS3 structure has been shown to have Zn^{+2} coordinated by Cys96, Cys98, Cys144, and His148 residues. Only a slight inhibition on NS3 protease activity was observed using EDTA suggesting that Zn^{+2} may perform a structural rather than a functional role such as stabilization of the active site orientation (Kim *et al.*, 1996; Lin and Rice, 1995).

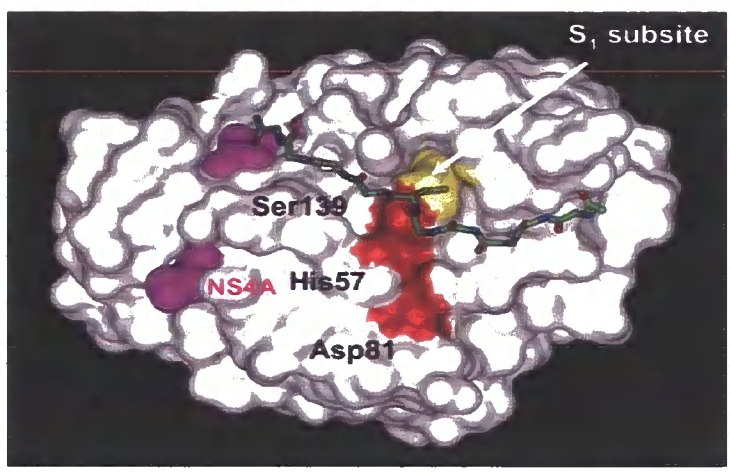

Fig. 1.23: 3D structure of NS3/4A protease. It is showing the three residues (His57, Asp81, and Ser139) that form the catalytic triad in red, the substrate site (S1) in yellow, and NS4A in purple (De Francesco and Carfi, 2007).

NS3 also possesses nucleoside triphosphatase (NTPase) and RNA helicase enzymatic activities located within the C-terminal domain similar to other *Flaviviridae* members (Choo *et al.*, 1991; Kim *et al.*, 1997; Suzich *et al.*, 1993). As NS3 contains a Asp-Glu-Cys-His motif, it is classified in the DEXH subfamily in the DEAD box RNA helicase family (Koonin, 1991; Linder *et al.*,

1989). NS3 was able to unwind up to 500 bp of RNA/DNA or RNA/RNA duplexes in the 3` to 5` direction. The hydrolysis of nucleotides or deoxyribonucleotides by NTPase activity possibly supplies the energy needed for the unwinding reaction (Suzich *et al.*, 1993). There is an interplay between the three enzymatic activities, serine protease, NTPase and helicase, that may regulate viral replication (Gwack *et al.*, 1996; Morgenstern *et al.*, 1997).

NS3 has been shown to limit expression of multiple host defence genes by inducing specific proteolysis of the Toll-like receptor 3 adaptor protein, TRIF, inhibiting the activation of IFN-regulatory factor 3 (IRF-3) and nuclear factor kappa B (NF-κβ) via the TLR3 signalling pathway, thereby facilitating establishment of a persistent infection (Li *et al.*, 2005). NS3 also inhibits the RIG-I signalling pathway through the cleavage of the interferon promoter stimulator-1 (IPS-1) adaptor protein resulting in further suppression of downstream activation of IRF-3 and NF-κB (Foy *et al.*, 2005; Johnson *et al.*, 2007; Loo *et al.*, 2006).

1.11.6 NS4 proteins

The HCV NS4 region encodes two non-structural proteins (NS4A and NS4B) that are cleaved by NS3 protease in *cis* at the NS3/4A junction and in *trans* at the NS4A/4B and NS4B/5A junctions (Lin *et al.*, 1994). NS4A (8 kDa) acts as a cofactor for NS3 that enhances its activity and metabolic stability by optimizing the orientation of the catalytic residues to form the substrate recognition site (Failla *et al.*, 1994). The first 20 amino acids of NS4A are hydrophobic and form a transmembrane domain that anchors NS3 to the ER outer surface facilitating formation of the replicase complex (Fig. 1.24) (Brass *et al.*, 2008). The next amino acids from 21 to 32 in the central region of NS4A are directly involved in the interaction with NS3, serving as a protease cofactor that is essential for proteolytic activity (La Torre *et al.*, 1998; Lin *et al.*, 1995; Tomei *et al.*, 1996).

Substitution mutations in the C-terminal domain of NS4A blocked NS5A hyperphosphorlylation and viral RNA replication suggesting that NS4A encodes one or more activities that are critical for NS5A hyperphosphorylation and viral replication (Lindenbach *et al.*, 2007). Anti-NS4A antibodies could be used to predict the IFN-treatment response of patients with chronic hepatitis C as these antibodies were observed at higher titers in individuals with sustained response (Desombere *et al.*, 2007).

NS4B is a hydrophobic 27-kDa protein localized to the ER membrane (Hugle *et al.*, 2001). NS4B induces a specific membrane rearrangement forming a membrane associated foci (MAF), commonly referred to as the membranous web, which can serve as scaffold for the formation of the viral replication complex (Egger *et al.*, 2002).

NS4B has four transmembrane domains with both N- and C-termini localized to the cytoplasm. After processing with NS3 at NS4A/4B junction, the N-terminus region is rearranged and translocated to form a fifth transmembrane domain with the N-terminus tail localized to the ER lumen by an unknown mechanism suggesting two possible orientations for NS4B (A and B, Fig. 1.24). This translocation may rearrange and prepare intracellular membranes for RNA replication and/or virus assembly (Lundin *et al.*, 2003). The translocation was reduced in the presence of NS5A and a mutation within the new transmembrane domain (labeled X in Fig. 1.24) failed to rearrange ER membranes to form the membranous web (Lundin *et al.*, 2006).

An amphipathic helix (AH) distinct from the other five TMDs was identified at the NS4B N-terminus which can mediate membrane association like the transmembrane domains. Disruption of this domain by mutagenesis inhibited RNA replication as the components of replication complex were found to be localized incorrectly. Interestingly, the AH is highly conserved among HCV genotypes suggesting an important role for this region in the HCV life cycle (Elazar *et al.*, 2004; Lundin *et al.*, 2006).

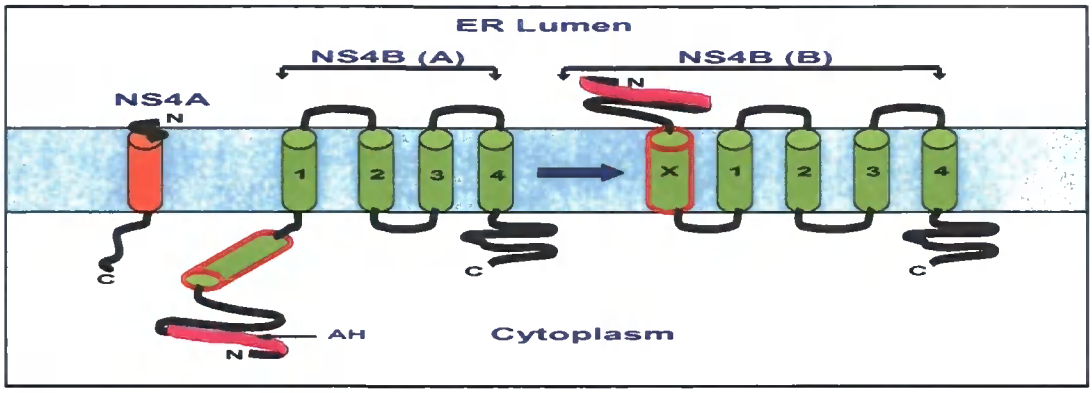

Fig. 1.24: NS4A and NS4B topology. The diagram is showing the two proposed orientation of NS4B. After processing with NS3 at NS4A/4B junction, the N-terminus region is rearranged and translocated to form a fifth transmembrane domain with N-terminus tail localized to the ER lumen. The AH region is indicated in red colour (Lundin *et al.*, 2006).

1.11.7 NS5 proteins

The NS5 region encodes two proteins, NS5A and NS5B, that are processed by NS3 (Grakoui *et al.*, 1993a). NS5A is present in two forms within infected cells; a 56 kDa phosphoprotein and 58-kDa highly phosphorylated protein (Kaneko *et al.*, 1994; Koch and Bartenschlager, 1999). NS5A consists of three domains (I, II and III) connected by two small loops and has an amphipathic α-helix at the N-terminus of domain I. Domain I contains a zinc binding site and by disrupting this site, RNA replication was inhibited suggesting that Zn binding is critical for viral replication (Fig. 1.25) (Brass *et al.*, 2002).

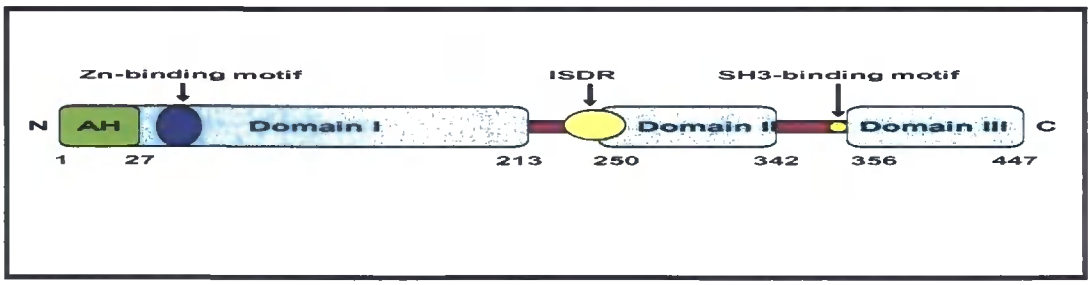

Fig. 1.25: NS5A main domains. NS5A consists of three domains (I, II and III) connected by two small loops with an amphipathic α-helix and zinc binding site at the N-terminus of domain I. Domain II has the ISDR region while the polyproline-rich region is localized at the stem loop II (Brass *et al.*, 2002).

The phosphorylation of NS5A is conserved in all flaviviruses suggesting an important role for this process in viral replication (Reed *et al.*, 1998). NS5A phosphorylation could have a role in regulation of viral replication as cell culture adaptive mutations of HCV replicons that increased RNA replication in Huh7 resulted in a reduction or disappearance of the hyperphosphorylated form (Blight *et al.*, 2000; Neddermann *et al.*, 2004). Hyperphosphorylation of NS5A was shown to disrupt the interaction with the human vesicle-associated membrane protein-associated protein A (hVAP-A), a cellular protein required for efficient replication of the genome suggesting that cellular adaptive mutations increase replication by reducing the phosphorylation-dependent dissociation of the RNA replication complex (Evans *et al.*, 2004). Furthermore, virus assembly and production were shown to be regulated by phosphorylation of NS5A at a specific serine residue (Ser457) without affecting RNA replication levels. This phosphorylation was shown to be mediated by casein kinase II (CKII). Targeting this site by chemical inhibitors or small interfering RNA (siRNA) impaired the release of infectious virus particles (Tellinghuisen *et al.*, 2008).

NS5A contains several proline-rich (PXXP) sequences in the loop region between domain I and II which are highly conserved among HCV species (Kaneko *et al.*, 1994). This polyproline region is able to interact with Src-homology 3 (SH3) domains of a variety of cellular proteins such as *Src*-family tyrosine kinases (e.g. Fyn, Lyn, Hck, and Lck) and perturb their signalling pathways which may contribute to the pathogenesis of the virus (Macdonald *et al.*, 2004; Shelton and Harris, 2008).

NS5A is able to inhibit the epidermal growth factor (EGF) or a virally induced phosphorylation of the mitogen-activated protein kinase (MAPK) or the extracellular signal-regulated kinase 1 and 2 (ERK1/2) (Macdonald *et al.*, 2005; Tan and Katze, 2001). This inhibition could be mediated by the interaction of NS5A with the growth factor receptor-bound protein 2 (Grb2) via SH3-binding domains interfering with normal transduction pathways in the infected cells (Fig. 1.26) (Tan *et al.*, 1999). It has been also shown that NS5A is able to inhibit the trafficking of EGFR to the late endosome attenuating the EGFR signalling (Mankouri *et al.*, 2008). MAPK mediated phosphorylation of STAT1 and 2 is

required for maximal transcriptional activity of IFN-stimulated gene factor 3 (ISGF3). MAPK function is essential for IFN-α-induced IFN-stimulated response element (ISRE)-dependent gene transcription (David *et al.*, 1995), a key step in setting up an antiviral status in a cell.

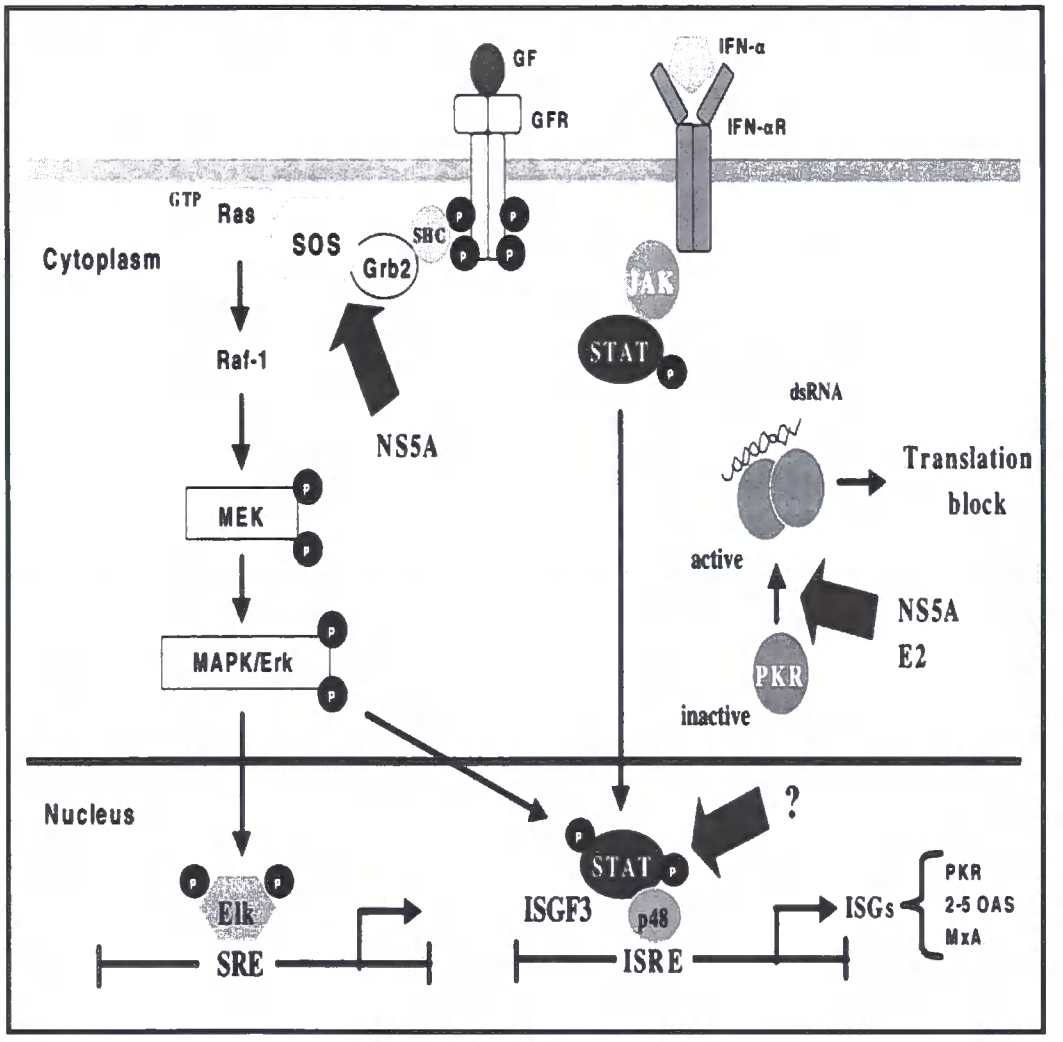

Fig. 1.26: Interference of NS5A with EGFR-mediated ERK and Jak-STAT signalling pathways (Tan and Katze, 2001). NS5A interferes with EGFR signalling pathway by interaction with Grb2 disturbing its interaction with SOS and inhibiting the ability of SOS to activate Ras and the down-stream MAPK/ERK signalling pathway. MAPK mediated phosphorylation of STAT is required for maximal transcriptional activity of ISGF3 and is essential for IFN-α-induced ISRE-dependent gene transcription. NS5A can also interact with PKR and inhibit its activity on the translation.

Interestingly DENV and WNV, similar to HCV, were shown to require c-Src like kinases for virus assembly at the site of RNA replication (Chu and Yang, 2007; Hirsch *et al.*, 2005). NS5A interacts with amphiphysin II, a tumor suppressor, via a SH3-binding region inhibiting its apoptotic effect on hepatic cells (Nanda *et al.*, 2006). Importantly, although mutations within the SH3-binding motif have a little effect on HCV RNA replication, mutation within this region in an HCV infectious genome resulted in a loss of infectivity suggesting that this region is involved in productive HCV infection (Nanda *et al.*, 2006; Zech *et al.*, 2003).

NS5A apparently contains an interferon sensitivity determining region (ISDR) (amino acids 237–276), the sequence of which could be correlated with increased resistance to IFN (Enomoto *et al.*, 1995; Kurosaki *et al.*, 1997; Maekawa *et al.*, 2004). Introduction of mutations into this region increases the efficiency of HCV replicon replication in Huh-7 cells (Maekawa *et al.*, 2004). Like E2, NS5A directly interacts with PKR preventing its dimerization and phosphorylation action on eIF2α which in turn fails to stop the translation of the viral proteins (Fig. 1.26). This interaction is mediated by the PKR binding domain (237 to 302 a.a.) that encompass the ISDR region (Gale *et al.*, 1998). Repression of PKR by NS5A affects many cellular processes such as PKR-dependent apoptosis and cell growth arrest that may have a role in HCV pathogenesis during chronic infection (Reed *et al.*, 1998).

NS5A also represses the transcription of the cell cycle regulatory gene $p21^{WAF1}$, a cyclin-dependent kinase (cdk) inhibitor, by an unknown mechanism promoting cell growth and tumour formation in nude mice (Ghosh *et al.*, 1999). NS5A also physically associates with the TATA box-binding protein (TBP) and the tumour suppressor p53 inhibiting their DNA-binding activities and allowing transcriptional regulation of the $p21^{WAF1}$ gene which can inhibit cyclin/cdk complexes formation required for the G1/S phase transition of the cell cycle (Lan *et al.*, 2002; Majumder *et al.*, 2001; May and May, 1999; Qadri *et al.*, 2002).

NS5A can interact with other cellular proteins such as the human vesicle-associated membrane protein A (hVAP-A) that is distributed in a broad range of tissues and membrane compartments of the cell suggesting a role in the association of the HCV replication complex with membranes (Tu *et al.*, 1999). NS5A is able to interact with NS5B by two discontinuous regions of amino acids (105-162 and 277-334) and modulates its polymerase activity (Shimakami *et al.*, 2004; Shirota *et al.*, 2002).

NS5B located at the C-terminal region of the HCV polyprotein is a 68 kDa highly conserved phosphorylated protein (Hwang *et al.*, 1997). NS5B contains a Glycine-Aspartate-Aspartate (GDD) motif that is commonly found in RNA-dependent RNA-polymerases (RdRp) (Kamer and Argos, 1984). Subsequently NS5B was shown to be the virus RNA polymerase (Behrens *et al.*, 1996).

NS5B contains a hydrophobic C-terminal region of 21 amino acids encompassing a putative membrane-anchoring domain localizing the protein to the prenuclear region (Fig. 1.27). NS5B lacking the terminal 21 amino acids is exclusively distributed within the nucleus although NS5B has no nuclear localization signal. Thus nuclear localization of the NS5B truncated form may be due to an ability to interact with cellular nuclear-localizing proteins (Yamashita *et al.*, 1998).

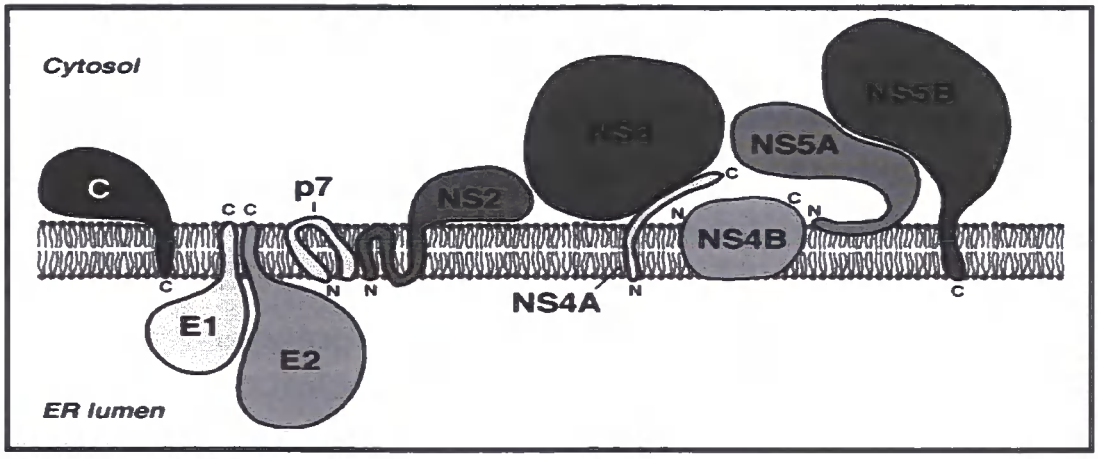

Fig. 1.27: HCV proteins positioning on the ER membrane (Moradpour *et al.*, 2003).

Four amino acid sequence motifs (A, B, C, and D) have been shown to be crucial for RdRp activity of NS5B. Motif A (amino acids 2640 to 2645) is involved in nucleotide binding and catalysis, while motif B (amino acids 2702 to 2711) is probably involved in template and/or primer positioning. Motif C (amino acids 2737 to 2739), which contains the GDD motif, is important for NTP binding and catalysis. Motif D (amino acids 2762 to 2766) could be also involved in nucleotide binding and catalysis (Lohmann *et al.*, 1997).

NS5B catalytic domain is similar to the human right hand composed of fingers, palm and thumb subdomains. The three-dimensional structure of NS5B determined that NS5B has a unique shape due to the extensive interactions between the finger and thumb polymerase subdomains that serve to encircle the enzyme active site (Fig. 1.28) (Ago *et al.*, 1999; Bressanelli *et al.*, 1999; Lesburg *et al.*, 1999). NS5B oligomerizes and this oligomerization is necessary for polymerase activity (Qin *et al.*, 2002).

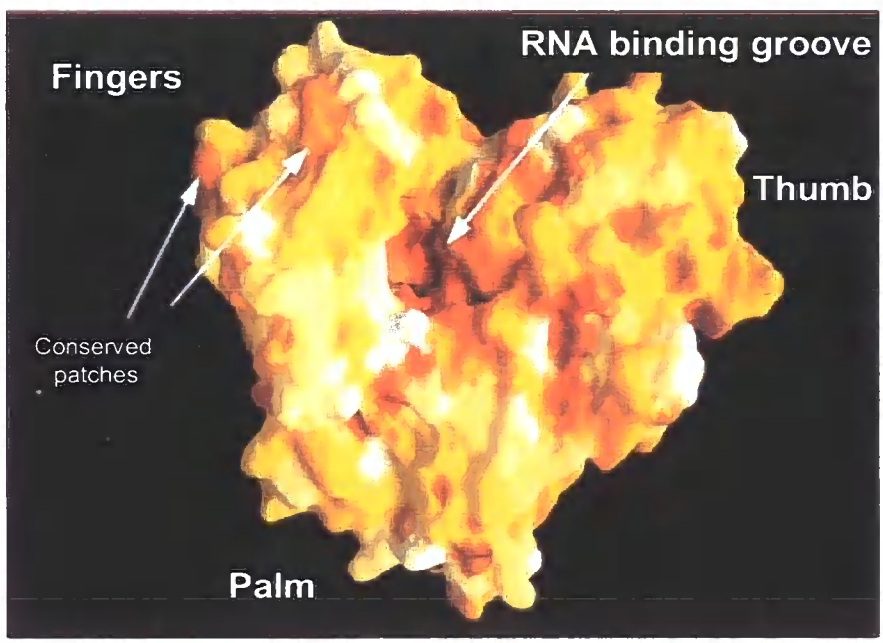

Fig. 1.28: NS5B crystal structure. The crystal structure of NS5B polymerase is showing the location of the finger, palm and thumb domains in addition to the RNA-binding groove (Bressanelli *et al.*, 1999).

NS5B interacts with the 3` terminus of the genomic RNA (Cheng *et al.*, 1999), with the core protein via the hydrophobic C-terminal region of NS5B (Uchida *et al.*, 2002), and with NS5A (Shimakami *et al.*, 2004). NS5B was shown to interact with all non-structural proteins NS2, NS3, NS4A, NS4B, and NS5A that suggested that all non-structural proteins may participate in the assembly of the replication complex (Dimitrova *et al.*, 2003). NS5B interacts with NS3 and modulates its helicase activity suggesting that the two proteins are functioning together during the viral genome replication (Jennings *et al.*, 2008).

Several cellular proteins have been shown to interact with NS5B and be involved in RNA replication such as human vesicle-associated membrane protein (VAP) subtype A, hVAP-A (Tu *et al.*, 1999), hVAP-B (Hamamoto *et al.*, 2005), nucleolin (Hirano *et al.*, 2003; Kusakawa *et al.*, 2007; Shimakami *et al.*, 2006), human homolog 1 of protein-linking intergrin-associated protein and cytoskeleton (hPLIC1) (Gao *et al.*, 2003), and a human RNA helicase (p68) (Goh *et al.*, 2004).

1.12 HCV Replication

HCV can only efficiently replicate in human and chimpanzee and therefore the study of HCV replication has been hampered by lack of a convenient cell culture system and suitable small animal models. Recently, the development of HCV replicons has improved the study of the full HCV life cycle *in vitro*. The life cycle of HCV can be summarized as such:

1.12.1 Virus Entry

HCV initiates the infection via the attachment to the cell which is mediated by the interaction of E2 with a cell membrane receptor molecule(s). A number of candidates have been implicated as a receptor for HCV such as CD81, LDL, VLDL and SR-BI receptors as described in section 1.10.2. In addition, other molecules were also suggested to be involved such as the dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and the related liver endothelial cell lectin L-SIGN (Lozach *et al.*, 2003), the asialoglycoprotein receptor (ASGP-R) (Saunier *et al.*, 2003) and glycosaminoglycans (GAGs) (Germi *et al.*, 2002).

A model has been proposed for the virus entry (Fig. 1.29) (Helle and Dubuisson, 2008). The virus initiates the attachment by an interaction with GAG and LDLR facilitated by the presence of lipoprotein associated with the virus envelope. Then, viral glycoproteins interact with SR-BI and CD81 co-receptors. These interactions are enhanced by HDL or inhibited by CD81 binding partner (EWI-2wint) which is not present in hepatic cells (Rocha-Perugini *et al.*, 2008). Following that, the virus envelope glycoproteins-receptors complex interacts with CLDN1 probably after a lateral migration to the tight junction (TJ) (Dubuisson *et al.*, 2008; Helle and Dubuisson, 2008).

After attachment, the virus is endocytosed by clathrin-mediated endocytosis, followed by a fusion step within an acidic endosomal compartment where a lowering in pH of the endosomal lumen induces a change in the conformation of the envelope proteins, notably E1, which in turn induces fusion of the viral membrane with the endosomal membrane resulting in viral uncoating and releasing the nucleocapsid into the cytoplasm. Then, the nucleocapsid associates with ribosomes, an interaction that is sufficient for releasing the genome from the capsid (Blanchard *et al.*, 2006; Flint *et al.*, 1999; Helle and Dubuisson, 2008).

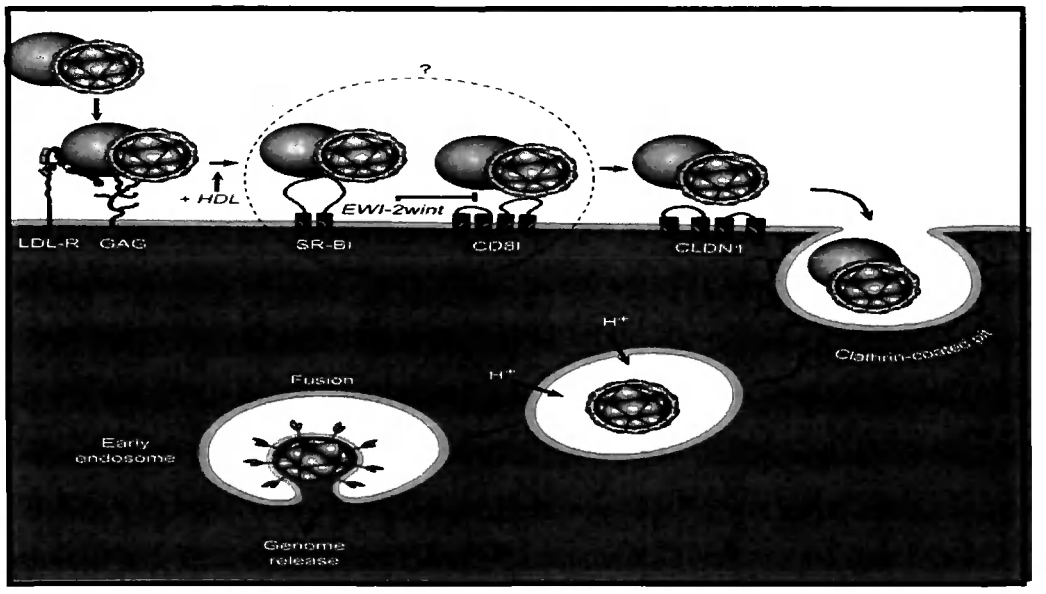

Fig. 1.29: Entry of HCV. A schematic diagram showing the attachment, penetration, entry, and uncoating of HCV virion (see the main text) (Helle and Dubuisson, 2008).

1.12.2 Translation of the Viral Genome

When the positive sense genomic RNA is released into the cytoplasm, it acts as mRNA and is translated into a single polyprotein by a cap-independent process using IRES-mediated translation (Wang *et al.*, 1993). Translation occurs on the ER membrane and the produced polyprotein undergoes co- and post-translational cleavages as previously described to give structural and non-structural proteins (Bartenschlager *et al.*, 1993; Grakoui *et al.*, 1993b; Hijikata and Shimotohno, 2004).

1.12.3 RNA Replication

The replication complex is assembled on the ER and/or ER-derived lipid raft (microdomains that are enriched in cholesterol and sphingolipid) membranes where NS5B associates with some cellular and non-structural viral proteins (Shi *et al.*, 2003). The multiprotein replication complex was also found to be associated with NS4B-induced membranous webs in the infected cells

that accommodates active RNA replication (Fig 1.30) (Aizaki *et al.*, 2004; Egger *et al.*, 2002; Gosert *et al.*, 2003; Schwartz *et al.*, 2004).

Plus-strand RNA is used as a template by NS5B polymerase to make a minus strand RNA copy. NS5B recognizes the 3` end of the genome and directs the synthesis in either a primer-dependent (Behrens *et al.*, 1996; Lohmann *et al.*, 1997) or in a primer-independent initiation process (Luo *et al.*, 2000). The minus-strand RNA is used solely as a template to generate many copies of positive-strand RNA molecules (10 fold higher than minus strand production) suggesting that NS5B interacts with minus strand promoter with a higher affinity (200 fold) than plus strand suggesting that the promoter activity on the antigenome for replication is more powerful than that on the genome (Dahari *et al.*, 2007). Within the membranous web, the HCV replication complex is protected from nuclease and proteinase attack (Miyanari *et al.*, 2003). A quantitative analysis estimated that an active replication complex consists of one minus-strand RNA, two to ten positive-strand RNA molecules, and several hundred copies of the non-structural proteins (Quinkert *et al.*, 2005).

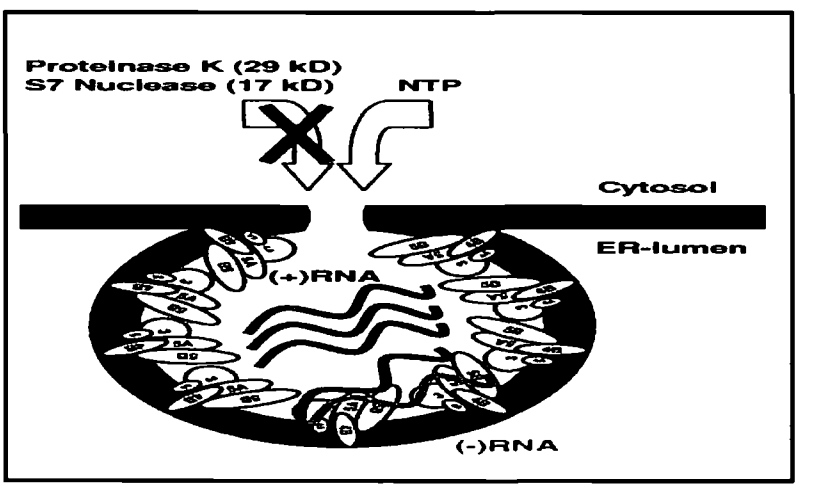

Fig. 1.30: HCV replication complex accommodated within an ER membranous web (Quinkert *et al.*, 2005). The viral genome and replication complex within the web are protected from the proteases and nucleases that are present in the cytoplasm. An active replication complex consists of one minus-strand RNA, two to ten positive-strand RNA molecules, and several hundred copies of the non-structural proteins.

1.12.4 Virus Assembly

The mature virion is composed of a nucleocapsid surrounded by an envelope formed by lipid membranes and viral glycoproteins. Assembly of the virus particle starts by interaction of the capsid protein with the RNA genome to form the nucleocapsid (Majeau *et al.*, 2004; Shimoike *et al.*, 1999). The nucleocapsid then interacts with the envelope proteins on the cytoplasmic surface of the ER membrane to acquire an envelope by budding forming a new virus progeny in the ER lumen (Cocquerel *et al.*, 1999).

Lipid droplets are involved in the virus assembly and budding as lipid droplet-associated core protein recruits the viral replication complex to the ER membrane that closely surrounds the lipid droplets. This recruitment is critical for production of infectious particles suggesting that some assembly steps take place around lipid droplets (Miyanari *et al.*, 2007).

Overexpression of core, E1, and E2 is sufficient to form virus-like particles in insect cells. HCV transcripts were selectively incorporated into these particles (Baumert *et al.*, 1998). Expression of all viral proteins produces infectious virus particles in mammalian cells (Huh7) (Heller *et al.*, 2005; Ishii *et al.*, 2008). Subgenomic JFH1 replicons lacking the entire core to NS2 coding region are efficiently encapsidated into virus-like particles indicating that packaging signals do not exist in the structural coding region which is similar to the flaviviruses (Ishii *et al.*, 2008; Steinmann *et al.*, 2008; White *et al.*, 1998).

1.12.5 Virus Release

After the new virus progeny acquires the envelope, it is released from the infected cell by budding through the cell membrane followed by exocytosis. Trafficking and release of the virus from the cell membrane could be mediated by microtubules networks (Boulant *et al.*, 2008a).

1.12.6 Summary of HCV Replication Cycle

The whole HCV life cycle can be summarized in Fig. 1.32. The virus enters the cell by endocytosis via binding to cell membrane receptors. Within the endosome, the virus uncoats by fusing with the endosome membrane releasing the nucleocapsid into the cytoplasm. Once released, the viral genome is translated to form viral proteins. A replication complex is constructed from viral and host cell proteins in a virus-induced membranous web at the ER surface. Viral structural proteins and the synthesized viral genome are assembled to form the progeny virus that is released from the cell as a mature virion by exocytosis (Racanelli and Rehermann, 2003).

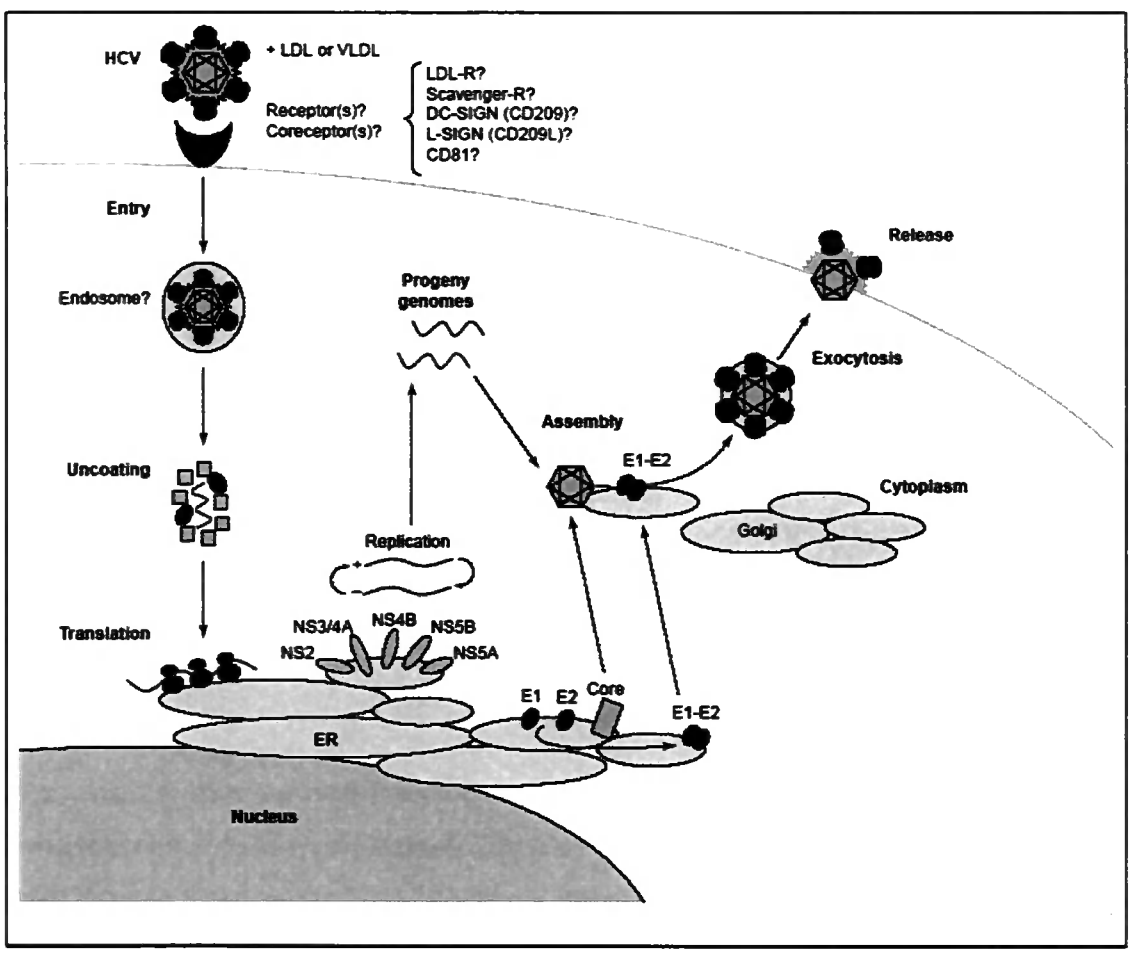

Fig. 1.32: HCV life cycle (Racanelli and Rehermann, 2003). A schematic diagram shows the main steps of HCV life cycle. Details of the life cycle are presented in the main text.

1.13 Aim of the Work

A molecular analysis of the HCV-host cell interaction is important to understand mechanisms necessary to maintain the viral infection. Therefore, the main aim of the work presented in this thesis is to identify host cell proteins that can interact with HCV RdRp (NS5B) and may play a role in viral replication and/or persistence. The discovery of novel interactions could help in the design and development of novel therapeutic strategies.

In order to achieve this goal, NS5B, a key enzyme in HCV replication, will be used as bait in two experimental approaches, a yeast two-hybrid system and a pulldown methodology combined with mass spectrometry, to identify the interacting host cell proteins. Several areas are targeted as being of interest and will be addressed within this thesis such as:

- Screening of a human cDNA library for potential cellular target proteins that can interact with NS5B and determining the nature of the interaction.
- Confirmation of the interactions *in vitro* and *in vivo* using pulldown assays and other *in vitro* assays such as enzyme-linked immunosorbent assay (ELISA) and far western blotting.
- Investigation of the biological role of interacting proteins and whether this may influence HCV replication using a replicon system.

In order to facilitate this we need to establish systems not currently in use within the laboratory. Primarily, the HCV replicon system needs to be developed as well as the lentivector gene delivery system for over-expression or for shRNA delivery to knock-down the expression of endogenous proteins.

CHAPTER 2

Materials and Methods

2.1 Chemicals, Antibiotics and Solutions

General chemicals, antibiotics and biological chemicals were mainly obtained from Sigma, Fluka, BDH, and Merck. Growth media were purchased from Oxoid, Difco and Clontech. Enzymes used in molecular biology were supplied by Promega, Roche Biochemicals, New England Biolabs and Invitrogen. A list of frequently used antibiotics and solutions is presented in Table 2.1. Other solutions used in this thesis are listed in the appendices.

Table 2.1: Common antibiotics and reagents used in thesis

REAGENTS	STOCK CONCENTRATION	WORKING CONCENTRATION
Ampicillin	100 mg/ml	100 µg/ml
Carbenicillin	100 mg/ml	100 µg/ml
Kanamycin	10 mg/ml	30 µg/ml
IPTG	200 mg/ml	Variable
X-Gal	50 mg/ml in DMF	4 mg/ml
Imidazole	1M	Variable
RNase	10 mg/ml	Variable
DNase	500 units/ml	Variable
Ethidium Bromide	10 mg/ml	0.5 µg/ml

2.2 Bacterial Strains and Plasmids

Various laboratory strains of *Escherichia coli* (*E. coli*) were employed during the course of this study. Strains of HB101 and DH5α were used generally for the purpose of plasmid DNA amplification and maintenance. BL21 (DE3) strain was used for expression of recombinant proteins.

2.2.1 Bacterial Strains

STRAIN	SOURCE	GENOTYPE
E. coli HB101	Promega	*thi*-1, *hsdS*20(r_B-, m_B-), *sup*E44, *rec*A13, *ara*-14, *leuB*6, *proA*2, *lacY*1, *gal*K2, *rpsL*20(str^r), *xyl*-5, *mtl*-1
E. coli DH5α	Invitrogen	φ80d*lacZ*ΔM15, *recA*1, *endA*1, *gyr*AB, *thi*-1, *hsdR*17(r_K-, m_K+), *supE*44, *relA*1, *deo*R, Δ(*lacZYA-arg*F) U169, *pho*A
E. coli BL21 (DE3)	Novagen	F–, *omp*T, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)
E. coli XL1-Blue	Novagen	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)]

2.2.2 Storage of Bacterial Stocks

All bacterial stocks were prepared for long term storage by combining fresh overnight culture with 80% (v/v) glycerol stock solution to give a 25% (v/v) final glycerol concentration. Samples were stored at -80°C.

2.2.3 Vectors Used in Thesis

VECTORS	SOURCE	APPLICATION
pCV-H77c (Access. No. AF011751)	J. Bukh (Bethesda, USA)	HCV cDNA (genotype 1a) clone
pFKi341PiLucNS3-3`dgET (pFK-luc) (Appendix 9.12)	R. Bartenschlager, (Heidelberg, Germany)	HCV sub-genomic replicon (genotype 1b)
pWPXL	Tronolab (Switzerland)	Lenti-vectors
psPAX2	Tronolab (Switzerland)	Lenti-vectors
pMD2G	Tronolab (Switzerland)	Lenti-vectors
pLVTHM (Appendix 9.13)	Tronolab (Switzerland)	Lenti-vectors
pLVTHM-shGFP	Tronolab (Switzerland)	Lenti-vectors
pGBKT7	Clontech	Yeast two-hybrid
pGADT7	Clontech	Yeast two-hybrid
pET21d	Novagen	Bacterial protein expression
pGEX-6P-3	GE Healthcare (Life Sciences)	Bacterial protein expression
pNTAP	Stratagene	Mammalian protein expression
pcDNA4His/Max	Invitrogen	Mammalian protein expression

2.3 Source of Antibodies

Antibodies used in this study and their sources were listed below:

ANTIBODY	ANTIBODY TYPE (DILUTION USED)	SPECIES	SOURCE
α-NS5B	Polyclonal (1:2000)	Sheep	Kindly provided by Mark Harris (Leeds University)
α-NS5B	Polyclonal (1:2000)	Rabbit	Abcam
α-His-HRP	Monoclonal (1:3000)	Mouse	Sigma
α-GST-HRP	Monoclonal (1:3000)	Mouse	Sigma
Anti-α-Tubulin	Monoclonal (1:1000)	Mouse	Calbiochem
Anti-β-Tubulin	Monoclonal (1:10)	Mouse	Kindly provided by Roy Quinlan (Durham University)
α-Mouse-HRP	Polyclonal (1:2000)	Goat	Sigma
α-Rabbit-HRP	Polyclonal (1:2000)	Sheep	Sigma
α-Sheep-HRP	Polyclonal (1:2000)	Donkey	Sigma

2.4 Centrifugation

Routine centrifugation of 1.5-ml and 0.5-ml tubes was performed using a bench top centrifuge (Eppendorf). Routine centrifugation of 15-50-ml Falcon tubes was carried out in (Boeco U320R). For larger volume and high speed, centrifugation was performed using Beckman's Avanti J-E centrifuge with either JA-20 or JA-10 rotors.

2.5 Annealing of Oligonucleotides

For RNA interference (RNAi) studies, two complementary DNA oligonucleotides were annealed together to form a DNA double-stranded insert (Chapter 5, Fig. 5.6) that was cloned into pLVTHM (Appendix 9.13) to create pLVTHM-shRNA that was used in Section 5.4.2 to down-regulate a target gene expression. The same annealing protocol was also used to create V5H insert (Appendix 9.10) to be cloned into pWPXL giving pWPXL-V5H (Appendix 9.11) that was used in Section 5.3.

COMPONENT	VOLUME	CONCENTRATION
Sense Sequence	10 µl	30 µg
Anti-sense sequence	10 µl	30 µg
10x PCR Buffer	10 µl	1x
dH_2O	970 µl	-
Total vol/Final conc.	1000 µl	60 ng/µl

The mix was incubated at 96 °C for 10 min, and then the temperature was decreased slowly to 60°C for 30 min. The mix was stored at -20°C until needed.

2.6 PCR (Polymerase Chain Reaction) Techniques

PCR using Techne TC3000 Thermal Cycler was frequently employed in this thesis for amplifying and screening the cloned ORFs. Generally, ProofStart

DNA Polymerase (Qiagen) was used for ORFs cloning, screening of successful cloning, and for semi-quantitative RT-PCR.

2.6.1 PCR

PCR was routinely carried out according to the instructions provided by the manufacturer of the amplifying polymerase. General reaction composition and condition used for cloning using ProofStart DNA polymerase (Qiagen) are described below.

The PCR mix was prepared as following:

COMPONENT	FINAL CONCENTRATION
10X PCR buffer	1X
dNTP mix (10 mM Each)	300 μM of each dNTP
Forward Primer	1μM
Reverse Primer	1μM
Polymerase Enzyme	2.5 units/reaction
Template DNA	100ng /reaction
dH_2O	Variable
Final Volume	25-50 μl

A thermal cycler (Techne) was programmed for the PCR reaction as follows:

CYCLE	TEMPERATURE	TIME
Initial activation of Polymerase	95°C	5 min
35 cycles:-		
• Denaturation	94°C	30 sec
• Annealing	55°C	30 sec
• Extension	72°C	1min/kb
Final extension	72°C	10 min
Hold	4°C	Indefinitely

The amplified DNA samples were used directly or stored at 4°C for later use.

2.6.2 Colony PCR Screening

For screening for successful cloning using colony PCR, one colony was isolated from LB medium plate (Appendix 9.1) containing colonies developed from cells transformed by a mixture of ligation reaction. The colony was used to inoculate a fresh numbered LB agar plate using a sterile tip and then the remaining amount of colony on the tip was mixed with PCR mixture in PCR tube. The composition of PCR mixture and the condition were performed as described earlier in PCR usually in a total volume of 25-40 µl.

2.7 DNA Agarose Gel Electrophoresis

Amplified DNA plus a 6x DNA loading buffer (Appendix 9.2) were loaded and electrophoresed on a 0.8-2% agarose gel prepared in TAE buffer (Appendix 9.2) using a gated mini-gel tank (BioRad) and containing ethidium bromide (0.5µg/ml) for 40 minutes at 100 Volts (V). Ready-Load 1kb DNA Ladder, 100 bp DNA Ladder (Invitrogen) or 1kb DNA Ladder (Promega) were routinely used as a molecular weight standard as a reference in the agarose gel. The gel was visualised on a UV transilluminator equipped with a camera (Syngene) to capture an image of the gel. Images were manipulated by GeneSnap software program.

2.8 DNA Isolation and Extraction from Agarose gel

The DNA fragment was excised and extracted from the agarose gel following the manufacturer's instructions using QIAquick® Spin Kit (Qiagen) or the Wizard SV Gel and PCR Clean-Up System (Promega).

2.9 Determination of Nucleic Acid Concentration

Nucleic acid concentration was determined by combining 5 µl of nucleic acid with 995 µl dH_2O in a quartz cuvette. H_2O was used as a reference. Absorbance at 260 nm (OD_{260}) was determined. The concentration of the nucleic acid was calculated follows:

$1OD_{260}$ = 50 µg ds DNA or 25 µg ss DNA or 40 µg RNA

2.10 Restriction Endonuclease Digestion

DNA digestion with restriction enzymes was performed to create the correct ends in plasmids and DNA fragments enabling ligation or to cut the ORF from one plasmid to sub-clone in another one. It was also used for screening for successful ligation and cloning. Most of enzymes were obtained from Promega. The following components were added to a single microcentrifuge tube.

COMPONENT	FINAL CONCENTRATION
10x Suitable Buffer	1x
Bovine Serum Albumin (BSA)	0.1mg/ml
Restriction Enzyme 1	5 units/µg
Restriction Enzyme 2	5 units/µg
dH_2O	variable
Template DNA	variable

The components were mixed well and incubated at 37°C for 3-4 hrs. If digestion with two enzymes using incompatible buffers was needed, the DNA was cut with the first one as a single cut followed by ethanol precipitation or gel extraction and then resuspended in dH_2O. After that, the DNA was cut with the second enzyme. The result of digestion was tested by loading on 0.8-2% agarose gel stained with ethidium bromide and visualized by UV trans-illuminator. All the restriction sites and primers used for cloning or screening in this study were listed in Appendix 9.11.

2.11 Ethanol Precipitation of Nucleic Acids

Ethanol precipitation was routinely used for removing salts and allowing buffer exchange. The following components were mixed.

COMPONENT	CONCENTRATION
DNA	1 Volume
3M Sod. Acetate, pH 5	1/10 Volume
100% Ethanol	2.5 Volume

The tube was incubated at -20°C for at least 2 hrs, and then centrifuged at 14.000 x g for 30 min at 4°C. The supernatant was discarded and the pellet washed once with cold 70% ethanol, and centrifuged at 14.000 x g for 5 min. The supernatant was discarded and the pellet left to dry in air for 15 min. The DNA pellet was resuspended in dH_2O and used directly or stored at -20°C.

2.12 Phenol/Chloroform Extraction of Nucleic Acids

Phenol/chloroform extraction was performed to remove contaminating proteins and lipids while isoamyl alcohol (IAA) was used to prevent foaming. DNA volume was adjusted 100 µl with dH_2O and then mixed with an equal amount of buffer-saturated phenol : chloroform (25 : 24). The sample was mixed well by vortexing for 30 sec then placed on ice for 1 min before centrifugation at 14,000 x g for 5 min at RT. The aqueous layer (upper layer) was carefully removed and transferred to a fresh tube. These steps were repeated for one more time to purify the remaining DNA from phenol. Collected aqueous layers were combined and mixed with an equal amount of chloroform/isoamyl alcohol (IAA) (24 : 1) and the procedure was repeated. DNA was precipitated from the aqueous layer by ethanol precipitation and then resuspended in dH_2O.

2.13 De-Phosphorylation Using Shrimp Alkaline Phosphatase (SAP)

Dephosphorylation was usually performed for removing the 5` phosphate from linearized vector DNA plasmid preventing self-ligation and recircularization of linearized cloning vector DNA. The following components were added to the microcentrifuge tube.

COMPONENT	VOLUME	CONCENTRATION
Linearized vector plasmid	20 µl	0.3 µg/µl
10x SAP Buffer	3 µl	1x
SAP	1 µl	1 unit
dH_2O	6 µl	
Total Volume	30 µl	

The components were mixed and incubated at 37°C for 1 hr, and then SAP enzyme was heat–inactivated at 75°C for 15 min. Dephosphorylated plasmid was ethanol precipitated and re-suspended in dH_2O before use in ligation reaction.

2.14 Ligation Reaction

For ligation reaction, 300 ng of the DNA fragment was combined with 100 ng of vector DNA in 3:1 ratio irrespective of the size of DNA fragment and vector. The following components were added to the microcentrifuge tube.

COMPONENT	VOLUME	FINAL CONCENTRATION
dH_2O	4 µl	
Plasmid Vector	1 µl	100 ng
DNA Insert	3 µl	300 ng
10x T4 Ligase Buffer	1 µl	1x
T4 Ligase (Promega)	1 µl	2.5 units
Total Volume	10 µl	

Plasmid, insert and dH_2O were mixed first and incubated at 65°C for 15 min. Then the ligase buffer and T4 ligase were added and incubated at 16°C for 30 min and left at 4°C o/n. The mix was used directly for bacteria transformation.

2.15 Preparation of Chemically Competent Bacterial Cells

A single colony of *E. coli* was selected from a fresh LB agar plate for inoculating a 10 ml LB overnight (O/N) starter culture. 100 ml of LB liquid media was inoculated with 1 ml of O/N culture and incubated at 37 °C with shaking at 200 rpm until OD_{600} of 0.8-1. The culture was incubated on ice for 20 min then centrifuged at 4000 rpm for 10 min at 4°C. The supernatant was poured off and the cells were resuspended in 50 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 30-60 min. The cells were centrifuged at 4000 rpm for 10 min at 4°C and then the supernatant was poured off and the cells were resuspended in 20 ml of ice cold 0.1 M $CaCl_2$. The last step was repeated two times reducing the volume of 0.1 M $CaCl_2$ by half each time and then the cells resuspended in 5 ml of 0.1 M $CaCl_2$ containing 20 % glycerol and dispensed in 80 µl aliquots and stored at -80°C.

2.16 Transformation of Chemically Competent Cells

1-5 µl of a plasmid or ligation mixture was added to a 40 µl aliquot of competent cells. The cells were incubated on ice for 30 min before heat shocked at 42°C in water bath for 42 seconds. The cells were incubated on ice for a further 2 min. 1 ml of LB liquid medium was added to the cells and incubated at 37°C with shaking at 250rpm for 1 hour. 100 µl of cells were spread onto LB agar plate containing an appropriate antibiotic and then the cells incubated at 37°C o/n.

2.17 Site-Directed Mutagenesis

Mutagenesis was performed using the QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer instructions to create carboxyl-terminal deletions of increasing size within PLSCR1 ORF in pGAD-SCR (pGAD-SCRD1, pGAD-SCRD2 and pGAD-SCRD3). A series of primers were designed to introduce a premature stop codon and a *SacI* restriction site at the end of each domain (Appendix 9.11). PfuTurbo® DNA

polymerase was used to amplify the mutants PLSCR1 ORFs using the designed primers. Components of a single mutagenic reaction were prepared as following.

COMPONENT	FINAL CONCENTRATION
10X Reaction buffer	1X
dNTP mix	1µl
Forward Primer	125 ng
Reverse Primer	125 ng
*Pfu*Turbo® DNA polymerase	2.5 units
Template DNA	50 ng
dH_2O	To 50 µl

The PCR reaction cycling parameters were adjusted as following:

CYCLE	TEMPERATURE	TIME
Initial activation of Polymerase	95°C	30 sec
18 cycles:-		
• Denaturation	95°C	30 sec
• Annealing	55°C	1 min
• Extension	68°C	9 min (1min/kb)
Final extension	68°C	5 min
Hold	4°C	Indefinitely

The products were then treated with *DpnI* restriction enzyme (10 units) that targets 5`-$Gm^6ATC$-3` sequence in the methylated parental strand of DNA causing its removal by digestion where DNA plasmids extracted from most of bacterial strains are *Dam* methylated and therefore susceptible to *DpnI* digestion. Mutated plasmids were then used to transform XL1-Blue supercompetent cells as described before for normal transformation protocol except using NZY^+ liquid medium in place of LB liquid medium (Appendix 9.1). Plasmids were extracted from some colonies growing on LB-ampicillin agar plates O/N. Plasmids were subjected to restriction analysis to confirm the presence of *SacI* restriction site. DNA sequencing was performed for successful mutants using AD-F and AD-R sequencing primers.

2.18 Extraction of Plasmids from Bacteria

Plasmid extraction from bacteria was usually performed using PureYield™ Plasmid Miniprep System (Promega) or Qiaprep Spin Miniprep Kit (Qiagen) according to the instructions provided by the manufacturers. When a large amount of DNA plasmids were required, Qiagen Plasmid Midi Kit or Qiagen Plasmid Mega Kit (Qiagen) was employed following the manufacturer's instructions.

2.19 Storage of the Recombinant Plasmids

Chemically competent bacterial cells were transformed by the recombinant plasmids. Plasmids were extracted and purified from the cells and stored at -20°C. Glycerol stocks of bacterial cells transformed with the plasmids were prepared and stored at -80°C for future reference.

2.20 DNA Sequencing

DNA sequencing was performed by Durham Biological Sciences (DBS) Genomics Unit using a 3730 DNA Analyser (Applied Biosystems).

2.21 Working with RNA

2.21.1 RNA Extraction

RNA extraction was performed using the SV Total RNA Isolation System (Promega) following manufacturer instructions. TRIzol® LS Reagent (Invitrogen) was also used to extract total cellular RNA from mammalian cells. For cells grown as a monolayer in 75 cm^2 tissue culture flasks, DMEM medium was removed from the flask and 0.75 ml of TRIzol reagent was added directly to the cells. Cells were pellet by centrifugation and incubated with TRIzol for 5 min at RT to permit the complete disruption of the cellular material. 0.2 ml of chloroform was added to the solution and mixed well by vigorous shaking by

hand for 30 sec then incubated at RT for 10 min followed by centrifugation at 12,000 x g for 15 min at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous layer which exclusively contains RNA was transferred to a clean tube and RNA precipitated by mixing with 0.5 ml isopropanol, incubated at RT for 10 min then centrifuged at 12,000 x g for 10 min at 4-8°C. The RNA precipitate was washed once with 0.5 ml 70 % ethanol and centrifuged at 7,000 x g for 5 min at 4°C. Ethanol was removed and RNA pellet was left to air-dry for 5 min at RT. RNA pellet was resuspended in appropriate volume of RNase-free water. In order to remove any contaminant DNA, 10 µl of RNA was treated with 1 unit of RNase-Free DNase (Promega) for 10 min at 37°C followed by heat inactivation of DNase for 10 min at 70°C. Concentration of RNA was determined at 260 nm as described before.

2.21.2 Reverse Transcription (RT)-PCR

RT-PCR was performed for isolation of full-length ORFs for cloning or for screening of gene expression using specific sets of primers. RT-PCR was carried out using SuperScript III Reverse Transcriptase (Invitrogen) or by using M-MLV Reverse Transcriptase (Promega) following the manufacturer's instructions.

First-Strand cDNA Synthesis

First strand cDNA was prepared in 20 µl total reaction volume. The following components were added to a nuclease-free microcentrifuge tube:

COMPONENT	VOLUME	CONCENTRATION
Reverse Primer (1µM)	2 µl	2 pmol
dNTPs Mix (10 mM)	1 µl	500 µM
RNA	1 µl	1 µg
dH_2O	9 µl	-
Total Volume	13 µl	

The components were mixed and incubated at 65°C for 5 min then put on ice for 1 min. The following components were added to the reaction mixture.

COMPONENT	VOLUME	CONC.
5X 1st Strand Buffer	4 µl	1X
0.1M DTT	1 µl	5 mM
RNaseOut RNase Inhibitor (Invitrogen)	1 µl	40 units
Reverse Transcriptase (Invitrogen)	1 µl	200 units
Total Volume	7 µl	

The components were mixed well, incubated at 25°C for 5 min then at 55°C for 45 min followed by inactivation of the reaction at 70°C for 15 min. The first strand (cDNA) was stored at 4°C for next day use or 2 µl was used directly as a template in PCR reaction as previously described.

In order to compare the induction of some genes using RT-PCR, band intensity of the test sample was divided on the β-actin band intensity of the same sample then the obtained value was divided on those obtained from the control sample to obtain the fold induction between the test and the control sample.

2.21.3 Nested RT-PCR

A nested RT-PCR was carried out to isolate the full-length sequences of PLSCR1, ZNF143 and β-Tubulin for cloning into different plasmids. Nested PCR increased the PCR product yield by involving two rounds of amplification reactions. Generally, the first-round of RT-PCR involved two primers designed for DNA sequences flanking the ORF sequence and was performed according to the previously described RT-PCR protocol. Subsequently, an aliquot of the first-round RT-PCR product was used as a template for the second-round of amplification reaction by PCR. The second-round PCR was performed with a new set of primers that hybridize to sequences internal to the first-round primer-target sequences and exactly amplify the target ORF sequence. The second-round primers were designed to contain restriction sites at both ORF ends to facilitate ORF cloning into the suitable vectors.

2.22 Protein Manipulation

2.22.1 Protein Quantification

Protein concentration was determined according to Bradford method (Bradford, 1976) using BioRad Protein Assay Dye Reagent (BioRad). Known concentrations of bovine serum albumin (BSA) were used as a reference. In 1 ml total volume, protein sample was added to dH_2O to a final volume of 800 µl. 200 µl of dye reagent was added and mixed well with the protein sample by inverting. Samples were transferred to plastic cuvettes and the absorbance was measured at 595 nm using water as a blank. Protein concentration of the sample was calculated by comparing with BSA standard curve.

2.22.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were mixed with 1X SDS sample buffer (Appendix 9.4.1) and boiled at 95°C for 5 min then separated by 10 % SDS-PAGE in 1X Tris-Glycine running buffer (Appendix 9.4.1) as previously described (Laemmli, 1970) using BioRad Proteon II minigel apparatus according to the manufacturer's instructions. All reagents used in the SDS-PAGE were purchased from Flowgen and listed in the appendix 9.4.1. The resolving gel of 10 % acrylamide percentage was prepared and immediately poured between two glass plates and left for 5-10 min for crosslinking and solidification. The stacking gel (5 %) was prepared and poured onto the top of resolving gel. Protein samples were electrophoresed at 50 V for 10 min then at 200 V for 40 min or until the bromophenol blue dye of the sample buffer reached the bottom of the gel. SeeBlue Pre-Stained Standard (Invitrogen) was used as guide for protein size. The gel was removed from the electrophoresis apparatus and the protein bands visualized by staining with coomassie blue stain for 2 hrs at RT then the background stain was reduced by incubating the gel in de-stain solution. Alternatively, the proteins were transferred from the gel to a nitrocellulose membrane for western blotting analysis.

2.22.3 Western Blot (WB) Analysis

Protein samples were resolved by 10 % SDS-PAGE as described below. Using western blotting apparatus (BioRad), proteins were transferred from the gel to the Enhanced chemiluminescence (ECL) grade nitrocellulose membrane (Amersham Bioscience). All reagents used in WB are listed in the appendix 9.4.2. Voltage of 100 V was applied for one hour. After stopping the run, the membrane was placed in blocking buffer (5 % (w/v) non-fat dry milk in TBST) O/N at 4°C to prevent non-specific background binding of primary and secondary antibodies to the membrane. After blocking, the membrane was washed three times with TBST 5 min each. The primary antibody was prepared in a suitable dilution in blocking buffer, and then incubated with the membrane for 1hr at RT with shaking. The primary antibody was removed and the membrane was washed three times before adding the secondary antibody conjugated to horseradish peroxidase (HRP) diluted in blocking buffer for 1hr at RT. The membrane was washed as described before and bands were developed using ECL solutions (A and B). An equal volume of both solutions were combined and added immediately to the membrane in a dark room and incubated for 1 min at RT. The membrane was removed and placed in a developing cassette then exposed to an X-ray film for appropriate time (seconds to minutes). The film was then developed using Developer and Fixer solutions.

2.23 Yeast Two-Hybrid System

Yeast two-hybrid screening was performed for detection of cellular proteins interactions with HCV NS5B using MATCHMAKER GAL4 Two-Hybrid System 3. Human HeLa MATCHMAKER cDNA library (Clontech) (HeLa S3 cell line, ATCC: CCL 2.2) was employed in this system. The cDNA library had been cloned into pGAD GH using *EcoRI/XhoI* cloning sites then amplified in *E. coli* DH10B and stored in LB medium with 25 % glycerol.

2.23.1 Yeast Strain, Genotype and Phenotype

STRAIN	SOURCE	GENOTYPE
AH109 (*Saccharomyces cerevisiae*)	Clontech	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3 : : MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ*

AH109 Phenotype

1. Nutritional Requirements: AH109 requires growing on YPDA medium supplemented with Tryptophan (Trp), Leucine (Leu), Histidine (His) and Adenine (Ade) amino acids as AH109 can't grow on Minimal Synthetic Dropout (SD) medium lacking one of the above amino acids.
2. Colony Colour: AH109 has Ade2-101 phenotype. In absence of GAL4, AH109 colony exhibits Ade2-101 phenotype that develops colony with reddish-brown colour. In the presence of protein interactions, Ade marker expression complements in *cis* the Ade2-101 phenotype and the colony develops white to pale pink colour.
3. MEL1 and LacZ Reporter Gene Expression: In response to GAL4 activation by protein interaction, AH109 secretes α-galactosidase and β-galactosidase (LacZ) that can be detected on medium containing X-α-Gal or X-Gal, respectively.

2.23.2 Control Vectors

- Positive control:
  - pCL1 plasmid encodes full-length, wild type GAL4 providing a positive control for α- and β-galactosidases.
  - pGADT7-T and pGBKT7-53 encode SV40 large T-antigen and murine p53 proteins in fusion with GAL4 DNA activation domain (DNA-AD, amino acids 1-147 of GAL4 protein) and GAL4 DNA binding domain (DNA-BD, amino acids 768-881 of GAL4 protein), respectively. Both proteins can interact together to provide a positive control.

- Negative control:

  pGBKT7-Lam plasmid encodes human lamin C protein in fusion with DNA-BD. The plasmid was used as control for a fortuitous interaction between an unrelated protein and the tested proteins.

2.23.3 Storage of AH109

AH109 cells were stored in YPDA liquid medium (Appendix 9.5) with 25 % glycerol at -80°C for later use. Transformed yeast cells were stored in the appropriate SD liquid medium (Appendix 9.5). To prepare working stock plate, a small portion of the frozen glycerol stock was streaked onto YPDA or the appropriate SD agar plate. The plate was incubated at 30°C for 3-5 days till yeast colonies were approximately 2 mm in diameter.

2.23.4 Preparation of Yeast Competent Cells

From a freshly prepared yeast plate (3-4 days), one colony was isolated and used to inoculate 50 ml of YPDA or SD liquid medium (for sequential transformation) then incubated at 30°C o/n with shaking at 250 rpm until OD_{600}>1.5. Overnight culture was used to inoculate 300 ml of YPDA or SD liquid medium to give an OD_{600} of 0.2-0.3. The culture was incubated at 30°C with shaking at 250 rpm for 3-4 hrs until an OD_{600} of 0.5 ± 0.1. Cells were collected by centrifugation at 1,000 x g for 5 min at RT. Cells were resuspended in 40 ml dH_2O and the centrifugation was repeated. Cell pellet was resuspended in 1 ml of 1X TE/LiAc solution (Appendix 9.5).

2.23.5 Yeast Transformation

Yeast transformation was performed by combining the following components.

COMPONENT	FINAL CONCENTRATION	
	SMALL SCALE	LARGE SCALE
DNA-BD/bait Plasmid	0.1 μg	20-100 μg
AD/library Plasmid	0.1 μg	50 μg
Herring Testes Carrier DNA	0.1 mg	2 mg
Yeast Competent Cells	0.1 ml	1 ml
PEG/LiAc Solution	0.6 ml	6 ml

These components were mixed well by vortexing at high speed then incubated at 30°C 30 min with shaking at 200 rpm. Dimethyl sulfoxide (DMSO) (70 μl for small scale and 700 μl for large scale) was added to the mixture and mixed by gentle inversion. Cells were heat shocked for 15 min at 42°C then kept on ice for 2 min. Cells were collected by centrifugation at 14,000 x g for 5 sec at RT. The supernatant was removed and the cells were resuspended in appropriate volume of YPDA liquid medium before plating onto the suitable SD plate(s) that was incubated for 4-5 days at 30°C. For low-stringency selection, the plating was performed onto SD/-Trp/-Leu medium plates. For medium-stringency selection, cells were plated onto SD/-Trp/-Leu/-His, while for high-stringency selection, cells were plated onto SD/-Trp/-Leu/-His/-Ade in addition to 4 mg/ml X-α-Gal when blue colour screening was required.

2.23.6 Preparation of Yeast Protein Extracts

Total cellular proteins were extracted from yeast to investigate the expression of some transformed plasmids. A single colony was isolated from fresh SD plate and used to inoculate 5 ml of the appropriate SD liquid medium then left o/n at 30°C. The entire o/n culture was used to inoculate 50 ml of YPDA liquid medium and then incubated at 30°C with shaking at 250 rpm until the OD_{600} reached 0.4-0.6. OD_{600} of the culture was determined and total number of OD_{600} was calculated by multiplying OD_{600} by the culture volume. The culture was chilled on ice then centrifuged at 1000 x g for 5 min at 4°C. The

cell pellet was resuspended in dH_2O and the centrifugation was repeated. The cell pellet was frozen in dry ice then thawed by the addition pre-warmed cracking buffer (100 µl of cracking buffer per 7.5 OD_{600} units of cells). The sample was transferred to a microcentrifuge tube containing 80 µl acid-washed glass beads (Sigma) per 7.5 OD_{600} units of cells. To free membrane-associated proteins, the cell suspension was heated at 70°C for 10 min then vigorously vortexed for 1 min and centrifuged at 14,000 rpm for 5 min at RT to remove cell debris and un-broken cells. The supernatant was transferred to a clean tube and used directly in WB analysis or stored at -80°C until needed.

2.23.7 Extraction of Plasmids from Yeast

Plasmid extraction from yeast was performed using Qiaprep Spin Miniprep Kit (Qiagen) following the manufacturer's instructions after some modifications. One colony was isolated from fresh prepared plate and used to inoculate 10 ml of appropriate selective medium at 30°C o/n. Cells were harvested by centrifugation at 5,000 x g for 5 min. Cell pellet was resuspended in Qiagen suspension buffer containing 100 µl of acid-washed glass beads (Sigma) and vortexed for 5 min to disrupt the yeast cell wall. Supernatant was transferred to a fresh microcentrifuge tube and the rest of the protocol was followed as the kit provider's instructions.

2.24 Over-Expression of Proteins in Bacteria

2.24.1 Over-expression and Purification of Histidine (His)-Tagged NS5B

For over-expression of NS5B in fusion with His-tag at its C-terminus in *E. coli* BL21 (DE3) cells a truncated form of the NS5B ORF (genotype 1a) lacking the last hydrophobic 21 amino acids was cloned into the bacterial expression pET21d plasmid to produce pET21-5B. pET21-5B was transformed into BL21 (DE3) cells. One colony was isolated and used to inoculate 10 ml LB-amp liquid medium then incubated at 37°C o/n. The overnight culture was used to inoculate 500 ml LB-amp liquid medium and incubated at 37°C with shaking at

250 rpm until an OD_{600} of 0.6-0.8. Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM o/n at 25°C. Cells were harvested by centrifugation at 3000 x g for 10 min at 4°C. The cell pellet was washed once with lysis buffer (50 mM Tris-HCl pH 8, 500 mM NaCl, 10 % glycerol, 1 % Triton-X100, 1 mM β-mercaptoethanol, lysozyme and protease inhibitor tablet (Roche)) then resuspended in 50 ml of lysis buffer and incubated for 10 min at RT. Cells were lysed by sonication on ice for 10 x 15 sec bursts with 5 sec intervals at 50% power level. DNase (2 U/ml) (Sigma) and RNase (160 µg/ml) (Sigma) were added to the lysate which was then incubated at 37°C for 30 min. Cell lysate was cleared from cell debris by centrifugation at 30,000 x g for 20 min at 4°C. The resulting supernatant was further cleared by passing through a 0.22 µm sterile syringe filter to remove any remaining solid debris. Imidazole was added to the cell lysate to give a final concentration of 10 mM.

NS5B-His protein was purified from the clarified lysate by affinity chromatography using HiTrap pre-packed 3 cm^3 column (Pharmacia Biotech). HiTrap column was equilibrated by passing 10 ml of equilibration buffer (lysis buffer containing 10 mM imidazole). The clarified lysate was then applied to the column and the column was washed once with equilibration buffer. NS5B-His was captured by HiTrap-Nickel ions, immobilized on a sepharose matrix, which can bind histidine residues of His-tag. Captured protein was then washed and eluted using different imidazole concentrations (50, 100, 200, 300, and 400 mM) in lysis buffer. Purified protein was collected in 1 ml fractions. Fraction eluted by 300 mM imidazole was dialysed against 50 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM β-mercaptoethanol, 10 % glycerol and protease inhibitors. The expression and purity of NS5B-His in the collected fractions were assessed by SDS-PAGE and western blotting (WB) analysis (Sections 4.2).

2.24.2 Over-expression of Glutathione-S-Transferase (GST)-Fusion Proteins

For expression of PLSCR1, RTN3, ZNF143, and β-Tubulin in fusion with GST protein tag, proteins ORFs were cloned into bacterial expression pGEX-6P-3 plasmid. The resulting constructs were transformed into BL21 (DE3). One colony was isolated and cultured in 5 ml LB-amp liquid medium then incubated o/n at 37°C. An overnight culture was used to inoculate 100 ml LB-amp liquid medium and incubated at 37°C for 3-4 hrs until an OD_{600} of 0.6-0.8. Protein expression was induced by the addition of IPTG to 1 mM. The culture was incubated at 25°C o/n. Cells were harvested by centrifugation at 3,000 x g for 10 min at 4°C. The cell pellet was washed once and then resuspended in 20 ml of lysis buffer (PBS, 500 mM NaCl, 10% glycerol, 1% Triton-X100, 1 mM β-mercaptoethanol, lysozyme and protease inhibitors) and left at RT for 10 min before sonication as previously described for NS5B-His protein expression. Cell debris was removed from the lysate by centrifugation at 15,000 for 20 min at 4°C. The supernatant was clarified by filtration through a 0.22 µm syringe filter. For purification of GST-fusion proteins, 10 ml of the clarified cell lysate was incubated with 300 µl of PBS-washed glutathioine-sepharose-4B beads (Amersham Bioscience) for 3hrs at 4°C with rotation using an end-over-end rotator. Beads were washed extensively with PBS to remove non-specific binding proteins. Proteins were kept bound to beads or eluted with 20 mM reduced glutathione in PBS. Protein expression and purity were investigated using SDS-PAGE and WB analysis (Sections 4.3 and 4.6).

2.25 *In Vitro* Assays for Protein-Protein Interaction

2.25.1 GST Pull-Down Assay

GST pull-down assays were performed to confirm the interaction of NS5B-His with GST-PLSCR, GST-RTN3 and GST-ZNF143 fusion proteins. 50 µl of GST or GST fusion proteins, bound to the beads, were mixed with 10 µg of soluble, purified NS5B-His. The mixture was adjusted to a 500 µl total volume

by PBS and incubated at 4°C for 3 hrs with mixing by end-over-end rotation. Beads were collected by centrifugation at 3,000 x g for 5 min at RT. Beads were washed extensively with PBS containing 0.1% Tween-20 (PBST) and then resuspended in 50 µl PBS. Beads (10 µl) were mixed with 1X SDS sample buffer and then boiled at 95°C for 5 min. The captured proteins were subjected to 10% SDS-PAGE and WB analysis using α-His-HRP antibody (Sigma) to detect NS5B-His or α-GST-HRP antibody (Sigma) for detection of GST-fusion proteins.

2.25.2 Far-Western

Far-western blotting was performed to confirm the interaction of NS5B with the full-length ZNF143. GST and GST-ZNF143 bound to beads were subjected to SDS-PAGE. Proteins were transferred to the nitrocellulose membrane. The membrane was washed three times with TBST. The membrane was probed with 10 ml of 10 µg/ml His-NS5B diluted in TBST for 3 hrs at 4°C. The membrane was washed three times with TBST and processed as previously described in the WB protocol. Proteins were detected using α-His-HRP for NS5B-His or α-GST-HRP for GST-fusion proteins.

2.25.3 Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA assays were performed to confirm the interaction of NS5B with PLSCR1. Soluble purified NS5B-His was diluted in bicarbonate/carbonate coating buffer (Appendix 9.4.3) to a final concentration of 16 µg/ml. Different concentrations of NS5B were made in coating buffer in the plate wells to give final concentrations of 1.6, 0.8, 0.2, 0.05, and 0.025 µg/well in 100 µl total volume per well. The plate was covered by adhesive plastic and incubated at RT for 2 hrs. The coating solution was removed and the plate washed 3-4 times with PBS. The remaining protein-binding sites of the coated wells were blocked by filling the wells with the blocking buffer, 5% non-fat dry milk in PBS containing 0.1% Tween-20, and incubated at 4°C o/n. The plate was washed 3-4 times with PBST. 100 µl of soluble purified GST-PLSCR1 or GST (50 µg/ml)

diluted in blocking buffer were added to each well. The plate was covered and incubated at RT for 2 hrs. Wells were washed 3-4 times with PBST before detecting the captured proteins by incubation with 100 µl of α-GST-HRP antibody diluted in blocking buffer for 1hr at RT. Wells were washed extensively with PBST before adding 25 µl of TMB (3,3',5,5'-tetramethylbenzidine) reagent for 15-30 min at RT in dark area. Colour was allowed to develop before stopping with 25 µl of 2M sulphuric acid (stop solution). Absorbance at 450 nm was determined. The assay was carried out in three separate experiments then the mean±standard error of the mean (SEM) of the three experiments were calculated and plotted against the absorbance at 450 nm.

2.26 Mammalian Tissue Culture

The following cell lines were frequently used throughout the work in this thesis.

Cell Line	
Huh7	Human Hepatoma Cell line
HEK 293T	Human Embryonic Kidney (HEK) 293T Cells
VERO	African Green Monkey Epithelial Kidney Cells

2.26.1 Maintenance of Cultured Cells

Mammalian cell lines were cultured as a monolayer in tissue culture flasks or plates. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, Invitrogen, Appendix 9.6) supplemented with 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin antibiotics, and 10% heat-inactivated fetal bovine serum (FBS) (Appendix 9.6). Cells were incubated at 37°C and 5% carbon dioxide (CO_2). Cells were passaged at 80-100% monolayer confluence. For sub-culturing the cells the monolayer was washed two times with 1X PBS. Three ml of trypsin solution (GIBCO, Invitrogen) supplemented with 1mM ethylenediamine tetraacetic acid

(EDTA) was added to the monolayer and incubated for 3-5 min at RT. Cells were resuspended in 7 ml of complete DMEM medium by pipetting up and down several times. 1-2 ml of cell suspension was added to a fresh tissue culture flask with medium and incubated at 37°C and 5% CO_2.

2.26.2 Calcium Phosphate Precipitation Transfection

One day before the transfection experiment, cells were cultured in complete DMEM to achieve 75-85% confluence at the transfection time. Next day, the medium was replaced with complete fresh medium two hours before transfection. For 75 cm tissue culture flask, the following components were prepared and mixed.

COMPONENT	VOLUME (OR CONCENTRATION)
Plasmid DNA	10-50 µg
1X Tris-HCl (TE) pH 8.8	330 µl
dH_2O	175 µl
2.5 M $CaCl_2$	56.5 µl

After mixing, 570 µl of 2X HBS buffer (Appendix 9.6) was added to the mixture dropwise under vortexing. The mixture was incubated at RT for 5 min then added dropwise to the cell monolayer and mixed by gentle rotation of the flask. Next day, the transfection medium was replaced with complete fresh DMEM medium. Investigation of protein expression was usually performed 48 hrs post-transfection.

2.26.3 Lentivirus-Based Vector (Lentivectors) Production

Lentivectors were used in this study to deliver NS5B, PLSCR1, ZNF143, short-hairpin (sh) PLSCR1, and shZNF143 open reading frames (ORFs) to Huh7 cells (Chapter 5). The system is summarized in 5 steps in Fig. 2.1 and as following: a) Lentivector plasmids were transfected into the packaging cell

(Human Embryonic Kidney (HEK) 293T cells), b) transfected cells express the main proteins required to form virus like particles (VLPs) containing RNA genome with the required ORF, c) Produced VLPs were used to infect the target cell (Huh7), d) Once inside, viral RNA with NS5B ORF is released from the capsid and transcribed to form DNA double strands, and e) DNA double strands then translocate to the nucleus and integrate with target cell chromosome to be translated by the host translational machinery expressing NS5B (Buchschacher and Wong-Staal, 2000).

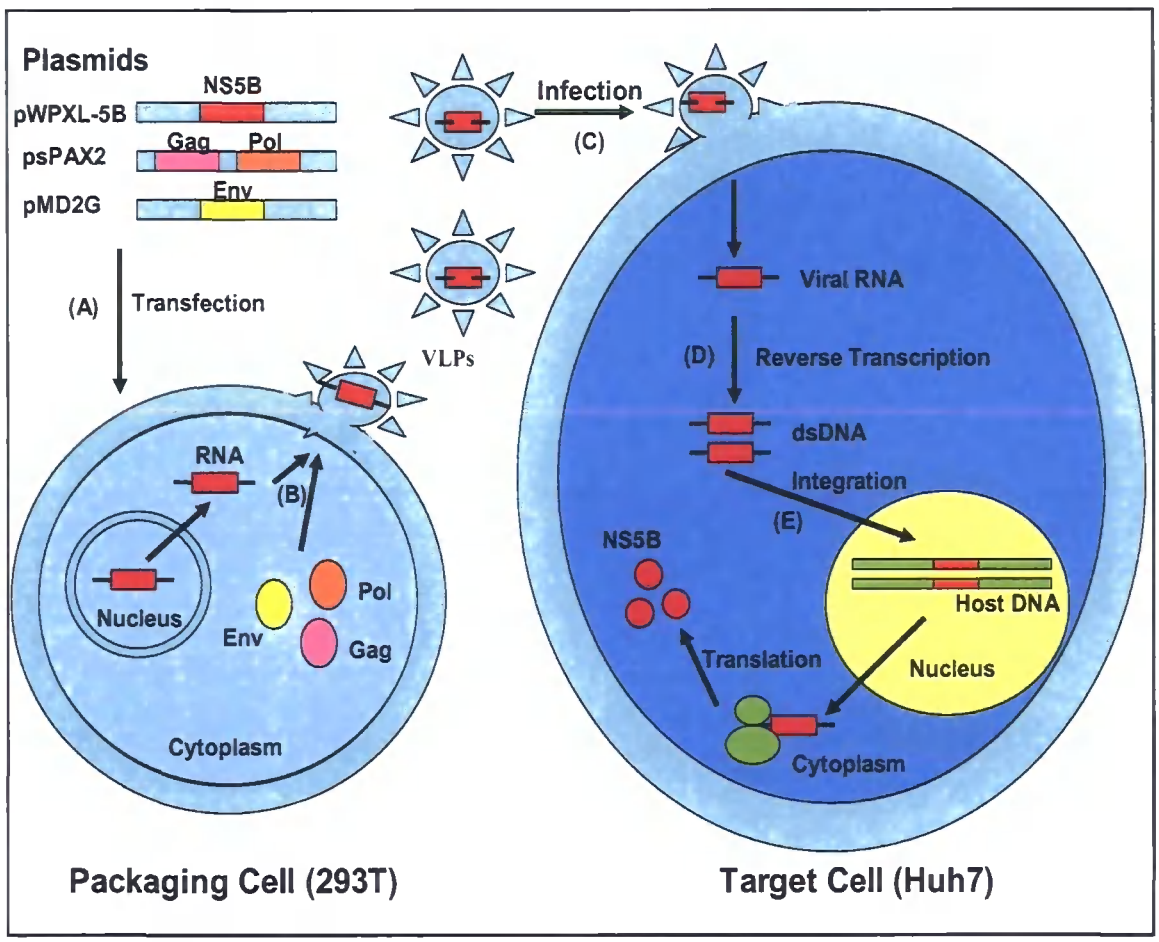

Fig. 2.1: Production of VLPs with NS5B ORF using lentivirus-based vector system (Buchschacher and Wong-Staal, 2000). (A) Lentivector plasmids were transfected into 293T cells, (B) Transfected cells express the main proteins required to form VLPs containing RNA genome with NS5B ORF, (C) Produced VLPs were used to infect Huh7 cells, (D) Once inside, viral RNA with NS5B ORF is released from the capsid and transcribed to form dsDNA, and (E) dsDNA then translocate to the nucleus and integrate with target cell chromosome that is translated by the host translational machinery expressing NS5B.

Lentivectors were produced in Human Embryonic Kidney (HEK) 293T cells (packaging cell line) by co-transfection of pWPXL (22.5 µg), psPAX2 (14.6 µg) and pMD2G (7.9 µg) into cells using calcium phosphate precipitation transfection method as described below. The virus-like particles (VLPs)-containing supernatant was collected 48 hrs and 72 hrs post-transfection. The collected supernatants were centrifuged at 1500 x g for 5 min to remove cell debris and then filtrated on 0.22 µm syringe filter. Supernatants containing VLPs were used directly for transduction protocol or stored at -80°C in aliquots. For titration of VLPs, a serial two-fold dilution of GFP-VLPs supernatant was made and used to transduce 293T cells cultured in 24 well plate in 1 ml/well total volume. GFP expression was detected 48 hrs post-transduction. The viral titer was expressed as a number of infected cells/ml determined by the number of GFP-expressing cells detected at the highest dilution. VLPs titre was optimized to produce 5 x 10^6 VLP/ml.

2.26.4 RNA Interference (RNAi)

shRNA was used to knock-down the expression of PLSCR1 and ZNF143 in Huh7 cells using lentivectors (kindly provided by Didier Trono, Tronolab, Swizterland). Two complementary oligonucleotides (19 nucleotides each from 94 to 112) targeting the coding sequence of PLSCR1 were synthesized by Sigma according to the previously published shPLSCR1 sequence (Dong *et al.*, 2004). For ZNF143 expression down-regulation, the ZNF143 target sequence (21 nucleotides from 885 to 905) was selected and designed using Block-iT RNAi Designer (Invitrogen) then synthesized by Sigma. The oligonucleotides were designed as recommended by Tronolab website (http://tronolab.epfl.ch) to contain the two complementary target sequences of 19-21 nucleotides separated by 9 nucleotides (loop) and encompassing *MluI* and *ClaI* restriction sites when annealed to facilitate the cloning (Fig. 2.1). The oligonucleotides were annealed as previously described then sub-cloned into pLVTHM (Tronolab, Appendix 9.13), a bicistronic plasmid that allows the expression of GFP under the control of EF1α promoter and shRNA under the control of H1 promoter.

VLPs carrying shRNA-encoding sequences were prepared by co-transfection of pLVTHM-shPLSCR1, –shZNF143, or pLVTHM-shGFP with psPAX2 and pMD2G into 293T cells by calcium phosphate precipitation method as previously described. VLPs-containing supernatant were harvested 48 and 72 hrs post-transfection. VLPs were used to transduce Huh7 cells and the expression was monitored by RT-PCR as described before. Virus titration was determined by monitoring GFP expression as described before using the serial dilution assay (Section 2.26.3).

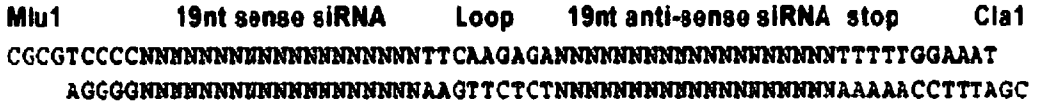

Fig. 2.2: General strategy for designing shRNA. Two oligonuceotides were designed to be annealed and cloned into pLVTHM. Upon transcription, they create shRNA targeting PLSCR1 or ZNF143 coding sequences.

2.26.5 *In Vitro* HCV Sub-Genomic Replicon RNA Transcription

Using HCV sub-genomic replicon plasmid (pFK i341 PiLucNS3-3` dg ET) (Appendix 9.12) as a template, RNA was synthesized *in vitro* using MEGAscript Transcription Kit (Ambion) following the manufacturer's instructions

2.26.6 RNA Agarose Gel Electrophoresis

In vitro transcribed-RNA was analyzed for its size and integrity on 1% (w/v) denatured MOPS-Formaldehyde agarose gel prepared with DEPC (diethylpyrocarbonate)-treated water (Appendix 9.3). Using a mini-gel electrophoresis apparatus (BioRad), RNA (1 µg) was mixed with the gel loading buffer (provided with the kit) and 1 µl of 0.5 µg/ml ethidium bromide. Electrophoresis was carried out at 100 V for 40 min or until the dye reached the bottom of the gel. RNA was visualized by UV-Trans-illuminator.

2.26.7 Electroporation of RNA

A Gene Pulser System (BioRad) was prepared for the transformation using electro-cuvette (Sigma) with gap width of 0.4 cm. The setting was adjusted for Huh7 cells (Voltage 270 V, Capacitance 960 µF) and the time constant was kept to approximately 20 msec. Huh7 cell monolayer cultured in 75 cm^2 tissue culture flask was trypsinized as described above. 7 ml of complete DMEM medium was added to stop the trypsin action then the cells were collected by centrifugation at 300 x g for 5 min. Cells were washed two times with PBS followed by centrifugation at 300 x g for 5 min. Cells were resuspended in PBS at concentration of 10^7/ml. 10 µg of *in-vitro* transcribed RNA was mixed well with 400 µl of cell suspension and then transferred to the electroporation cuvette. One pulse was delivered to the cell suspension that immediately transferred to 10 ml of complete DMEM medium. Cells were seeded in 24 well plate or cells of several electroporations were combined and seeded in when more plates were required.

2.26.8 Luciferase Assay

Luciferase expression and activity were measured using One-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. To measure the luciferase activity in cells cultured in 24 well plate, medium was removed 48 hrs post-transfection or –transduction and 100 µl of fresh medium was added. An equal volume of One-Glo reagent was added to the cells and mixed well for 10 min before transferring the cell lysate to an opaque walled 96-well plate. Luminescence signal was then measured using a plate reader (MicroBeta 1450, PerkinElmer). The assay was carried out in three separate experiments and each sample was measured in triplicate then the mean±standard deviation (SD) or the mean±standard error of the mean (SEM) of the three experiments were calculated.

2.26.9 *In Vitro* NS5B-His Pull-Down Assay

NS5B-His bound to Ni-beads were used for pull-down cellular proteins that can interact with NS5B. For cell lysate preparation, Huh7 or 293T cells were solubilized in cell culture lysis reagent (CCLR; Promega) containing protease inhibitor cocktail (Sigma). The solubilized cell lysates were frozen and thawed three times. Unbroken cells and cellular debris were removed by centrifugation at 30,000 x g at 4°C for 10 min. After incubation on ice for 15 min, the lysates were then incubated with tNS5B-His fusion protein bound to the beads for 3 hrs at 4°C with rotation. The beads were washed three times with PBS and the bound proteins were eluted by adding 1X SDS sample loading buffer then boiled for 5 min. The samples were subjected to 10% SDS-PAGE and proteins were detected by WB using anti-α or β-tubulin antibodies or α-His for detection of His-tag fusion proteins.

2.26.10 *In Vivo* NS5B-His Pull-Down Assay

His-NS5B or His-LacZ fusion proteins were expressed in Huh7 cells by transfection of pcDNA4-5B or pcDNA4-LacZ, respectively, using calcium phosphate precipitation method as previously described. 48 hrs post-transfection, cells were harvested and lysed with CCLR containing protease inhibitors. Cell lysates were centrifuged at 30,000 x g at 4°C for 10 min and then incubated with PBS-washed Ni beads for 3 hrs at 4°C with rotation. Beads were collected and washed extensively with PBS before subjected to SDS-PAGE and WB as described before.

2.26.11 Protein Mass Spectrometry

Samples were prepared by resolving on 10% SDSPAGE and staining with Coomassie blue R-250. A protein band was carefully excised and cut into 1 × 1-mm pieces. The piece of gel was sent to the Proteomic Unit (Durham University) for sequencing analysis after trypsin digestion using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry

using a PE Biosystems ABI Voyager DE-STR mass spectrometer. The data were processed using Applied Biosystems Analyst and BioAnalyst™ software. MASCOT search tools were used for searching against all publicly available databases for protein identification. The method for calculation of the Score parameter is described at Website http://www.matrixscience.com.

2.26.12 Statistics

Each assay was carried out in three separate experiments and each sample was measured in triplicate then the mean±standard deviation (SD) or the mean±standard error of the mean (SEM) of three experiments were calculated according to the following equations:

$$SD = \sqrt{\frac{\sum(X - \bar{X})^2}{n - 1}} \qquad SEM = \frac{SD}{\sqrt{n}}$$

Where SD is the standard deviation, $\bar{X}$ is the mean of three observations and n is the number of observations. *p*-value was calculated from three separate experiments using the online student's two-tailed t-test calculator (http://www.graphpad.com/quickcalcs/ttest1.cfm).

CHAPTER 3

Identification of Host Cell Proteins that Interact with NS5B

3.1 Introduction

The interaction with the host cell is critical for HCV to replicate and develop persistence (Grakoui, 2004). NS5B interacts with viral and host cell proteins to drive viral RNA replication but host cell proteins implicated in viral replication and/or persistence are still largely unknown (Gosert *et al.*, 2003). Therefore, there is a need to identify cellular proteins that could contribute to viral replication and persistence that may help in the development of anti-HCV therapies. To achieve this aim, the yeast two-hybrid system was employed as this system has been widely used and has a proven capability in identifying new interactions between NS5B and cellular proteins such as upiquitin-like protein (hPLIC1), RNA helicase p68 and the scaffold protein, septin 6 (Gao *et al.*, 2003; Goh *et al.*, 2004; Kim *et al.*, 2007).

The MATCHMAKER GAL4 two-hybrid system 3 (Clontech) was used in a screening protocol for potential host factors that can interact with NS5B under high stringency conditions to exclude the possibility of false positive interactions. It is an advanced GAL4-based two-hybrid system that provides a transcriptional assay for detecting even relatively weak and transient protein-protein interactions *in vivo* in yeast. In this system, NS5B was used as a fusion with the GAL4 DNA-Binding Domain (DNA-BD), while a human cDNA library produced from HeLa cells was expressed as fusions with the GAL4 DNA-activation domain (DNA-AD). When NS5B and cDNA library fusion proteins interact, DNA-BD and DNA-AD form an active transcription complex that can activate transcription of four downstream reporter genes, *HIS3, ADE2, LacZ,* and *MEL1* (Fig. 3.1).

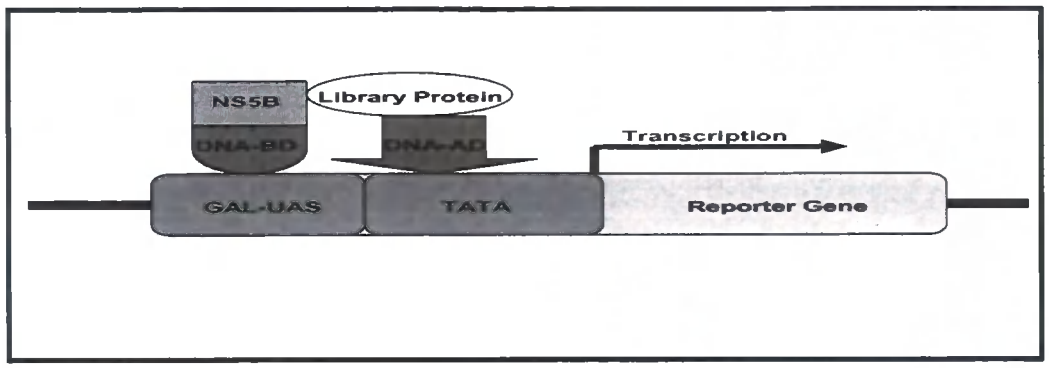

Fig. 3.1: Yeast two-hybrid system principle. The DNA-BD is amino acids 1–147 of the yeast GAL4 protein, which binds to the GAL upstream activating sequence (UAS) upstream of the reporter genes. The AD is amino acids 768–881 of the GAL4 protein and functions as a transcriptional activator. The four reporter genes are under the control of distinct GAL4 upstream activating sequences (UASs) and TATA boxes.

The four reporter genes are under the control of heterologous GAL4-responsive upstream activating sequences (UASs) and TATA box promoters that give strong and specific responses to GAL4. *HIS3* and *ADE2* provide strong nutritional selections for yeast cells that contain interacting proteins. *MEL1* or *LacZ* encode α- and β-galactosidase, respectively, which provide additional colour indicators for isolation of positive yeast clones containing interacting proteins (Fig. 3.2).

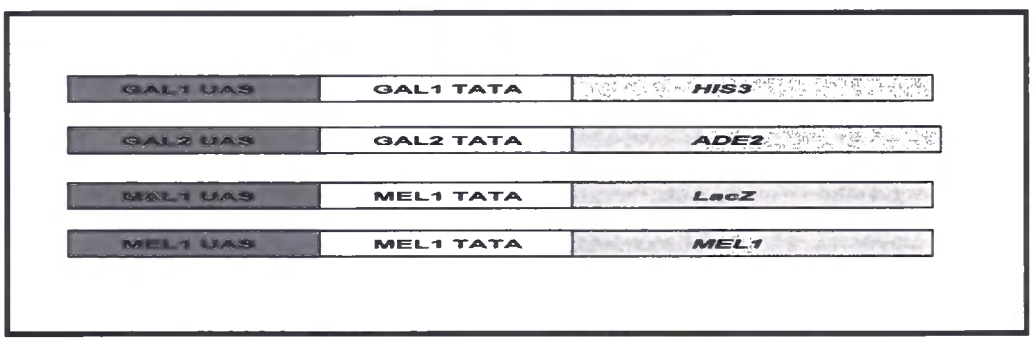

Fig. 3.2: Four reporter genes in AH109 yeast strain. A schematic diagram showing that expression of four reporter genes in AH109 is under the control of three different UAS (GAL1, GAL2 and MEL1) and TATA sequences.

3.2 Expression of GAL4 BD-NS5B in Yeast

Full-length HCV NS5B ORF (Appendix 9.7.1) was amplified from a cDNA clone of HCV-H77c (genotype 1a; accession number AF011751, kindly provided by Jens Bukh, NIH, Bethesda, USA). Primers (Appendix 9.11) were designed to facilitate in-frame cloning of NS5B ORF (1776 bp) with the DNA-BD sequence of the bait vector, pGBKT7, at its N-terminus. Sequencing of potential positive clones was performed and a clone with NS5B, in-frame with the GAL4 BD was isolated for the subsequent work. This new plasmid was termed pGBK-5B.

pGBK-5B was transformed into competent *Saccharomyces cerevisiae,* strain AH109, and plated onto SD/-Trp medium plate and incubated for 4-6 days at 30°C. A clone was isolated and cultured in 5 ml SD/-Trp liquid medium and incubated at 30°C O/N. The cells were lysed and total protein was extracted. Expression of BD-NS5B was confirmed by western blotting using sheep anti-NS5B antibodies (kindly provided by Mark Harris, Leeds University) and anti-sheep-HRP (Sigma). A band of ~80 kDa, equivalent to the size of GAL4 DNA-binding domain fused to NS5B, BD-NS5B, was detected confirming BD-NS5B expression in AH109 cells (Fig. 3.3).

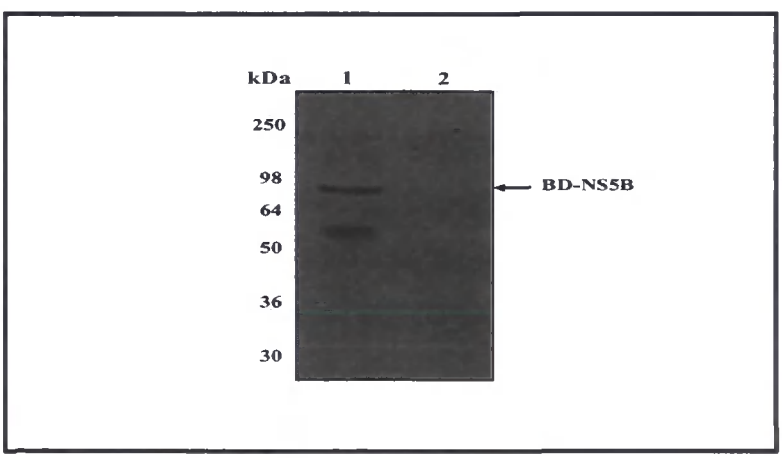

Fig. 3.3: Expression of BD-NS5B in AH109 yeast cells. Western blotting analysis was performed for NS5B detection using sheep α-NS5B primary antibodies and α-sheep secondary antibodies. Where 1- Total protein extracted from AH109 cells transformed by pGBK-5B or 2- pGBKT7.

3.3 Screening for Interactions with NS5B Using HeLa cDNA Library

Initially to determine that NS5B did not activate the reporter genes by itself, AH109 cells were transformed by pGBK-5B and plated onto SD/-Trp/X-α-Gal and incubated for 4-6 days at 30°C. There was no activation of reporter genes indicating that NS5B was suitable for use with the yeast two-hybrid system.

Freshly prepared AH109 cells containing pGBK-5B were sequentially transformed with a human HeLa cDNA library using a large-scale transformation protocol (Section 2.23.5). The transformed AH109 cells were plated onto 5 large medium-stringency plates (SD/-Trp/-Leu/-His) and incubated for 6 days at 30°C. A large number of colonies (~500) were obtained in this initial screen but as it was a medium-stringency screen it was likely that a number of false positives were present. Further screening of colonies under high-stringency conditions, was performed by replating clones onto a high-stringency plate (SD/-Trp/-Leu/-His/-Ade) supplemented with X-α-Gal in a second round of screening. A number of colonies survived this second high-stringency protocol. Blue colonies (positive colonies) were isolated and re-streaked again onto high-stringency plate for retesting and so confirming the phenotype (Fig. 3.4).

Positive colonies were isolated and cultured in SD/-Leu liquid medium under a selective pressure to maintain pGAD-cDNA library plasmids within the yeast but with no pressure to retain pGBK-5B. Cells were incubated O/N at 30°C then a small inoculum from these cell cultures were incubated again in fresh liquid SD/-Leu medium O/N at 30 °C. This process was repeated for a third time to facilitate the removal of pGBK-5B. AD/cDNA plasmids were subsequently isolated from the cultured AH109 cells.

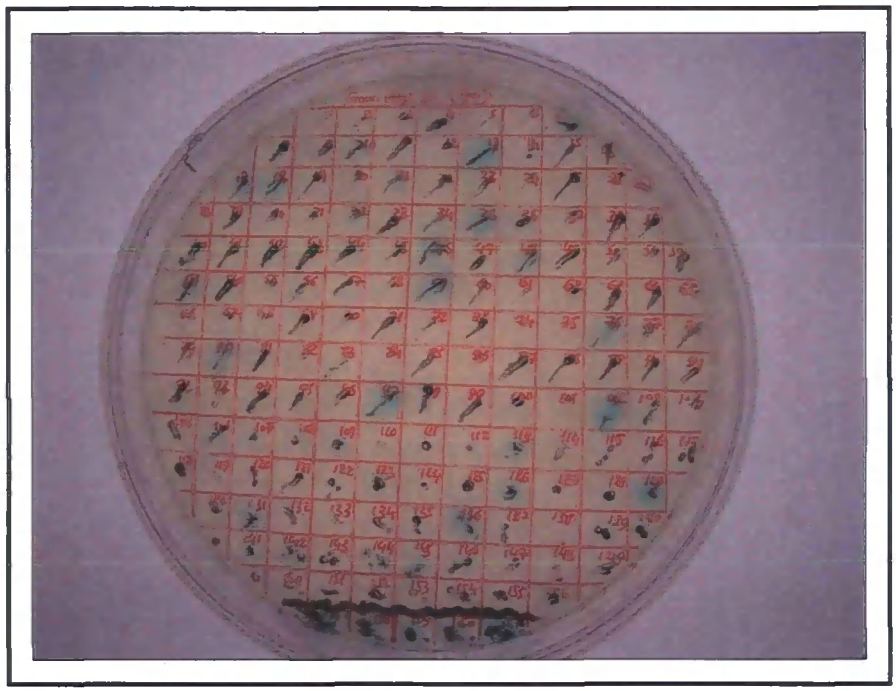

Fig. 3.4: Yeast two-hybrid screening for positive interactions with NS5B. Transformed cells were plated onto high-stringency plate containing X-α-Gal for α-galactosidase activity detection. Positive interactions develop blue colour colonies.

3.4 Confirmation of Positive Interactions with NS5B

Isolated AD/cDNA plasmids from yeast were used to transform chemically competent *E. coli* which was plated onto LB plates containing ampicillin (LB-amp). The plates were incubated O/N at 30°C. One colony, from each plate, was isolated and cultured in 50 ml LB-amp O/N at 30°C. Plasmids were extracted and the presence of an insert was confirmed by *EcoRI-XhoI* restriction.

Purified positive AD/cDNA library plasmids were reintroduced into AH109 cells containing either a negative control plasmid (pGBKT7-Lam) or pGBK-5B and plated onto SD/-Leu/-Trp medium plates to isolate transformants that were subsequently transferred to high-stringency plates. pGBKT7-Lam, which encodes the human lamin C protein in fusion with GAL4-BD, was used as a

non-specific protein control for non-specific interactions between NS5B and AD/library proteins. pGBKT7-53 and pGADT7-T were used as a positive control for expression of two interacting proteins. No false positive interactions were observed confirming the specificity of the interaction of the isolated library clones and BD-NS5B, at least in the yeast.

Positive clones were sequenced and subjected to a Blast search on the National Centre for Biotechnology Information (NCBI). Blast identified seven potential candidate proteins that interacted with NS5B:

- Clone 7: Phospholipid Scramblase 1 (PLSCR1) (NCBI Accession Number AF008445).

- Clone 14: Oxysterol Binding Protein 8 (OSBP8) (NCBI Accession Number NP_001003712).

- Clone 17: Reticulon 3 Isoform A (RTN3) (NCBI Accession Number NP_006045).

- Clone 31: HS1 (Haematopoietic Lineage Cell-Specific Protein 1)-Associated Protein X-1 (HAX1) (NCBI Accession Number NP_006109).

- Clone 59: Zinc Finger Protein 143 (ZNF143) (NCBI Accession Number NM_003442).

- Clone 71: Translocase of Inner Mitochondrial Membrane 50 (TIMM50) (NCBI Accession Number AAH50082).

- Clone 93: Splicing Factor, Arginine/Serine-Rich 10 (SFRS10) (NCBI Accession Number NP_004584.1).

3.5 Protein Sequence Blast Search of Clone Number 7

Blast confirmed that clone 7 encoded 192 amino acids, from a total of 318 amino acids, from the N-terminal region of PLSCR1 (Fig. 3.5).

```
                    1                                                    50
pGADGH-SCR1     (1) MDKQNSQMNASHPETNLPVGYPPQYPPTAFQGPPGYSGYPGPQVSYPPPP
  FL-PLSCR1     (1) MDKQNSQMNASHPETNLPVGYPPQYPPTAFQGPPGYSGYPGPQVSYPPPP
                    51                                                   100
pGADGH-SCR1    (51) AGHSGPGPAGFPVPNQPVYNQPVYNQPVGAAGVPWMPAPQPPLNCPPGLE
  FL-PLSCR1    (51) AGHSGPGPAGFPVPNQPVYNQPVYNQPVGAAGVPWMPAPQPPLNCPPGLE
                   101                                                   150
pGADGH-SCR1   (101) YLSQIDQILIHQQIELLEVLTGFETNNKYEIKNSFGQRVYFAAEDTDCCT
  FL-PLSCR1   (101) YLSQIDQILIHQQIELLEVLTGFETNNKYEIKNSFGQRVYFAAEDTDCCT
                   151                                                   200
pGADGH-SCR1   (151) RNCCGPSRPFTLRIIDNMGQEVITLERPLRCSSCCCPCCLQE--------
  FL-PLSCR1   (151) RNCCGPSRPFTLRIIDNMGQEVITLERPLRCSSCCCPCCLQEIEIQAPPG
                   201                                                   250
pGADGH-SCR1   (193) ----------------------------------------------------
  FL-PLSCR1   (201) VPIGYVIQTWHPCLPKFTIQNEKREDVLKISGPCVVCSCCGDVDFEIKSL
                   251                                                   300
pGADGH-SCR1   (193) ----------------------------------------------------
  FL-PLSCR1   (251) DEQCVVGKISKHWTGILREAFTDADNFGIQFPLDLDVKMKAVMIGACFLI
                   301            318
pGADGH-SCR1   (193) ------------------
  FL-PLSCR1   (301) DFMFFESTGSQEQKSGVW
```

Fig. 3.5: Protein sequence of clone 7 aligned with full-length PLSCR1 protein sequence. Clone 7 encoded 192 amino acids (highlighted), from a total of 318 amino acids, from the N-terminal region of PLSCR1.

PLSCR1 is a multiply palmitoylated, lipid-raft-associated plasma membrane protein that mediates the accelerated ATP-independent bidirectional flipping of phospholipids upon binding calcium ions which results in a loss of phospholipid asymmetry in the plasma membrane (Sims and Wiedmer, 2001). PLSCR1 is encoded by an interferon-stimulated gene (ISG) and enhances the IFN response through increased expression of other potent downstream antiviral genes (including ISG15, ISG54, p56, and guanylate binding proteins) (Dong *et al.*, 2004).

3.6 Protein Sequence Blast Search of Clone Number 14

Clone 14 encoded 40 amino acids from the OSBP8 C-terminal region (Fig. 3.6).

```
                        1                                                  50
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8      (1)  MSQRQGKEAYPTPTKDLHQPSLSPASPHSQGFERGKEDISQNKDESSLSM
                       51                                                 100
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8     (51)  SKSKSESKLYNGSEKDSSTSSKLTKKESLKVQKKNYREEKKRATKELLST
                      101                                                 150
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (101)  IIDPSVIVMADWLKIRGTLKSWTKLWCVLKPGVLLIYKTQKNGQWVGTVL
                      151                                                 200
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (151)  LNACEIIERPSKKDGFCFKLFHPLEQSIWAVKGPKGEAVGSITQPLPSSY
                      201                                                 250
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (201)  LIIRATSESDGRCWMDALELALKCSSLLKRTMIREGKEHDLSVSSDSTHV
                      251                                                 300
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (251)  TFYGLLRANNLHSGDNFQLNDSEIERQHFKDQDMYSDKSDKENDQEHDES
                      301                                                 350
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (301)  DNEVMGKSEESDTDTSERQDDSYIEPEPVEPLKETTYTEQSHEELGEAGE
                      351                                                 400
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (351)  ASQTETVSEENKSLIWTLLKQVRPGMDLSKVVLPTFILEPRSFLDKLSDY
                      401                                                 450
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (401)  YYHADFLSEAALEENPYFRLKKVVKWYLSGFYKKPKGLKKPYNPILGETF
                      451                                                 500
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (451)  RCLWIHPRTNSKTFYIAEQVSHHPPISAFYVSNRKDGFCLSGSILAKSKF
                      501                                                 550
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (501)  YGNSLSAILEGEARLTFLNRGEDYVMTMPYAHCKGILYGTMTLELGGTVN
                      551                                                 600
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (551)  ITCQKTGYSAILEFKLKPFLGSSDCVNQISGKLKLGKEVLATLEGHWDSE
                      601                                                 650
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (601)  VFITDKKTDNSEVFWNPTPDIKQWRLIRHTVKFEEQGDFESEKLWQRVTR
                      651                                                 700
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (651)  AINAKDQTEATQEKYVLEEAQRQAARDRKTKNEEWSCKLFELDPLTGEWH
                      701                                                 750
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (701)  YKFADTRPWDPLNDMIQFEKDGVIQTKVKHRTPMVSVPKMKHKPTRQQKK
                      751                                                 800
pGADGH-OSBP8      (1)  --------------------------------------------------
    FL-OSBP8    (751)  VAKGYSSPEPDIQDSSGSEAQSVKPSTRRKKGIELGDIQSSIESIKQTQE
                      801                                              847
pGADGH-OSBP8      (1)  ------MALRNHLVSSTPATDYFLQQKDYFIIFLLILLQVIINFMFK
    FL-OSBP8    (801)  EIKRNIMALRNHLVSSTPATDYFLQQKDYFIIFLLILLQVIINFMFK
```

Fig. 3.6: Protein sequence of clone 14 aligned with full-length OSBP8 protein sequence. Clone 14 encoded 40 amino acids (highlighted) from the OSBP8 C-terminal region.

OSBP8 is a member of the oxysterol-binding protein-related protein (ORP) family which includes 12 mammalian proteins involved in vesicle transport, lipid metabolism, and cell signalling (Jaworski *et al.*, 2001; Lehto *et al.*, 2001; Lehto and Olkkonen, 2003). They are characterized by the presence of the C-terminal OSBP-homology domain responsible for binding oxysterols and cholesterol

(Suchanek *et al.*, 2007). OSBP8 is highly expressed in liver and localizes to the ER via its C-terminal transmembrane motif (Lehto and Olkkonen, 2003; Yan *et al.*, 2008). It has a suppressive effect on the expression of the ATP-binding cassette transporter A1 (ABCA1) affecting cholesterol efflux to apolipoprotein A1 (Yan *et al.*, 2008).

3.7 Protein Sequence Blast Search of Clone Number 17

Clone 17 encoded 142 amino acids, from a total of 236 amino acids from the C-terminal region of RTN3 (Fig. 3.7).

```
                    1                                                  50
pGADGH-RTN3     (1) --------------------------------------------------
    FL-RTN3     (1) MAEPSAATQSHSISSSSFGAEPSAPGGGGSPGACPALGTKSCSSSCAVHD
                   51                                                 100
pGADGH-RTN3     (1) ----------------------------------------------ISFRIY
    FL-RTN3    (51) LIFWRDVKKTGFVFGTTLIMLLSLAAFSVISVVSYLILALLSVTISFRIY
                  101                                                 150
pGADGH-RTN3     (7) KSVIQAVQKSEEGHPFKAYLDVDITLSSEAFHNYMNAAMVHINRALKLII
    FL-RTN3   (101) KSVIQAVQKSEEGHPFKAYLDVDITLSSEAFHNYMNAAMVHINRALKLII
                  151                                                 200
pGADGH-RTN3    (57) RLFLVEDLVDSLKLAVFMWLMTYVGAVFNGITLLILAELLIFSVPIVYEK
    FL-RTN3   (151) RLFLVEDLVDSLKLAVFMWLMTYVGAVFNGITLLILAELLIFSVPIVYEK
                  201                                 236
pGADGH-RTN3   (107) YKTQIDHYVGIARDQTKSIVEKIQAKLPGIAKKKAE
    FL-RTN3   (201) YKTQIDHYVGIARDQTKSIVEKIQAKLPGIAKKKAE
```

Fig. 3.7: Protein sequence of clone 17 aligned with full-length RTN3 protein sequence. Clone 17 encoded 142 amino acids (highlighted), from a total of 236 amino acids, of the C-terminal region of RTN3.

RTN3, one of the four membered reticulon family of proteins, is an ER-localized transmembrane protein apparently involved in membrane trafficking between the ER and the Golgi that may play a role in membrane trafficking in the early secretory pathway (Wakana *et al.*, 2005). Over-expression of RTN3 caused an ER-overload response leading to the release of ER Ca^{2+} stores that resulted in an elevation of cytosolic Ca^{2+} which can trigger apoptotic pathways (Kuang *et al.*, 2005). RTN3 has previously been shown to be an important component of the replication complex of enterovirus 71, a picornavirus (Tang *et al.*, 2007).

3.8 Protein Sequence Blast Search of Clone Number 31

Clone 31 encoded 124 amino acids from the HAX1 C-terminal region (Fig. 3.8).

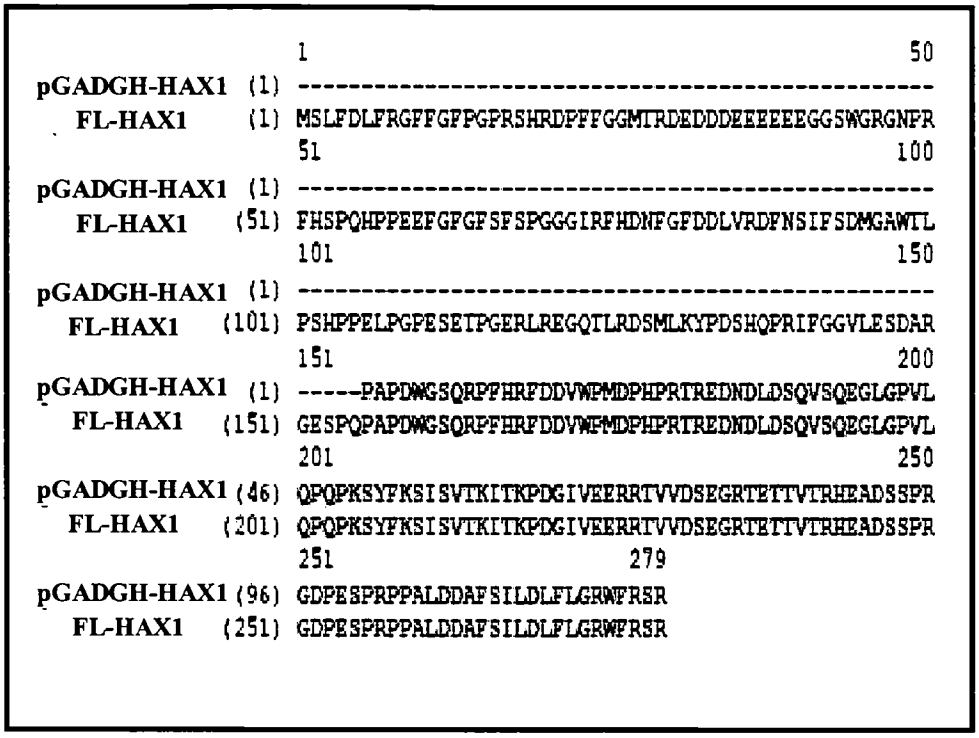

Fig. 3.8: Protein sequence of clone 31 aligned with full-length HAX1 protein sequence. Clone 31 encoded 124 amino acids (highlighted) from the HAX1 C-terminal region.

HAX1 is a multifunctional factor involved in apoptosis, cell migration, endocytosis and mRNA transport (Szwarc *et al.*, 2007). It is localized mainly to the mitochondria, but is also found in the ER and the nuclear envelope (Suzuki *et al.*, 1997). The Epstein-Barr virus (EBV) nuclear-antigen leader protein (EBNA-LP), which plays a critical role in EBV-induced transformation, affects apoptosis in EBV-infected cells by an interaction with the cellular anti-apoptotic Bcl2 protein through HAX1 (Matsuda *et al.*, 2003). HAX1 was also observed to inhibit the binding of HIV-1 rev to the rev-responsive element (RRE) by altering its sub-cellular localization (Modem and Reddy, 2008).

3.9 Protein Sequence Blast Search of Clone Number 59

Blast analysis of protein sequence from clone 59 showed that it encoded 277 amino acids, from a total of 638 amino acids, from the ZNF143 C-terminal region (Fig. 3.9).

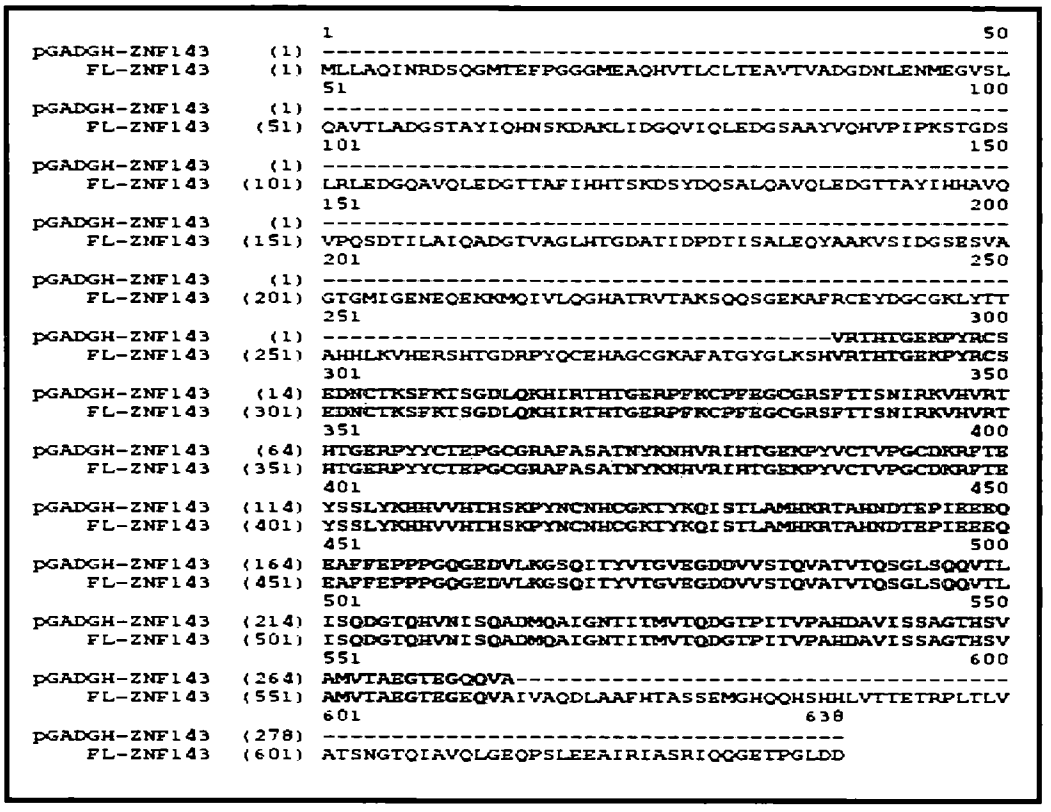

Fig. 3.9: Protein sequence of clone 59 aligned with full-length ZNF143 protein sequence. Clone 59 encoded 277 amino acids (highlighted) from a total of 638 amino acids at the ZNF143 C-terminal region.

ZNF143 is a transcriptional-activating factor and is the human homolog of the *Xenopus* selenocysteine tRNA transcription-activating factor, *Staf* (Myslinski *et al.*, 1998). ZNF143 was shown to enhance the transcription of small nuclear RNAs (snRNA) and snRNA-type genes by RNA Pol III. Significantly, ZNF143 also possesses the capacity to stimulate the expression from a RNA Pol II promoter, notably that of the IFN regulatory factor-3 (IRF3) gene which is required for induction of IFN-β after viral infection (Mach *et al.*, 2002; Myslinski *et al.*, 1998; Schuster *et al.*, 1995).

3.10 Protein Sequence Blast Search of Clone Number 71

Clone 71 encoded 271 amino acids from the central region of TIMM50 (Fig. 3.10).

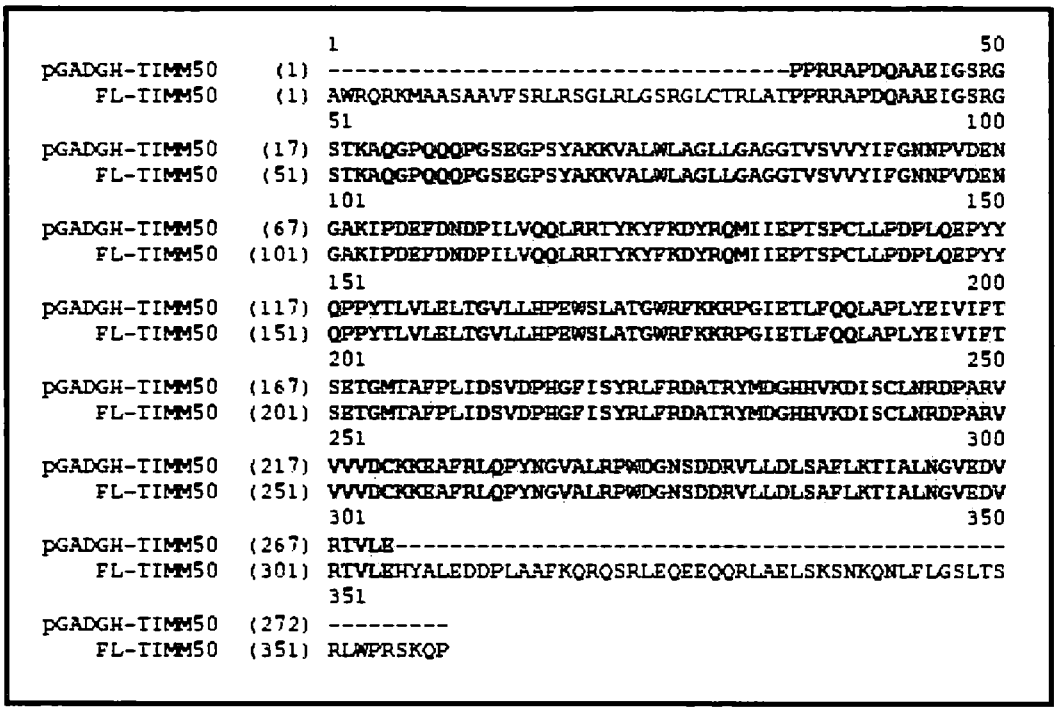

Fig. 3.10: Protein sequence of clone 71 aligned with full-length TIMM50 protein sequence. Clone 71 encoded 271 amino acids (highlighted) from the central region of TIMM50.

TIMM50 is located mainly in the inner mitochondrial space and represents the human functional homolog of the yeast protein TIM50. It has three domains, a mitochondrial targeting domain at the N-terminus, a central transmembrane domain and a C-terminal phosphatase domain (Guo *et al.*, 2004). It functions as a receptor for the regulation of the trafficking of proteins between the outer and inner mitochondrial membranes and interference with its expression in yeast caused growth arrest and loss of cell viability (Geissler *et al.*, 2002; Mokranjac *et al.*, 2003; Yamamoto *et al.*, 2002). It has a phosphatase activity on some anti-apoptotic factors such as *Bcl2* family members suggesting a role in the regulation of apoptosis. Loss of TIMM50 activity led to mitochondrial dysfunction and cell death (Guo *et al.*, 2004; Meinecke *et al.*, 2006; Sugiyama *et al.*, 2007).

3.11 Protein Sequence Blast Search of Clone Number 93

Clone 93 encoded 168 amino acids from SFRS10; the sequence was discontinuous when compared to the native sequence SFRS10 (Fig. 3.11). This may represent a splice variant although there is no published data to confirm this assumption.

Fig. 3.11: Protein sequence of clone 93 aligned with full-length SFRS10 protein sequence. Clone 93 encoded 168 amino acids (highlighted) of SFRS10.

SFRS10 was found to be over-expressed in invasive breast cancer. Induction of this splicing factor might be responsible for splicing of CD44 isoforms associated with tumour progression and metastasis suggesting an important role for this factor in tumour progression (Watermann *et al.*, 2006). It can regulate apoptosis induction by modulating the activity of the p53 tumour-suppressor protein, a critical mediator of cell growth arrest (Huang *et al.*, 2004).

3.12 Discussion

NS5B, in addition to NS3, represents a good target for designing a novel HCV antiviral therapy as it is the viral RNA polymerase that is essential for viral replication (Behrens *et al.*, 1996). The HCV replication complex is a multiprotein complex that consists of the viral non-structural proteins (NS3 to NS5B) in addition to a number of cellular proteins (Gosert *et al.*, 2003). To gain more insight into host cell proteins that can interact with NS5B, full-length NS5B was used as bait by the yeast two-hybrid system to identify novel interactions with host cell proteins which may be involved in viral replication, persistence or pathogenicity.

The yeast two-hybrid system successfully identified seven proteins that can interact with NS5B *in vivo* in yeast. The interactions were confirmed by more than three rounds of testing in yeast under high-stringency conditions to eliminate false positives and back testing against control proteins to show the specificity of the interaction with NS5B. All the identified proteins are expressed in hepatic and non-hepatic cells. Most have a role in cellular signalling pathways such as apoptosis and IFN signalling pathways.

PLSCR1 is plasma membrane protein enriched in lipid rafts and traffics between the cytoplasm and nucleus to perform multiple functions in such cell proliferation, apoptosis, gene regulation, IFN response, EGFR signalling pathway and the trans-bilayer movement of plasma membrane phospholipids (Dong *et al.*, 2004; Li *et al.*, 2006; Nanjundan *et al.*, 2003; Sun *et al.*, 2002; Yu *et al.*, 2003; Zhou *et al.*, 2005). PLSCR1 was identified with other ISGs to play a major role in decreasing HCV titre during the early phase of interferon treatment (Brodsky *et al.*, 2007). In addition, PLSCR1 was shown to have an antiviral activity against VSV, EMCV *in vitro* (Dong *et al.*, 2004) and DENV *in vivo* in rhesus macaques (Sariol *et al.*, 2007). Recently, PLSCR1 was shown to interact with CD4 at the cell surface of T lymphocytes. This interaction was important for HIV-1 entry as the disruption of the CD4/PLSCR1 interaction by the secretory leukocyte protease inhibitor (SLPI) blocked HIV-1 infection (Py *et al.*, 2009).

Our results have shown that NS5B could interact with OSBP8 which is involved in lipid transport and metabolism. It could also regulate the efflux of cholesterol from the cell by modulating the activity of ABCA1 (Bowden and Ridgway, 2008; Yan *et al.*, 2008). Interestingly, HCV infection alters VLDL and LDL molecular composition and changes cellular lipid metabolism leading to the accumulation of intracellular lipid (Jarmay *et al.*, 2005; Napolitano *et al.*, 2007; Zejc-Bajsarowicz *et al.*, 2005).

NS5B also interacted with the C-terminus of RTN3. This region encompassed most of the RTN homology domain (RHD) (60-236 a.a.) which is highly conserved throughout family members (He *et al.*, 2007). RTN3 is required for cellular survival and for the optimal anti-apoptotic activity of Bcl-2 (Wan *et al.*, 2007). RTN3 plays an important role in Alzheimer pathogenesis by interacting with, and inhibiting the activity of, β-secretase β-site APP cleaving enzyme 1 (BACE1), a membrane-bound aspartic protease essential for the generation of amyloid β-protein, which accumulates in the brains of Alzheimer's patients (Murayama *et al.*, 2006). RTN3's C-terminus, as with NS5B, mediates the interaction with BACE1 (He *et al.*, 2006). It has been suggested that RTN3 is a common factor for the replication of *Picornaviruses* such as enterovirus 71, poliovirus and coxsackie virus A16 (Tang *et al.*, 2007).

HAX1, a 35 kDa anti-apoptotic protein localized in mitochondria, is involved in mRNA transport, cell migration, and cancer progression (Ramsay *et al.*, 2007; Szwarc *et al.*, 2007). HAX1 was required for maintaining and protecting the inner mitochondrial membrane against apoptosis in myeloid cells. Its deficiency caused a severe congenital neutropenia, a primary immunodeficiency syndrome associated with increased apoptosis in myeloid cells (Klein *et al.*, 2007). The HAX1 C-terminus, which mediated the interaction with NS5B, also mediated the interaction with HS1, a substrate of the *Src* family tyrosine kinases (Suzuki *et al.*, 1997). HS1 and its tyrosine phosphorylation are necessary for the nuclear localization and the transduction of downstream signalling such as B cell antigen receptor-mediated apoptosis (Yamanashi *et al.*, 1997).

NS5B interacted with a domain localized between amino acids 288 and 564 of ZNF143. ZNF143 is a transcription activator that enhances the activity of RNA polymerases II and III (Schaub *et al.*, 1997). Its expression is induced by DNA damaging agents such as gamma-irradiation and cisplatin, a chemotherapy drug that is used to treat various cancers such as ovarian cancer (Torigoe *et al.*, 2005). ZNF143 mediates cisplatin resistance by regulating DNA repair genes expression and binding to cisplatin-modified DNA (Ishiguchi *et al.*, 2004). This process may be modulated by the interaction with the tumour-suppressor protein p73 (Ishiguchi *et al.*, 2004; Torigoe *et al.*, 2005; Wakasugi *et al.*, 2007). ZNF143 can also regulate the metabolic network controlling cell survival and differentiation via controlling the basal and tissue specific expression of transaldolase that regulates redox-dependent apoptosis via the pentose phosphate pathway (Grossman *et al.*, 2004).

NS5B was shown to interact with TIMM50 which is important for the function of mitochondria as reducing its expression by RNA interference enhances mitochondrial membrane permeability causing cytoplasmic release of cytochrome c (Guo *et al.*, 2004; Meinecke *et al.*, 2006). NS5B was also found to interact with SFRS10 which is expressed in brain, liver, testis, and weakly in kidney (Nayler *et al.*, 1998). It is involved in the modulation of p53 activity regulating cell growth and proliferation (Huang *et al.*, 2004).

In a recent study, a proteome-wide screening for interactions between HCV and cellular proteins has shown that PLSCR1 can interact with HCV core protein. It was suggested that PLSCR1 is essential for the inter-functionality of Jak/Stat and insulin pathways. Therefore, interaction of core with PLSCR1 may interfere with these pathways (de Chassey *et al.*, 2008). RTN3 was also shown to interact with NS4B, a component of HCV replication complex (Liu *et al.*, 2005; Piccininni *et al.*, 2002). The other proteins identified here have not been previously reported to interact with HCV proteins. The interaction of NS5B with these proteins may have a role in modulation of their respective function which may in turn be involved in HCV replication, persistence and/or pathogenesis.

CHAPTER 4

Interaction of NS5B with PLSCR1, RTN3 and ZNF143 *In Vitro*

4.1 Introduction

In order to confirm some of interactions identified by the yeast two-hybrid screen, alternative assays were utilized to test whether the binding of NS5B to these proteins could be demonstrated by other systems. It was not possible to work on all candidates so a decision was made to limit the investigation to three targets, PLSCR1, RTN3 and ZNF143. These proteins are multifunctional proteins involved in many cellular pathways such as IFN signalling pathways, protein expression, protein trafficking and apoptosis as previously described in Chapter 3. These pathways may be important for HCV to modulate to facilitate replication and persistence.

4.2 Expression and Purification of Truncated NS5B

The HCV NS5B ORF, that lacked the coding region for the last 21 hydrophobic C-terminal amino acids which was required to enhance the solubility of the expressed protein in bacteria, was amplified from pCV-H77c. The truncated NS5B (tNS5B) ORF was successfully cloned into pET21d (Novagen) in-frame with T7- and His-tag sequences at the N- and C-termini, respectively (Fig. 4.1), as confirmed by restriction analysis and DNA sequencing. The new plasmid was termed pET21-5B (Appendix 9.11).

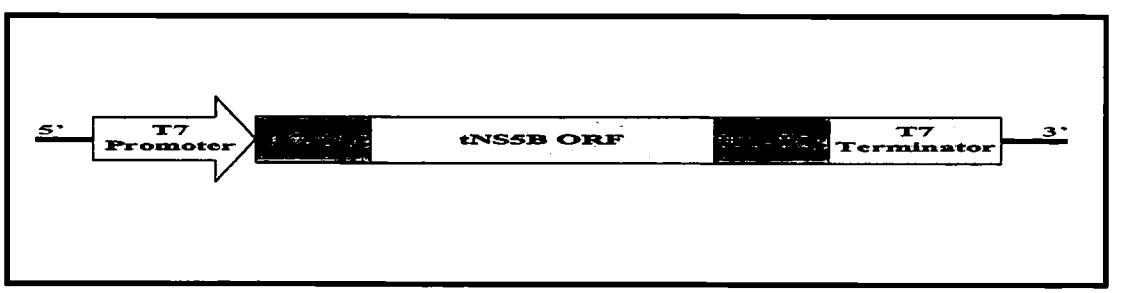

Fig. 4.1: A schematic diagram of tNS5B ORF in pET21d. Truncated NS5B ORF (tNS5B) is cloned into pET21d in-frame with T7-tag and His-tag at the N- and C-terminus, respectively. Expression of NS5B is driven by T7 promoter.

After preliminary expression experiments (not shown) that demonstrated the expression of a truncated NS5B (tNS5B) as a His-tag fusion protein in a soluble form, a large scale preparation was undertaken. Purification of tNS5B was performed by Ni-affinity chromatography (HiTrap metal ion chelating column) (Section 2.24.1). The protein was eluted with increasing concentrations of imidazole in lysis buffer. Expression and purification of tNS5B were investigated by SDS-PAGE stained with coomassie blue stain (Fig. 4.2, A) and by western blotting analysis using α-His-HRP antibody for detecting the His-tag (Fig. 4.2, B). The fraction eluted with 300 mM imidazole was relatively pure and used for subsequent experiments after dialysis.

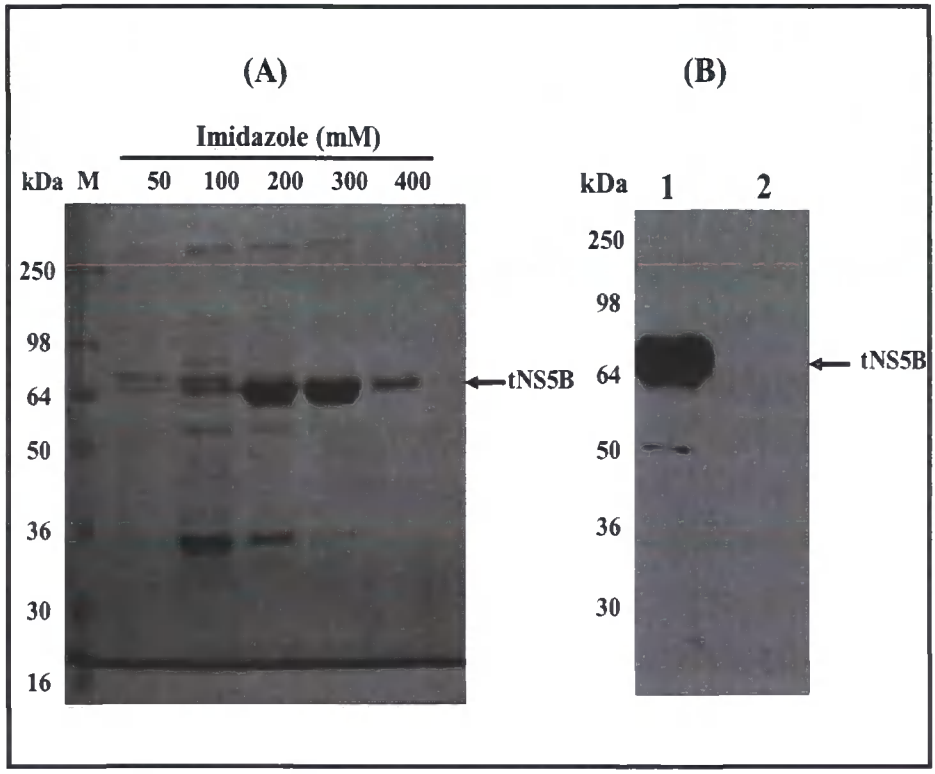

Fig. 4.2: Expression of tNS5B in fusion with His-tag. (A) Over-expression and Purification of tNS5B. tNS5B was expressed in *E. coli* BL21 (DE3) and captured using a HiTrap metal ion chelating column. The protein was eluted with increasing concentrations of imidazole. (B) Detection of tNS5B was performed using α-His-HRP antibody for His-tag. 1- tNS5B purified protein and 2- Total protein extracted from *E. coli* BL21 (DE3) cells transformed by empty pET21d.

4.3 Expression of GST-PLSCR1, -RTN3 and -ZNF143 ORFs

PLSCR1, RTN3, and ZNF143 partial ORFs were obtained from the library AD/cDNA plasmids by digestion with *EcoRI-XhoI*. The resulting ORFs were successfully sub-cloned into pGEX-6P-3, in-frame with the glutathione S-transferase (GST)-tag sequence at the N-terminus, as confirmed by DNA sequencing (see Fig. 4.3 for a schematic of such constructs).

GST-PLSCR1, -RTN3, and -ZNF143 fusions were successfully expressed and purified from *E. coli* BL21 (DE3) cells induced with 1 mM IPTG at 25°C for 16-24 hrs using Glutathioine-Sepharose-4B beads (Section 2.24.2). Expression of each GST fusion protein was confirmed by SDS-PAGE and Western blotting analysis using α-GST-HRP antibody (Figs. 4.4 and 4.5).

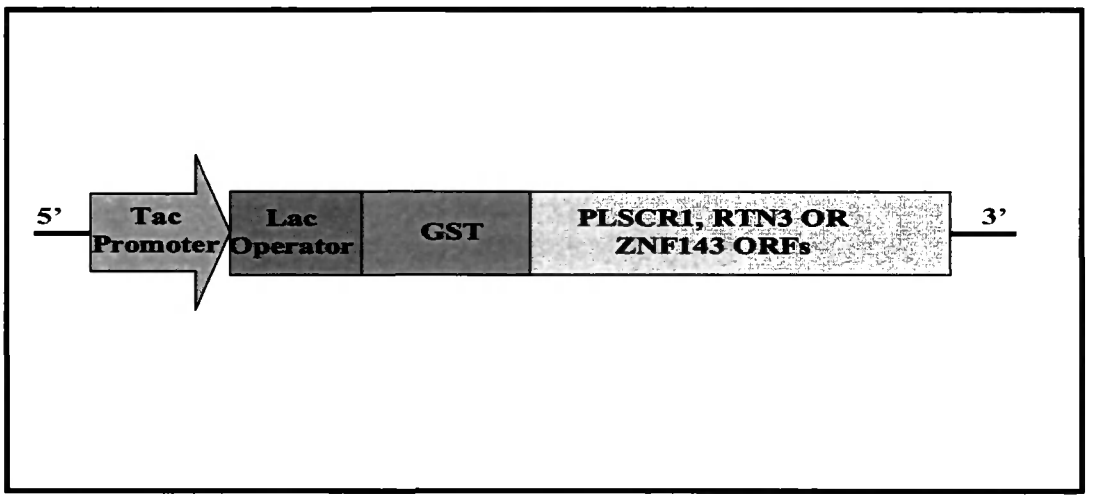

Fig. 4.3: A schematic diagram of PLSCR1, RTN3 or ZNF143 in pGEX-6P-3. The ORFs were cloned in-fusion with GST at their N-termini and the expression was driven by tac promoter.

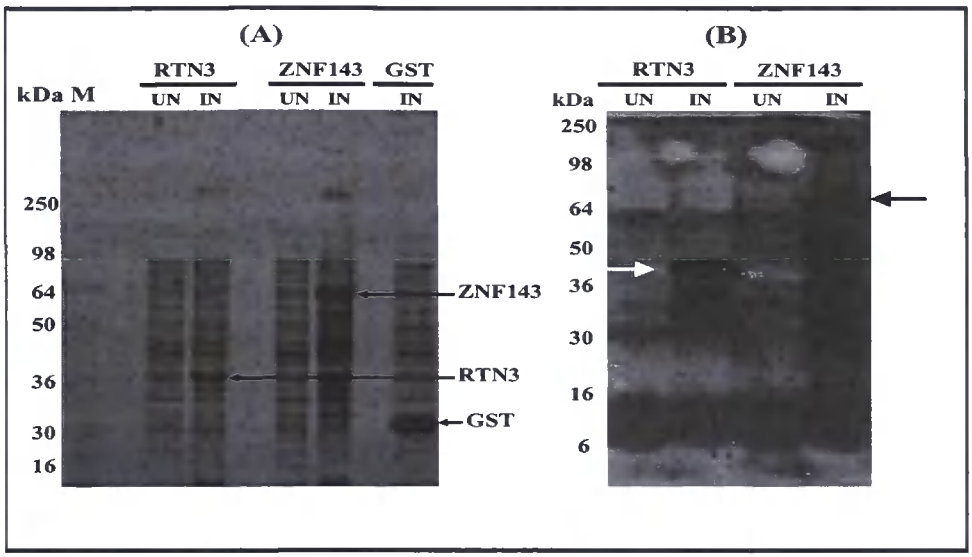

Fig. 4.4: Expression of RTN3 and ZNF143 ORFs in fusion with GST. (A) RTN3 and ZNF143 domains in fusion with GST were over-expressed in *E. coli* BL21 (DE3) cells. Un-induced (UN) and induced (IN) cells for RTN3, ZNF143, and GST expression were indicated. (B) Western blotting analysis indicating the expression of GST-RTN3, and GST-ZNF143. Detection was carried out using mouse α-GST-HRP antibody. White arrow indicates RTN3 while black arrow indicates ZNF143.

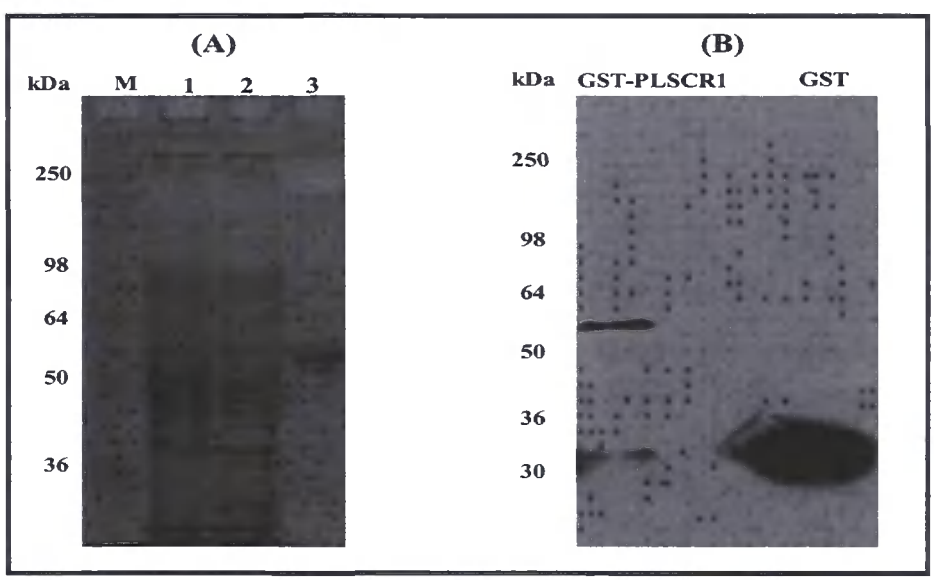

Fig. 4.5: Expression of GST-PLSCR1. (A) Detection of PLSCR1 purification by SDS-PAGE where 1- Cell lysate of un-induced cells, 2- Cell lysate from induced cells, and 3- Purified PLSCR1 using glutathioine-sepharose-4B beads. (B) WB analysis indicating expression of GST-PLSCR1 or GST in *E. col.* BL21 (DE3) cells using mouse α-GST-HRP antibody.

4.4 *In Vitro* Confirmation of the Interactions with NS5B

In order to confirm the interaction of tNS5B with PLSCR1, RTN3, and ZNF143, GST pull-down assays were performed as detailed in Section 2.25.1. Soluble purified tNS5B protein was mixed with either GST-PLSCR1, GST-RTN3, or GST-ZNF143 fusion proteins loaded on glutathione-sepharose beads; GST was used as a negative control. The beads were collected by centrifugation and washed extensively with PBS containing 0.1% Tween-20 (PBST). The captured products were analyzed by western blotting analysis using α-His-HRP antibody to detect tNS5B or α-GST-HRP antibody for detection of GST-fusion proteins.

Results demonstrated that tNS5B was pulled down by GST-PLSCR1, GST-RTN3, and GST-ZNF143; there was no evidence of binding of tNS5B to the GST moiety (Fig. 4.6).

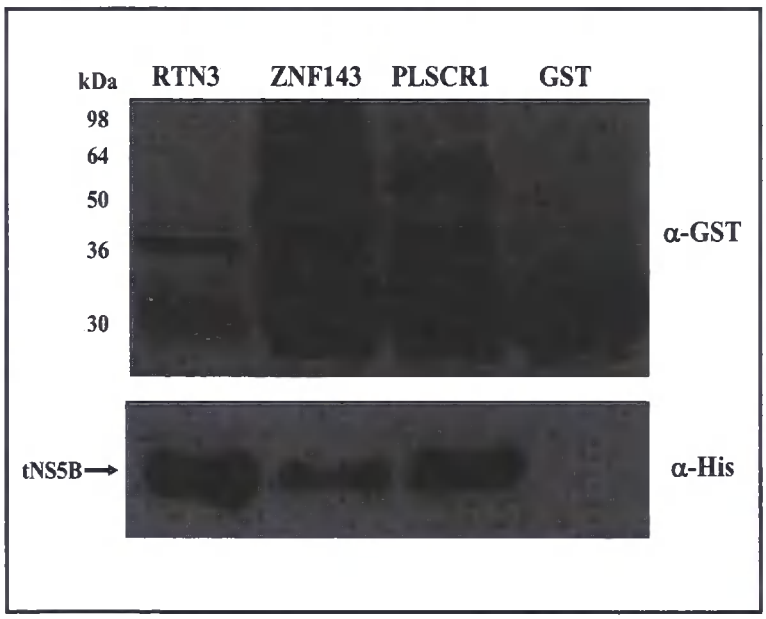

Fig. 4.6: Interaction of tNS5B with RTN3, ZNF143 or PLSCR1 *in vitro*. Soluble tNS5B was incubated with GST-RTN3, GST-ZNF143, GST-PLSCR1, or GST bound to beads. SDS sample buffer was added to the washed beads before resolving on SDS-PAGE and subjected to WB analysis.

4.5 Interaction of NS5B with Full-Length PLSCR1 and ZNF143 in Yeast

For further investigation of the biological significance of these interactions, PLSCR1 was selected as the main subject of this study as it had an antiviral activity and was involved in the reduction of HCV infection by the innate immune response (Brodsky *et al.*, 2007) in addition to its role in EGFR signalling pathway and phospholipids transbilayer movement. PLSCR1 was also enriched in lipid rafts that accommodate the HCV replication complex and RNA synthesis (Shi *et al.*, 2003; Sun *et al.*, 2002). In addition to PLSCR1, ZNF143 was also subjected for further characterization to identify the biological significance of its interaction with NS5B. ZNF143 is a transcription factor involved in the activation of IRF3 transcription that activates the expression of IFN α and β (Mach *et al.*, 2002). ZNF143 could also modulate cell survival by interacting with p73 and transaldolase (Grossman *et al.*, 2004; Wakasugi *et al.*, 2007).

It was necessary to clone the full-length PLSCR1 (FL-PLSCR1) and ZNF143 (FL-ZNF143) ORFs to determine if the binding to NS5B still occurring. It was also important for subsequent biological assays that may need full-length proteins for functional activities. FL-PLSCR1 and FL-ZNF143 ORFs (NCBI Accession Number AF008445 and NM_003442, respectively) were successfully amplified from total RNA extracted from Huh7 cells (Section 2.21.1). Nested RT-PCR was performed as an initial attempt to isolate ORFs from a direct RT-PCR failed to give sufficient product (Section 2.21.3). External primers flanking PLSCR1 and ZNF143 ORFs were designed (Appendix 9.11). Internal (nested) sets of primers for PLSCR1 and ZNF143 were used for the nested PCR. The obtained full-length PLSCR1 and ZNF143 ORFs (954 bp and 1917 bp, respectively) were cloned into pGADT7 in-frame with DNA-BD at their N-termini (Appendix 9.11).

In order to confirm that NS5B can interact with FL-PLSCR1 or FL-ZNF143, a yeast two-hybrid assay was performed using pGBK-5B and pGAD-FL-PLSCR1 or pGAD-FL-ZNF143. Results demonstrated clearly that NS5B could interact with both full-length proteins, at least in yeast (Fig. 4.7).

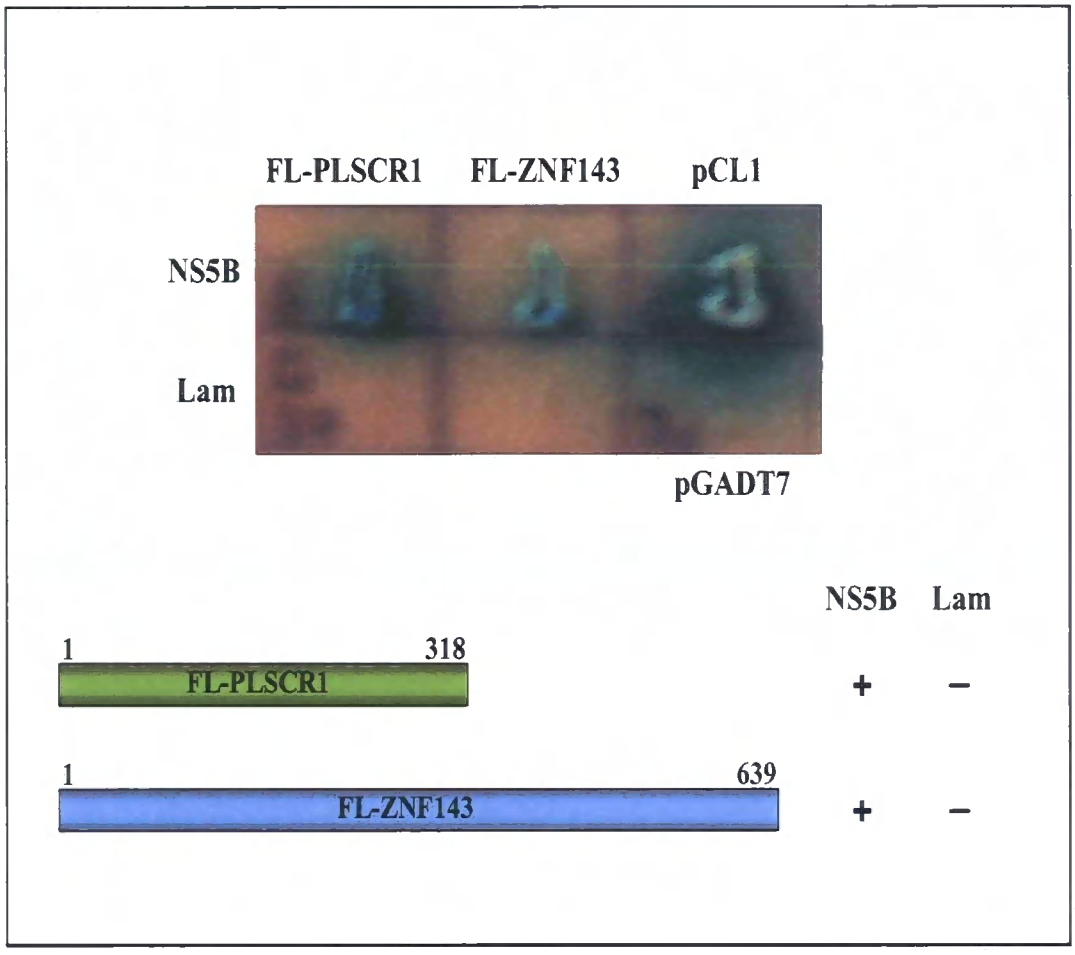

Fig. 4.7: Yeast two-hybrid assay to confirm the interaction of NS5B with FL-PLSCR1 and FL-ZNF143. AH109 yeast cells were transformed with pGBK-5B or pGBK-Lam with pGAD-FL-PLSCR1 or pGAD-FL-ZNF143. pCL1 plasmid was used as a positive control and pGBK-Lam with pGADT7 were used as negative control plasmids.

4.6 Expression of FL-PLSCR1 and FL-ZNF143 *In Vitro*

Full-length PLSCR1 and ZNF143 ORFs were cloned into pGEX-6P-3 in-frame with GST-tag sequence at the N-terminus (Section 9.11). pGEX-FL-PLSCR1 and pGEX-FL-ZNF143 were introduced into *E. coli* BL21 (DE3) cells. Protein expression was carried out as previously described in Section 2.24.2 and then analyzed by SDS-PAGE and western blotting. Proteins were purified using glutathioine-sepharose-4B beads and elution was performed with reduced glutathione.

The majority of GST-PLSCR1 (63 kDa) and GST-ZNF143 (96 kDa) preparations were partially pure (Fig. 4.8). Some contaminating bands, possibly breakdown products, were observed to co-purify with the target proteins which proved difficult to remove. However, the level of purity achieved was considered acceptable for subsequent experimental procedures.

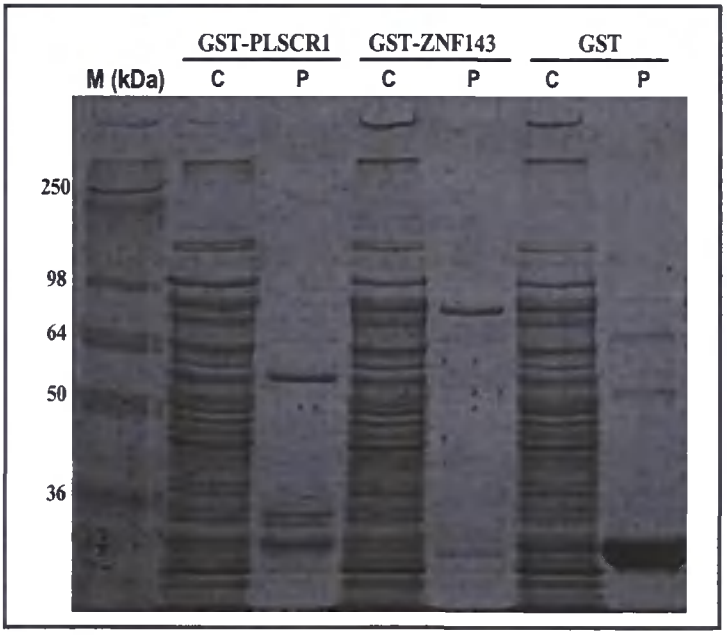

Fig. 4.8: Expression of full-length GST-PLSCR1 and GST-ZNF143 fusions. PLSCR1, ZNF143, and GST were expressed in *E. coli* BL21 (DE3) cells induced with 1 mM IPTG at 25°C for 16-24 hrs. Expressed proteins were captured by glutathioine-sepharose-4B beads and eluted with 20 mM Glutathione containing buffer. (C) Cell lysate from induced cells, and (P) Purified protein.

4.7 Interaction of NS5B with FL-PLSCR1 *In Vitro*

To confirm the NS5B/FL-PLSCR1 interaction, an ELISA assay was performed (Section 2.25.3). A microtitre plate was coated with soluble purified tNS5B in increasing concentration series. After blocking non-specific sites, soluble GST-PLSCR1 or GST was added to the coated wells. After extensive washing, the captured proteins were detected using α-GST-HRP antibody.

It was observed that the amount of captured FL-PLSCR1 was proportional to the concentration of tNS5B, whilst no significant change was noted with GST which confirmed the specificity of the interaction of tNS5B with FL-PLSCR1 *in vitro* (Fig. 4.9).

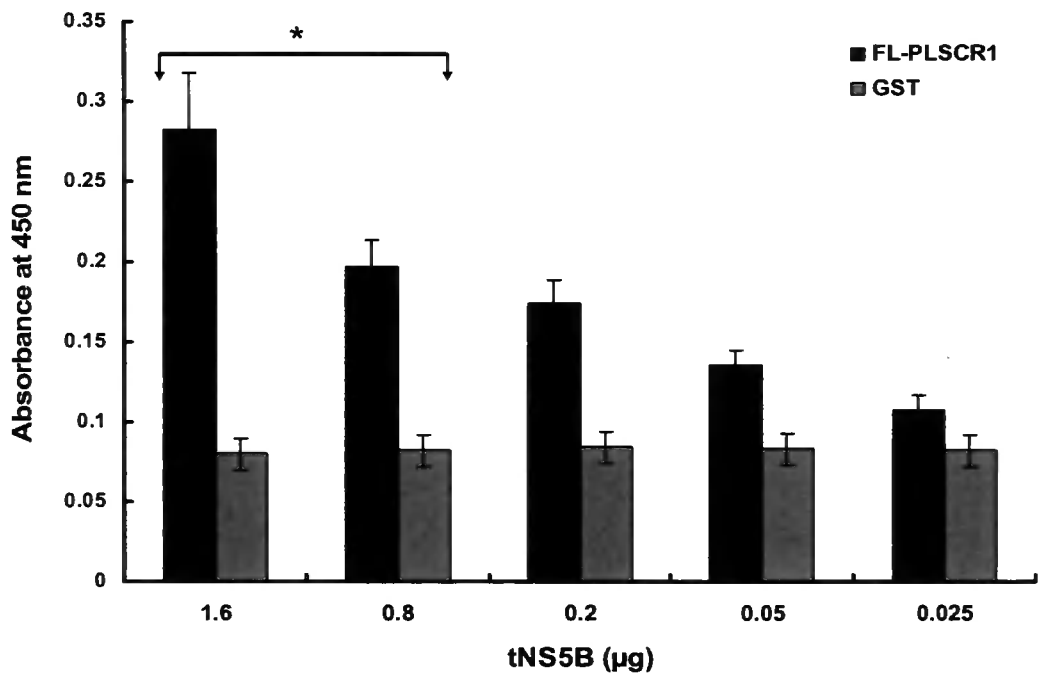

Fig. 4.9: Interaction of tNS5B with FL-PLSCR1 *in vitro*. An ELISA assay was performed using soluble purified tNS5B and FL-PLSCR1 or GST. The captured proteins were detected using α-GST-HRP antibody. Each sample was measured in triplicates in three separate experiments then the means±SEM were calculated and plotted on the graph. * The significance of binding of FL-PLSCR1 with two different amounts of tNS5B (1.6 and 0.8 µg) was determined (p-value ≤ 0.05).

4.8 Interaction of NS5B with FL-ZNF143 *In Vitro*

Far-western blotting was used to confirm the interaction of tNS5B and FL-ZNF143 (Section 2.25.2). Purified FL-ZNF143 or GST was resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with NS5B-His. After washing, captured tNS5B was detected using α-His-HRP antibody. Only FL-ZNF143 captured tNS5B at a point consistent with the mass of GST-ZNF143, 96 kDa (Fig. 4.10).

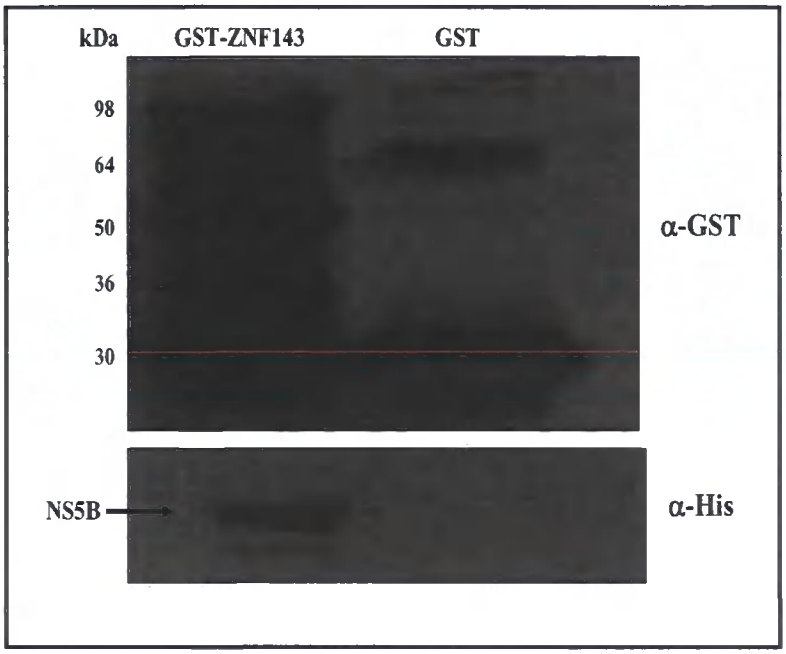

Fig. 4.10: Interaction of tNS5B with FL-ZNF143 *in vitro*. FL-ZNF143 or GST were resolved on SDS-PAGE then transferred to nitrocellulose membrane. Nitrocellulose membrane was blocked and probed with soluble purified tNS5B protein. The membrane was washed extensively before detection of the bound tNS5B with α-His antibodies conjugated with HRP.

4.9 Mapping the NS5B Interacting Domain on PLSCR1

To determine the binding domain(s) on PLSCR1 for NS5B, a series of carboxy co-terminal truncated mutants were generated using the Quick-Change Site-Directed Mutagenesis kit (Stratagene) by introducing stop codons after amino acids 60, 137 and 192 of PLSCR1 in pGAD-FL-PLSCR1. These deletion mutants were subsequently tested for an interaction with NS5B using the yeast two-hybrid assay. The results, summarized in Fig. 4.11, demonstrated that NS5B failed to bind to PLSCR1 after removal of amino acids 61-137 suggesting that this region was the binding site for NS5B, or at least contained sequences required for the interaction.

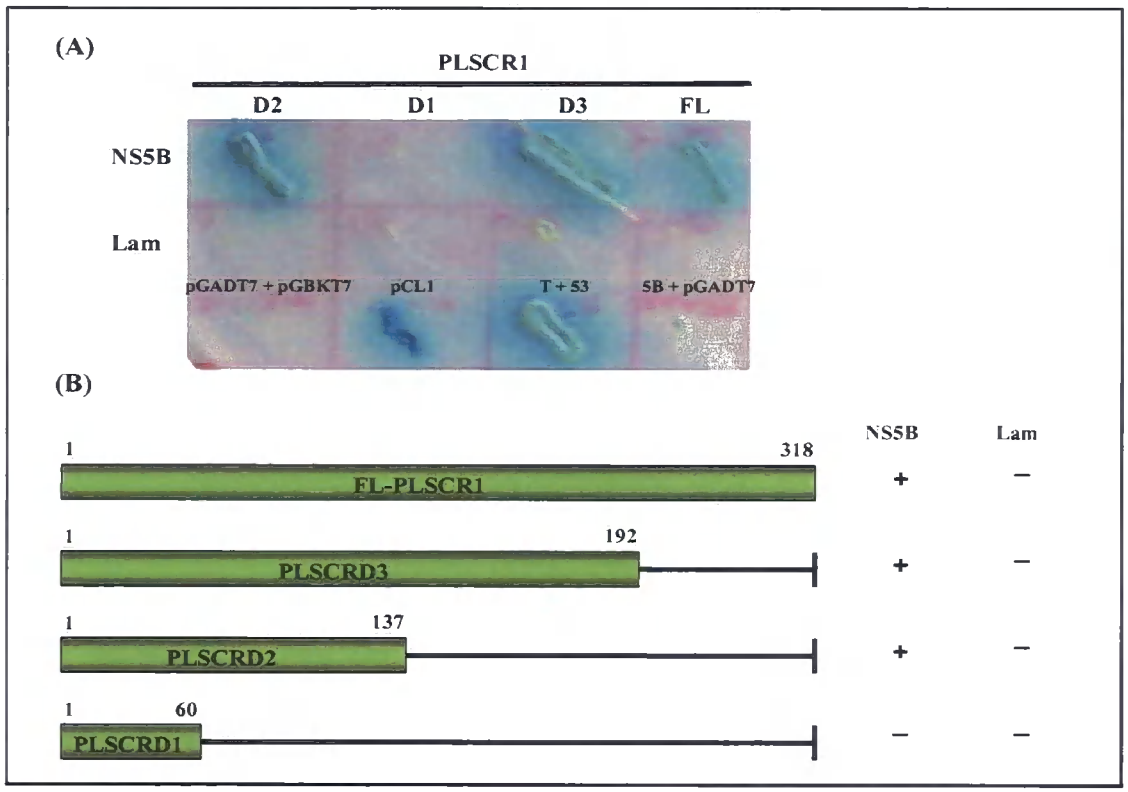

Fig. 4.11: Mapping the interacting domain on PLSCR1. (A) AH109 was transformed with pGBK-5B or pGBKT7-Lam and pGADT7 containing full-length or deletion mutants of PLSCR1. Transformation of pGBKT7-53 with pGADT7-T was used as a positive control while transformation by pGADT7 with pGBK-5B or pGBKT7 was used as a negative control for the assay. (B) Diagram showing the interaction of NS5B with PLSCR1's domains. The number of amino acid at the start and end of each PLSCR1 deletion mutant's domains is indicated. The positive interaction is expressed as (+) while the lack of interaction is expressed as (-).

4.10 Mapping the PLSCR1 and ZNF143 Interacting Domain(s) on NS5B

In order to map the PLSCR1 and ZNF143 interacting regions on NS5B, a number of NS5B C-terminal deletion mutants were created using PCR, $NS5B^{\Delta 154-591}$ (NS5BD1), $NS5B^{\Delta 302-591}$ (NS5BD2) and $NS5B^{\Delta 449-591}$ (NS5BD3). The truncated ORFs were cloned into pGBKT7 for expression as fusions with the GAL4 DNA-BD at the N-terminus. NS5B and its deletion mutants were tested for the interaction with FL-PLSCR1 or FL-ZNF143 using the yeast two-hybrid system (Fig. 4.12). The assay demonstrated that all NS5B deletion mutants could interact specifically with full-length PLSCR1 and ZNF143 in yeast. These results indicated that the binding domain of NS5B for both proteins was likely to be present within the amino-terminus of NS5B (amino acids 1-153), or this region contained elements that by themselves were sufficient to support the interactions.

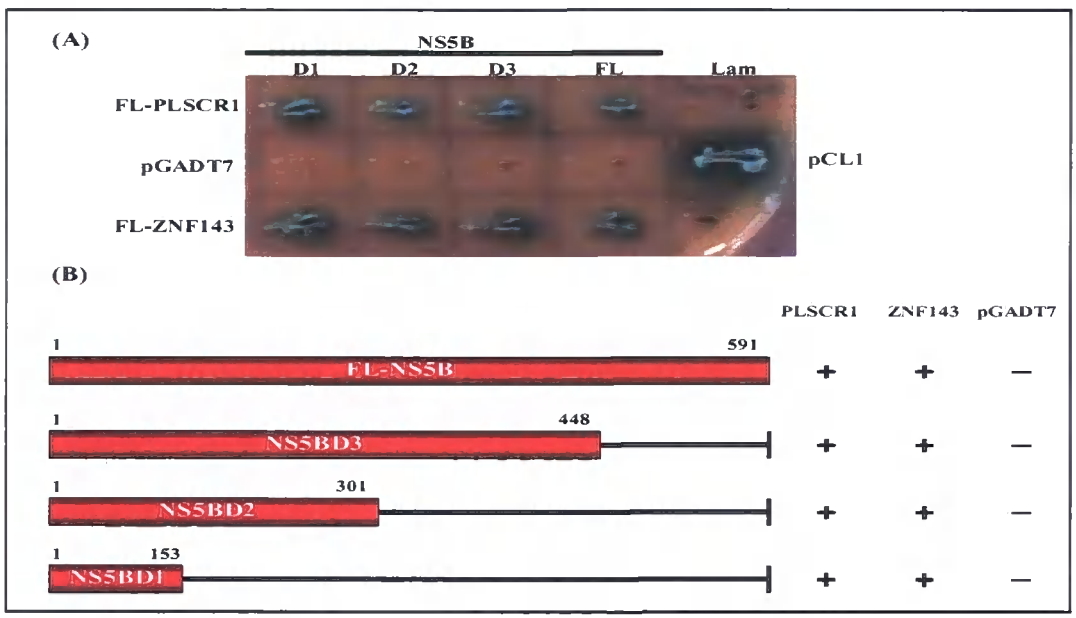

Fig. 4.12: Mapping the PLSCR1 and ZNF143 interacting domain on NS5B. (A) AH109 was transformed with pGBK-5B, -NS5BD3, -NS5BD2, -NS5BD1 or pGBKT7-Lam in combination with pGAD-FL-PLSCR1, -ZNF143, or pGADT7. Transformed cells were tested for α-galactosidase activity. (B) Diagram showing the interaction of NS5B or its deletion mutants with FL-PLSCR1 or FL-ZNF143. The number of amino acid at the start and end of each domain is indicated. The positive interaction is expressed as (+) while the lack of interaction is expressed as (-).

4.11 *In Vivo* Pulldown Analysis of the Interaction of NS5B with PLSCR1 and ZNF143

Full-length NS5B ORF was cloned into pNTAP (Stratagene), a mammalian-expression plasmid, in-frame with a streptavidin binding peptide-tag at the N-terminus to create pNTAP-5B (Appendix 9.11). Expression of NS5B was confirmed by WB using rabbit α-NS5B (Abcam) and α-rabbit-HRP antibodies (Sigma) (Fig. 4.13).

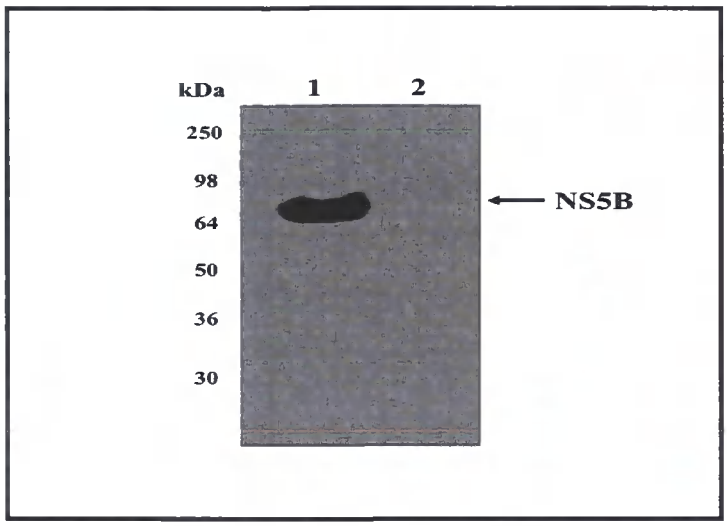

Fig. 4.13: Expression of NS5B in Huh7 cells. Huh7 cells were transfected with pNTAP-5B (1) or pNTAP (2). NS5B expression was detected 48 hrs post-transfection using rabbit α-NS5B and α-rabbit-HRP antibodies.

FL-PLSCR1 and FL-ZNF143 ORFs were subcloned into pcDNA4His/Max (Invitrogen) in-frame with a His-tag at the N-terminus to facilitate purification and identification (Appendix 9.11). Expression of histidine-tagged proteins was confirmed by western blotting using α-His antibody. In order to investigate the interaction of NS5B with PLSCR1 or ZNF143 *in vivo*, pNTAP-5B with either pcDNA4-PLSCR1 or pcDNA4-ZNF143 was co-transfected into Huh7 cells. Expression was allowed to proceed for 48 hrs before harvesting cells. Cell lysates were incubated with nickel (Ni) beads for 3 hrs at 4°C to capture PLSCR-His or ZNF143-His with the associated proteins. After washing, samples were resolved by SDS-PAGE and subjected to WB analysis. Sheep α-NS5B and α-sheep-HRP antibodies were used for detecting NS5B.

Under our experimental conditions a pull-down assay was unable to detect a direct interaction between NS5B and either PLSCR1 or ZNF143 that suggested that the interactions may be weak and easily disrupted under our assay conditions. The interactions may also need special conditions such as IFN or EGF stimulation to create a favourable environment for interactions.

4.12 Discussion

The main objective of this chapter was to confirm the interaction of NS5B with some of proteins identified by yeast two-hybrid screening using alternative assays. To confirm the interactions *in vitro*, NS5B ORF minus the C-terminal hydrophobic amino acid sequence, to increase the solubility of NS5B, was successfully cloned and expressed as a fusion with a His-tag at its C-terminus to facilitate purification and identification. Large amounts of recombinant protein in a soluble form were obtained and considered sufficiently pure for subsequent applications (Fig. 4.2). Using a pull-down assay, the interactions of NS5B with truncated forms of PLSCR1, RTN3, and ZNF143 obtained from the yeast screening were confirmed *in vitro* (Fig. 4.6).

The study subsequently focussed on PLSCR1 and ZNF143, and in particular PLSCR1, as they are involved in IFN signalling pathways and apoptosis which are significant antiviral pathways that HCV must negate to establish a productive infection. Yeast two-hybrid (Fig. 4.7) and other supportive *in vitro* assays confirmed the interaction of NS5B with full-length PLSCR1 and ZNF143 proteins *in vivo* in yeast and *in vitro* by ELISA (Fig. 4.9) or by far western blotting (Fig. 4.10).

PLSCR1 is a membrane protein that contains multiple important functional domains such as a transmembrane helix ($PLSCR1^{288-306}$) at the C-terminus, an extracellular C-terminal tail ($PLSCR1^{307-318}$) and an N-terminal cytoplasmic domain (Fig. 4.14). The cytoplasmic domain contains an N-terminal proline-rich (PXXP) region (SH3 binding domain, $PLSCR1^{1-97}$), a DNA-binding domain ($PLSCR1^{86-118}$), a cysteine palmitoylation motif ($PLSCR1^{184-189}$), a nuclear

localization signal (NLS) ($PLSCR1^{257-266}$), and a Ca^{2+} binding domain ($PLSCR1^{273-284}$) (Ben-Efraim *et al.*, 2004; Wiedmer *et al.*, 2003; Wiedmer *et al.*, 2000; Zhou *et al.*, 2005).

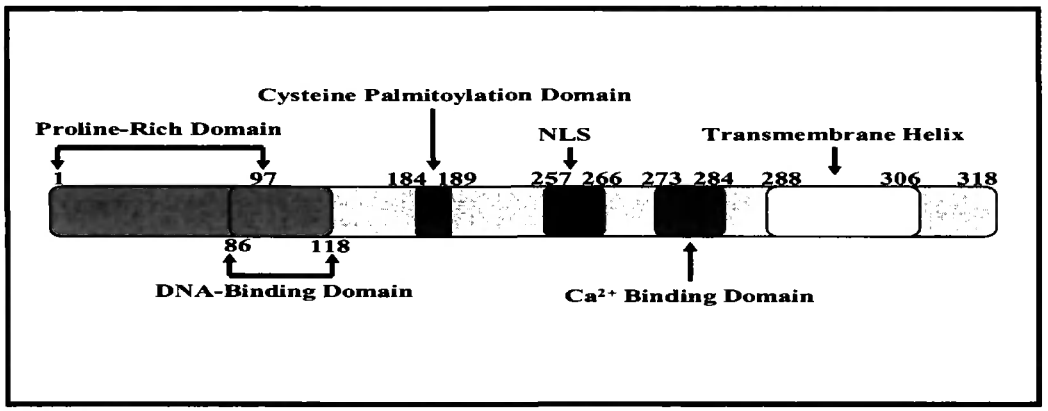

Fig. 4.14: Main PLSCR1 domains. Amino acids numbers are indicated at the beginning and the end of each domain (Sahu *et al.*, 2007).

The mapping studies showed that the PLSCR1 domain that mediated the interaction with NS5B was located between amino acids 60 and 137 (Fig. 4.11). Interestingly, this region is part of the PLSCR1 proline-rich domain ($PLSCR1^{1-97}$), that is involved in the interaction with other cellular proteins that contain SH3 domains (Wiedmer *et al.*, 2000). This region also contains the DNA binding domain of PLSCR1, $PLSCR1^{86-118}$ (Zhou *et al.*, 2005).

It has been shown that the c-Abl tyrosine kinase phosphorylated PLSCR1 at tyrosine residues (Tyr^{69}/Tyr^{74}) within the SH3-like domain (Sun *et al.*, 2001). The same region was responsible for the physical association of PLSCR1 with the Src homology 2 containing (Shc) adaptor protein and EGFR upon stimulation by EGF (Sun *et al.*, 2002). Moreover, PLSCR1 mediated the EGF-dependent recruitment and activation of c-Src tyrosine kinase suggesting that PLSCR1 has a role in the EGFR signalling pathway (Nanjundan *et al.*, 2003; Sun *et al.*, 2002). PLSCR1 was also shown to traffic into the nucleus under specific circumstances such as IFN induction and bind directly to the 5`-promoter region of the inositol 1,4,5-triphosphate receptor type 1 gene (IP3R1)

through its DNA-binding domain to enhance expression of the receptor (Zhou *et al.*, 2005).

To gain a better understanding of the molecular basis of the interaction of NS5B with FL-PLSCR1 or FL-ZNF143, carboxyl deletion mutants of NS5B, $NS5B^{\Delta 154-591}$ (NS5BD1), $NS5B^{\Delta 302-591}$ (NS5BD2) and $NS5B^{\Delta 449-591}$ (NS5BD3), were generated to map the interacting domains on NS5B. Results demonstrated that NS5BD1, i.e. the N-terminal domain of NS5B, was the target for both proteins, in yeast, suggesting that amino acids 1 to 153 mediated the interaction of NS5B with PLSCR1 and ZNF143, or at least contained sequences required for these interactions (Fig. 4.12).

$NS5B^{1-187}$ domain interacted with, and was phosphorylated by, protein kinase C-related kinase 2 (PRK2) and this phosphorylation was shown to regulate HCV RNA replication (Kim *et al.*, 2004). $NS5B^{84-85}$ also mediated the interaction of NS5B with α-actinin which was suggested to be part of the viral replication complex (Lan *et al.*, 2003). It was found that $NS5B^{83-194}$ has RNA-binding activity (Cheng *et al.*, 1999). Notably, targeting the N-terminus of NS5B with monoclonal antibodies disrupted the enzymatic function of NS5B (Kang *et al.*, 2008). Taken together, it is clear that the NS5B N-terminal region is involved in many cellular interactions and is important for the viral RNA replication. However the interaction of PLSCR1 or ZNF143 with this region may affect its role in these interactions and/or the viral RNA replication.

CHAPTER 5

Investigation of the Biological Significance of the Interaction of NS5B with PLSCR1 or ZNF143

5.1 Introduction

PLSCR1 and ZNF143 potentiate the cellular response to IFN by increasing the expression of some interferon-stimulated and -regulatory genes important for establishing an antiviral state (Dong *et al.*, 2004; Mach *et al.*, 2002). Huh7 cells accommodating a HCV replicon have a decreased expression of IFN and associated ISGs suggesting that down-regulation of the IFN response is important in the establishment of a persistent infection (Itsui *et al.*, 2006; Zhang *et al.*, 2005). As PLSCR1 and ZNF143 interact with NS5B, it may interfere with their activity on ISGs expression that could modulate the cellular response to IFN. In order to determine whether there is a biological significance for the interactions between NS5B and PLSCR1 or ZNF143 *in vivo*, the effect of co-expression of NS5B with either PLSCR1 or ZNF143 on the cell's response to IFN treatment was investigated.

5.2 Effect of NS5B Interaction with PLSCR1 or ZNF143 on Mammalian Cell Response to Interferon

To investigate if the interaction of NS5B with PLSCR1 or ZNF143 had an effect on the cellular response to IFN-α, Huh7 or VERO cells were treated with 50 U/ml IFN-α for 5 hrs before transfection with the pISRE-Luc reporter plasmid and pcDNA4-5B (for expression of full length NS5B in fusion with His-tag at the N-terminus, Appendix 9.11) with or without pcDNA4-PLSCR1 or pcDNA4-ZNF143. Empty pcDNA4 plasmid was used to keep an equal total amount of transfected DNA per well (1 μg/well). Cells were harvested 48 hrs post-transfection and *luc* activity was determined (Section 2.26.8). pISRE-Luc, a reporter plasmid expressing the firefly luciferase (*luc*) under the control of the interferon-stimulated response element (ISRE) which is

found in the promoters of most interferon-stimulated genes (ISGs), was used to monitor the effect of NS5B, PLSCR1 and ZNF143 on IFN signalling.

In both cell lines, expression of NS5B alone had no effect on *luc* expression (Section 2.26.8). In Huh7, *luc* expression was markedly increased, by 1.7 and 1.3 fold, in the presence of plasmids expressing PLSCR1 or ZNF143, respectively. In VERO cells, which do not produce an endogenous interferon but do respond to exogenous IFN (Diaz *et al.*, 1988), expression of PLSCR1 or ZNF143 had marked enhancing effects on *luc* expression, of 4.5 and 3 fold increase over the control, respectively (Fig. 5.1). Interestingly, co-expression of NS5B with either PLSCR1 or ZNF143 significantly reduced *luc* expression in both cell lines (p-values ≤ 0.05) suggesting an anti-interferon role for NS5B.

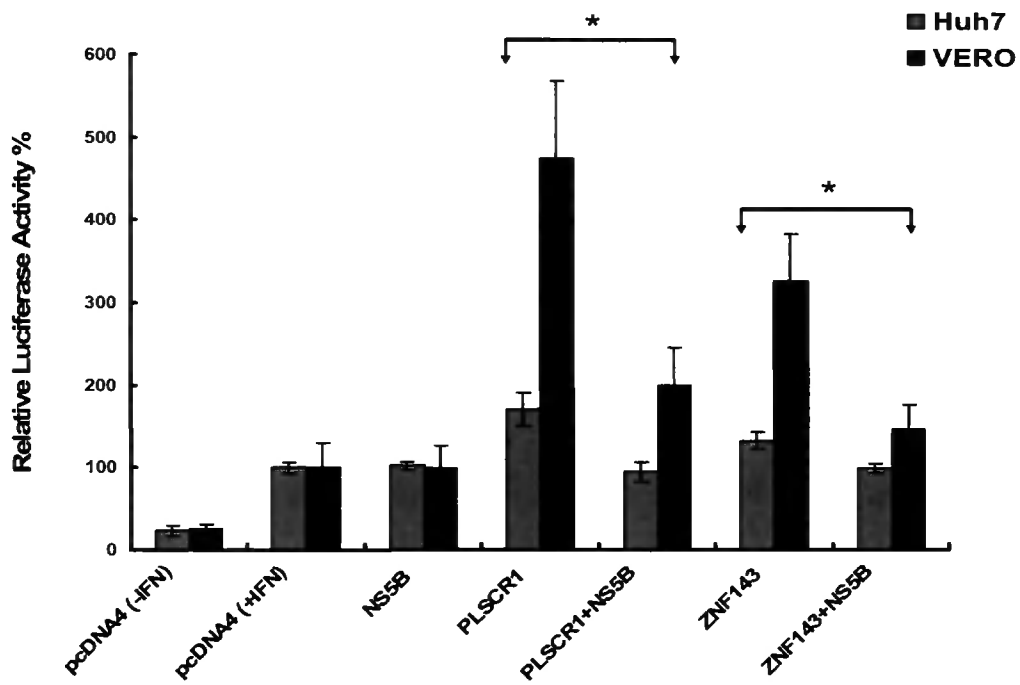

Fig. 5.1: Effect of co-expression of NS5B with PLSCR1 or ZNF143 on IFN ability to induce the expression from ISRE in Huh7 and VERO cells. Cells were pre-treated with 50U/ml IFN-α for 5 hrs before transfection with pISRE-Luc, pcDNA4-5B, and/or pcDNA4-PLSCR1 or pcDNA4-ZNF143. Each sample was measured in triplicate in three separate experiments. Error bars represent the SEM of three separate experiments. * Co-expression of NS5B with either PLSCR1 or ZNF143 significantly reduced *luc* activity in both cell lines with p-values ≤ 0.05.

5.3 Effect of NS5B on the Expression of Cellular ISRE-Driven Down-Stream Interferon-Stimulated Genes (ISGs)

To determine If NS5B had an effect on the response to interferon, the effect on the expression of some interferon stimulated genes (ISGs), such as interferon stimulated gene 15 (ISG15), ISG54, 2', 5'-oligoadenylate synthetase 2 (OAS2), and interferon regulatory factor 7 (IRF7), was tested. The choice of ISGs was based on those previously reported to be induced by PLSCR1 (Dong *et al.*, 2004). To this aim we utilized a lentivirus-based vector system (2nd generation, kindly provided by Didier Trono, Tronolab, Switzerland) to deliver the NS5B ORF sequence into Huh7 cell to ensure expression in the majority of cells (Section 2.26.3). The NS5B ORF was cloned into pWPXL to produce pWPXL-5B (Appendix 9.11) which was subsequently used to produce VLPs. Production of high titre VLP stocks was achieved by transfection of 293T cells with psPAX2, pMD2G and pWPXL or pWPXL-5B. Supernatant's containing VLPs were collected and used to transduce Huh7 cells. Cells were harvested 48 hrs post-transduction and total cellular RNA or protein lysates were prepared and NS5B expression was confirmed by RT-PCR (not shown) and western blotting (Fig. 5.2).

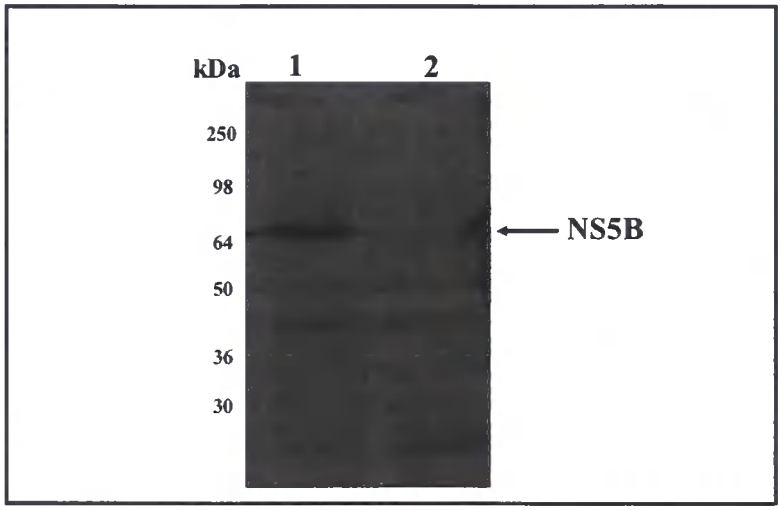

Fig. 5.2: Expression of NS5B in Huh7 cells transduced with NS5B VLPs. Huh7 cells were transduced with (1) NS5B VLPs or (2) GFP VLPs. NS5B expression was detected by rabbit α-NS5B and α-rabbit-HRP antibodies.

Similarly, PLSCR1 and ZNF143 ORFs were cloned into pWPXL-V5H, a modified version of pWPXL containing His-tag specifically generated for this study to facilitate detection, in-frame with His-tag at the N-terminus (Appendix 9.10). The resulting plasmids, denoted pWPXL-V5H-PLSCR1 and pWPXL-V5H-ZNF143 respectively (Appendix 9.11), were used to produce VLPs in 293T cells. VLPs were used to transduce Huh7 cells and the expression of His-tagged proteins was confirmed by western blotting using α-His antibody (data not shown).

Huh7 cells were treated with 50 U/ml IFN for 24 hrs after transduction with NS5B VLP either alone or in combination with PLSCR1 or ZNF143 VLPs. Total cellular RNA was extracted 48 hrs post-transduction and semi-quantitative RT-PCR was performed for monitoring the expression of ISG15, ISG54, OAS2, IRF7, and β-actin using primers specific for the first ~500 bp of each ORF sequences (Appendix 9.11). No significant difference was observed (in three experiments) in the expression of ISGs in Huh7 cells expressing PLSCR1 or ZNF143 and cells co-expressing NS5B with both proteins (Fig. 5.3).

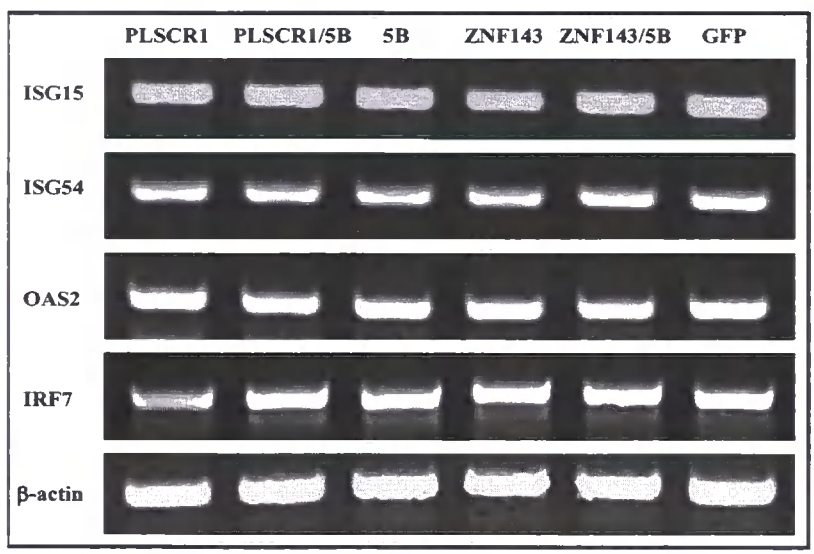

Fig. 5.3: Expression of ISGs in Huh7 cells expressing NS5B, PLSCR1 and ZNF143. RT-PCR was carried out for detection of the expression of ISG15, 54, OAS2, IRF7 and β-actin in Huh7 cells expressing PLSCR1, PLSCR+NS5B, NS5B, ZNF143, ZNF143+NS5B or GFP.

5.4 Effect of PLSCR1 and ZNF143 on HCV Sub-Genomic Replicon Replication

The effect of PLSCR1 or ZNF143 on HCV replication was monitored using a sub-genomic replicon (FKi341PiLucNS3-3'dgET, FK-Luc, Appendix 9.12), kindly provided by Ralf Bartenschlager (University of Heidelberg, Germany). pFK-Luc contains in order: T7 RNA polymerase promoter (for *in vitro* transcription) followed by nucleotides 1 - 341 of the HCV 5` consensus sequence (required for replication of the RNA), the poliovirus (PV) IRES element (for translation of *luc*), the firefly-luciferase ORF (to monitor replicon replication), EMCV IRES (for translation of NS3-NS5B), the NS3 to NS5B coding sequence and the 3` UTR of HCV genotype 1b and finally a T7 RNA polymerase terminator sequence (to terminate *in vitro* transcription) (Fig. 5.4).

Replicon RNA was synthesized *in vitro* with pFK-Luc as a template using the MEGAscript T7 Transcription Kit (Ambion). A preliminary experiment was carried out to investigate replication levels of the replicon at various time points. The ability of the replicon to replicate was confirmed by IFN treatment as this treatment resulted in a loss of a luciferase signal which was consistent with *luc* expression being due to genuine replicon replication (data not shown).

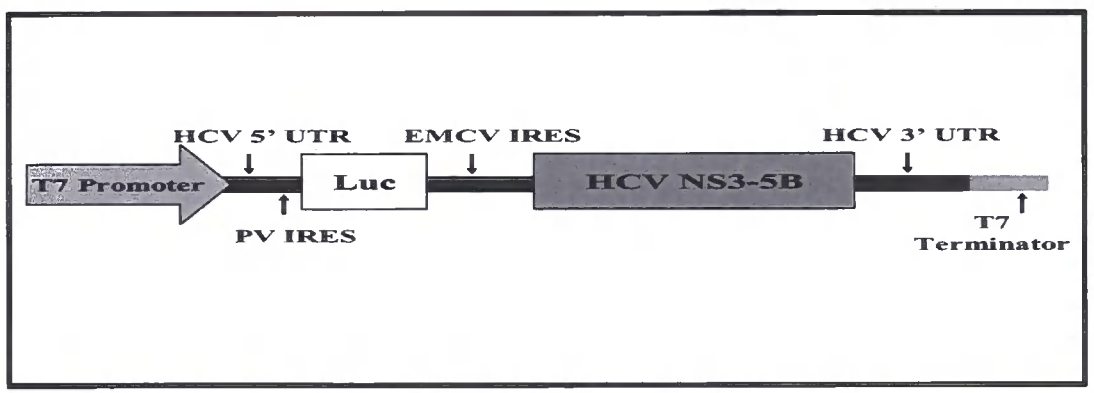

Fig. 5.4: A schematic diagram showing the overall structure of the pFK-Luc containing the HCV sub-genomic replicon. *luciferase* transcription is driven by HCV 5` UTR while HCV NS3-5B expression is driven by EMCV IRES.

5.4.1 Effect of PLSCR1 or ZNF143 Overexpression on Replicon Replication

In order to investigate the effect of PLSCR1 or ZNF143 on HCV replicon replication, Huh7 cells were transfected with *in vitro* transcribed pFK-Luc RNA. Transfected cells were subsequently transduced with PLSCR1, ZNF143 or GFP VLPs 24 hrs post-transfection. Cells were incubated for 48 hrs before determining *luc* activity. No significant change was observed in cultures expressing PLSCR1, while a reduction (30 %) in replication was observed in cultures expressing ZNF143 over the GFP control (Fig. 5.5) suggesting an antiviral role for this protein.

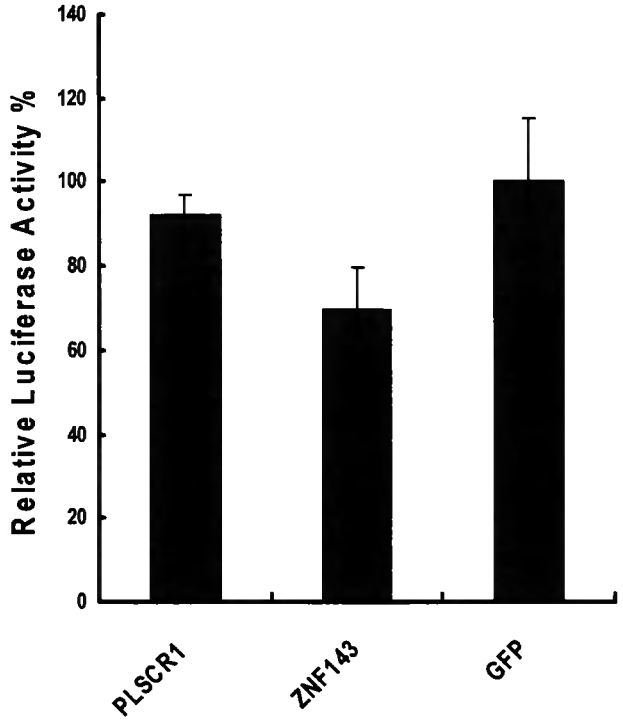

Fig. 5.5: Effect of PLSCR1, ZNF143, and GFP on the replicon replication. Huh7 cells were transfected by *in vitro* transcribed pFK-Luc RNA. Transfected cells were transduced with VLPs of PLSCR1, ZNF143 or GFP. Cells were incubated for 48 hrs before determining *luc* activity. The assay was carried out one time and each sample was measured in triplicates then the mean±SD values were calculated and plotted as a percentage of the GFP control sample. Error bars represent SD values.

5.4.2 Effect of Down-regulating PLSCR1 or ZNF143 Expression on Replicon Replication

To determine the effect of down-regulating PLSCR1 or ZNF143 expression on replicon replication, shRNA constructs to deplete PLSCR1 or ZNF143 were generated (Fig. 5.6). Oligonucleotides, designed in accordance with previous protocols, were inserted into pLVTHM (a kind gift from Didier Trono, Switzerland, Appendix 9.13) giving pLVTHM-shPLSCR1 and pLVTHM-shZNF143 (Appendix 9.11).

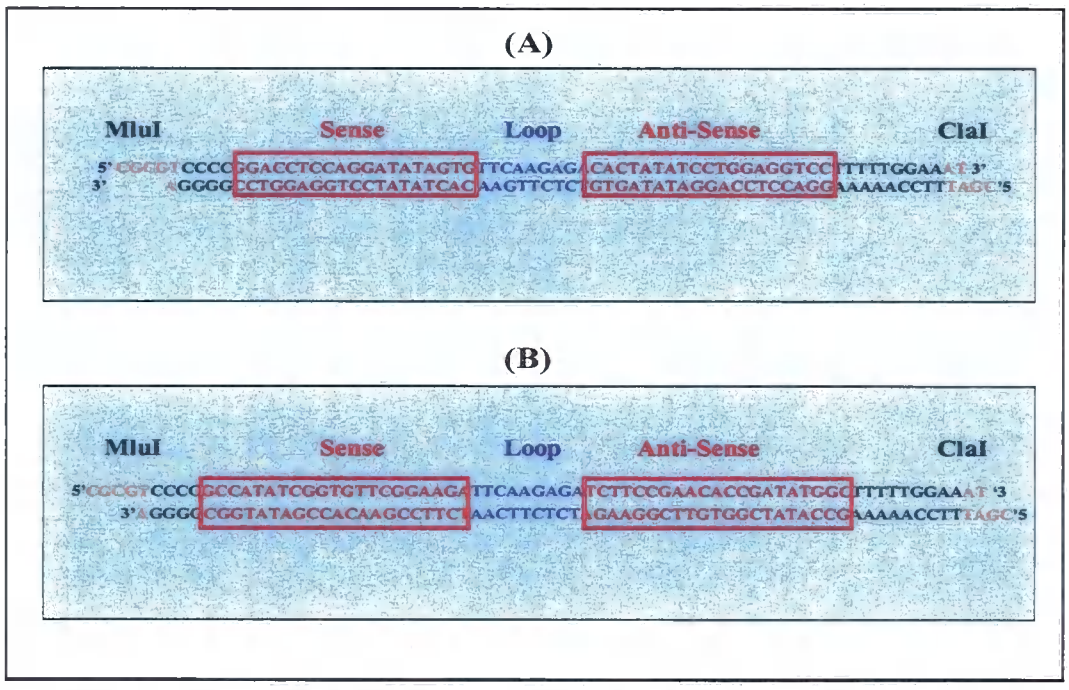

Fig. 5.6: shRNA oligonucleotides against PLSCR1 or ZNF143. Annealed oligonucleotides designed for cloning into pLVTHM to form shRNA targeting PLSCR1 (nucleotides 94-112) or (B) ZNF143 (nucleotides 885-905) when transfected into cells.

To negate PLSCR1 expression, VLPs were prepared using pLVTHM-shPLSCR1 or pLVTHM-shGFP (a control plasmid containing shRNA against GFP). Huh7 cells were transduced with these VLPs and the total cellular RNA was extracted 48 hrs post-transduction. PLSCR1 expression was examined using semi-quantitative RT-PCR (Section 2.21.2). It was observed that cultures transduced with the shRNA against PLSCR1 had a reduced expression of PLSCR1 mRNA by ~40 % (observed in two experiments) (Fig. 5.7).

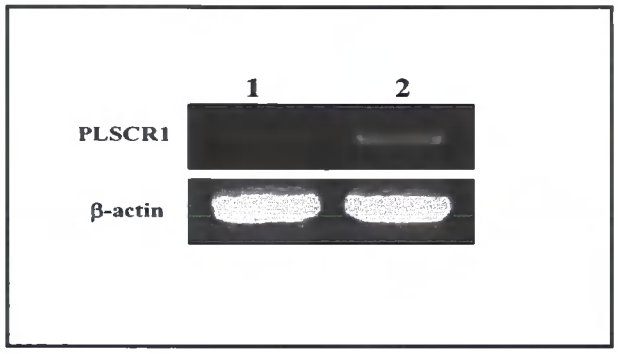

Fig. 5.7: Down-regulation of PLSCR1 expression. RT-PCR quantisation of the expression of PLSCR1 in Huh7 cells transduced with VLPs expressing 1- shPLSCR1, and 2- shGFP. β-actin mRNA was used to normalize the levels.

Similarly, for down-regulation of ZNF143 expression, Huh7 cells were transduced with VLPs prepared by using pLVTHM-shZNF143 or pLVTHM-shGFP. Total RNA was extracted 48 hrs post-transduction and ZNF143 mRNA levels were examined using semi-quantitative RT-PCR (Fig. 5.8). Compared with control cells, expression of ZNF143 was reduced by 25 % in cultures transduced with shZNF143 VLPs (repeated twice).

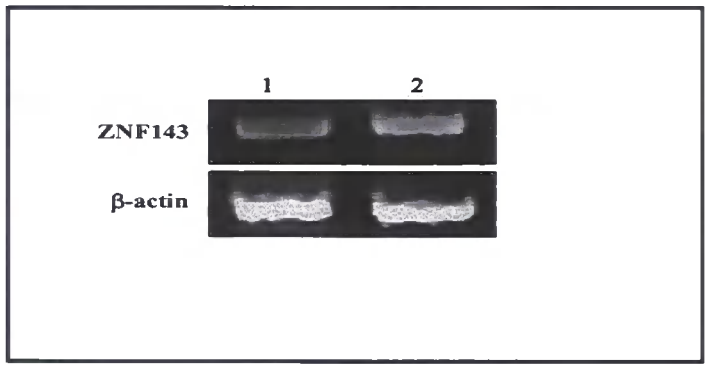

Fig. 5.8: Down-regulation of ZNF143 expression. Semi-quantitative RT-PCR for detection of ZNF143 expression in Huh7 cells transduced with VLPs expressing 1- shZNF143 and 2- shGFP as a control. Actin mRNA was used to normalize levels between samples.

In a preliminary experiment, Huh7 cells harbouring the HCV sub-genomic replicon were transduced with shPLSCR1, shZNF143, or shGFP VLPs. Replication was monitored using *luc* activity 48 hrs post-transduction.

Reduction of PLSCR1 expression had the effect of increasing *luc* activity by more than 35 % over the control which may suggest that endogenous PLSCR1 may have an antiviral role on HCV replication. There was no effect when ablating expression of ZNF143 suggesting that a contribution to the antiviral status of the cell was not evident under these conditions (Fig. 5.9).

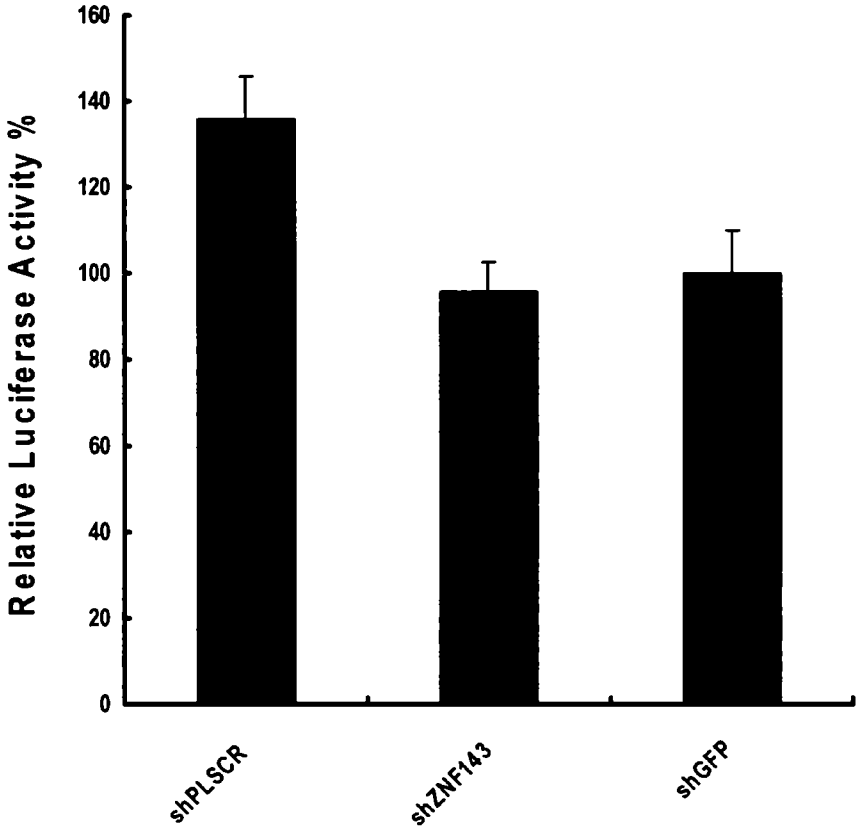

Fig. 5.9: Effect of repression PLSCR1 and ZNF143 expression on HCV replicon replication. Expression of PLSCR1, ZNF143 or GFP was knocked-down in Huh7 cells harbouring HCV replicon. Expression of *luc* was determined 48 hrs post-transduction as indication for replication. Light units were expressed as a percentage relative to those obtained from the control (shGFP). The assay was performed in triplicates for once. The mean±SD values were calculated and plotted as a percentage of the shGFP control sample. Error bars represent SD values.

5.5 Discussion

In this chapter we tried to investigate whether any biological significance could be ascribed to the interaction of NS5B with either PLSCR1 or ZNF143. Expression of PLSCR1 or ZNF143 significantly enhanced the ability of IFN to induce the expression from an ISRE element which confirmed their roles in amplifying the response to IFN (Fig. 5.1) (Dong *et al.*, 2004; Mach *et al.*, 2002). However, co-expression of NS5B with either protein reduced this activity. These results implicated NS5B in an anti-interferon response by interacting with PLSCR1 or ZNF143 supporting the observation that NS5B has some form of interaction with both proteins.

Using RT-PCR, no significant changes in ISGs expression levels were observed in cells transduced by NS5B, PLSCR1, ZNF143 or GFP VLPs (Fig. 5.3). Possibly other ISGs may be involved in affecting the IFN activity or more sensitive techniques such as real time RT-PCR or northern blotting are required to investigate the effect of these proteins on ISGs expression.

The effect of PLSCR1 on HCV sub-genomic replicon replication was tested in absence of IFN. It was observed that PLSCR1 over-expression had no effect on replicon replication (Fig. 5.5) while a reduction in its expression enhanced replication (Fig. 5.9). This may suggest that endogenous PLSCR1 had a maximal effect on the antiviral state; however increasing PLSCR1 expression could not increase the effect. Conversely ZNF143 over-expression, reduced replication by 30 % while reducing its expression had no effect on replication which suggested that a basal level of ZNF143 expression may not contribute to an antiviral state but by increasing its levels within the cell by over-expression, ZNF143 may participate in the antiviral state and reduce replicon replication. From these results we hypothesized that PLSCR1 and ZNF143 may have different strategies to contribute to the antiviral state of the cell. Further work is required to further these observations. As mentioned previously they represented a limited number of experiments performed in the laboratory of Dr A Patel (MRC Virology Unit, Institute of Virology) within a limited time.

In support with the role of PLSCR1 in the viral infection, a previously published work had shown that PLSCR1 expression was down-regulated by ~50 % in Huh7 cells stably expressing NS5A (Girard *et al.*, 2002) and also in cells harbouring HCV sub-genomic replicon (genotype 1b) (Sumpter *et al.*, 2004). Conversely it was up-regulated during a persistent HCV infection *in vivo* in chimpanzees (Bigger *et al.*, 2004). This suggests that regulation of PLSCR1 expression during HCV infection has a role in the development of the persistent infection. ZNF143 has not previously been reported to be involved in viral replication or persistence but HCV could interfere with its role in the transcription of IRF3 or other ISGs which would be needed to develop and maintain a persistence infection. Results presented in this chapter suggested that PLSCR1 and ZNF143 are not likely to be part of the viral replication complex but may contribute to the host cell response to the viral infection. The data also suggested that NS5B may participate in negating the response to IFN in part by the interaction with these proteins.

CHAPTER 6

Identification of Cellular Proteins that Can Interact with NS5B Using Pulldown Methodology and Mass Spectrometry

6.1 Introduction

In order to further improve our knowledge about the range of cellular proteins that can interact with NS5B, a second approach was undertaken using a pulldown methodology coupled with mass spectrometry. This methodology has been extensively used in recent years as it is a highly sensitive and powerful tool for rapidly identifying proteins from a complex set of proteins such as that from a cell (Lai *et al.*, 2008).

His-tagged tNS5B bound to nickel beads was used as bait for capturing proteins from Huh7 cell lysates. NS5B-associated proteins were analyzed by SDS-PAGE and identified by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry by the University of Durham's in-house proteomic service.

6.2 Pull-Down NS5B-Associated Proteins from Huh7 Lysate

Truncated NS5B (tNS5B) was expressed in BL21 (DE3) using pET21-5B construct as described in Section 4.2. Purified protein bound to Ni-NTA beads was washed extensively with PBS containing 20 mM imidazole to remove non-specific binding proteins. Beads were subsequently washed with PBS without imidazole. Expression and purity of NS5B were determined by SDS-PAGE and WB (not shown).

A Huh7 cell lysate was prepared from approximately 1 x 10^7 cells in cell culture lysis reagent (CCLR, Promega) followed by centrifugation to clarify the lysate which was then passed through a 0.45 µm filter. An equal amount of

NS5B-beads, or Ni-beads without NS5B, was added to the cell lysate. Imidazole was added to a final concentration of 20 mM to inhibit non-specific binding. The mixtures were incubated for 3 hours at 4°C. A parallel set of samples were also prepared that were incubated for 24 hrs. Beads were collected by centrifugation and washed extensively with washing buffer and then boiled in 1X SDS loading buffer and subjected to SDS-PAGE (Fig. 6.1).

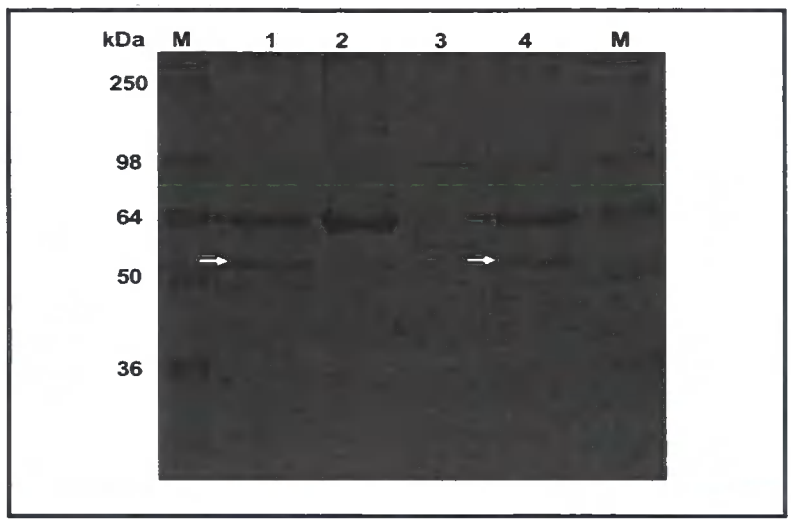

Fig. 6.1: SDS-PAGE analysis of NS5B-associated proteins derived from Huh7 cell lysate. (1) and (4) are NS5B-beads incubated for 3 and 24 hrs with the cell lysate, respectively. (2) tNS5B-beads without incubation with cell lysate. (3) Ni-beads incubated with cell lysate for 24hrs. Black arrows indicate NS5B, while the white arrows indicate the protein band isolated for identification by mass spectrometry. Gel stain was coomassie blue R-250.

An obvious major band (indicated by the white arrow, Fig. 6.1) of ~50 kDa was pulled down by NS5B (in both 3 and 24 hr samples) but not with Ni-beads suggesting that this band represented a protein that was specifically pulled down with NS5B. The presence of a large band on the gel suggested that this protein may have a strong interaction with NS5B; alternatively it may be due to its abundance within cell. This finding was confirmed in several independent experiments with different lots of lysate and NS5B.

6.3 Identification of NS5B-Associated Protein by MALDI-TOF

In order to identify the putative NS5B-associated protein, the band was excised from the gel. The gel fragment was sent for sequencing and identification by mass spectrometry. The protein candidate was prepared for MALDI-TOF mass spectrometry by destaining and in-gel digestion with sequencing grade trypsin. MALDI-TOF mass spectrometry analysis was carried out using PE Biosystems ABI Voyager DE-STR mass spectrometer. AutoMSfit database searches were all carried out under the control of Proteomics Solution 1 (PS1) software from PE Biosystems. Candidate protein mass spectrum was obtained after data acquisition and spectral processing using the Applied Biosystems Analyst and BioAnalyst™ software (Fig. 6.2).

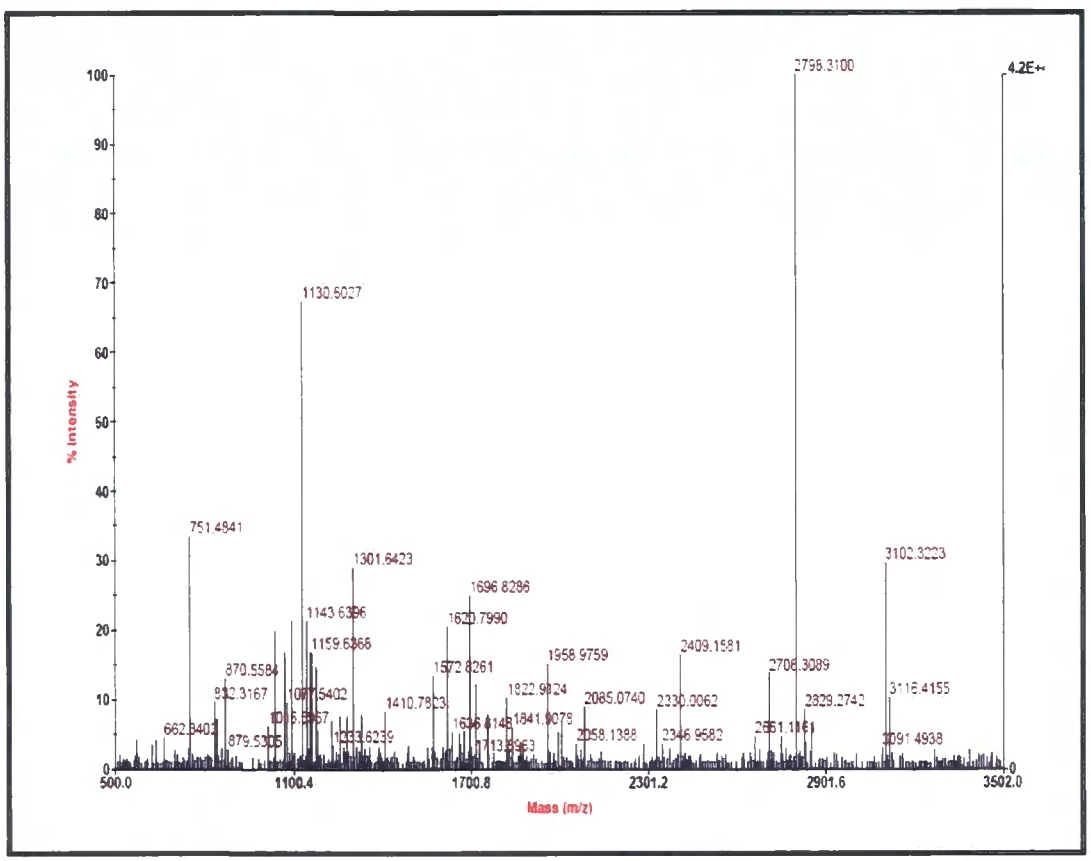

Fig. 6.2: Mass spectroscopy of NS5B-associated protein. The spectrum is showing the mass to charge (*m/z*) ratio and the % abundance of the precursor ion fragments on X and Y axes, respectively.

Protein identification was performed using MASCOT search tools for searching against all publicly available databases. Mascot search tools successfully identified that the novel band was *Homo sapiens* β-tubulin (Accession Number BC020946). The sequence coverage was 52 % and covered the whole sequence of the β-tubulin protein that made the identification statistically significant ($p < 0.05$) (Fig. 6.3).

```
  1 MREIVHIQAG QCGNQIGAKF WEVISDEHGI DPTGTYHGDS DLQLDRISVY
 51 YNEATGGKYV PRAILVDLEP GTMDSVRSGP FGQIFRPDNF VFGQSGAGNN
101 WAKGHYTEGA ELVDSVLDVV RKEAESCDCL QGFQLTHSLG GGTGSGMGTL
151 LISKIREEYP DRIMNTFSVV PSPKVSDTVV EPYNATLSVH QLVENTDETY
201 CIDNEALYDI CFRTLKLTTP TYGDLNHLVS ATMSGVTTCL RFPGQLNADL
251 RKLAVNMVPF PRLHFFMPGF APLTSRGSQQ YRALTVPELT QQVFDAKDMM
301 AACDPRHGRY LTVAAVFRGR MSMKEVDEQM LNVQNKNSSY FVEWIPNNVK
351 TAVCDIPPRG LKMAVTFIGN STAIQELFKR ISEQFTAMFR RKAFLHWYTG
401 EGMDEMEFTE AESNMNDLVS EYQQYQDATA EEEEDFGEEA EEEA
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Fig. 6.3: The amino acid sequence of β-tubulin depicted as an example for sequence coverage. The peptide fragments produced by trypsin digestion of the NS5B-associated band were used to identify the protein identity. The uniquely identified peptide sequences are marked in red. The protein sequence coverage shown above is 52 %.

6.4 Interaction of NS5B with β-Tubulin *in Vitro*

In order to confirm the mass spectrometry identification of β-tubulin as a possible NS5B-associated protein, a pulldown assay was performed for Huh7 cell lysate using NS5B-beads or Ni-beads as described before. The pulldown assay was followed by SDS-PAGE and WB analysis using α-His for detection of NS5B and mouse monoclonal anti-β-tubulin (kindly provided by Roy Quinlan, Durham University) and α-mouse-HRP for detection of β-tubulin. Results showed that NS5B can pulldown β-tubulin, confirming the MS identification (Fig. 6.4, A).

The specificity of the pulldown experiment using a non-specific protein, LacZ, was tested. Pulldown assays using NS5B or LacZ were carried out as described before. NS5B-His, LacZ-His were detected using α-His while anti-β-tubulin was used for β-tubulin (Fig. 6.4, B). Only NS5B was able to pulldown β-tubulin; there was no evidence for an equivalent pull-down of β-tubulin with LacZ.

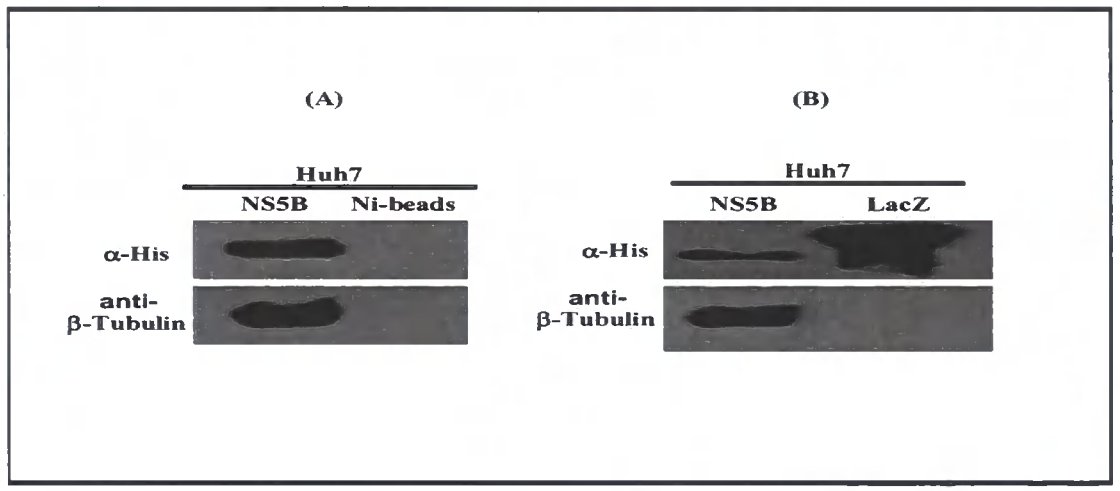

Fig. 6.4: Interaction of NS5B with β-Tubulin *in vitro*. (A) NS5B-beads or Ni-beads and (B) NS5B or LacZ previously bound to Ni-beads were incubated with Huh7 cell lysate for 3 hrs at 4°C. Beads were washed extensively before resolving on SDS-PAGE followed by WB analysis. Detection of NS5B or LacZ was performed using α-His while β-tubulin detection was carried out using anti–β-tubulin antibodies.

6.5 Interaction of NS5B with β-Tubulin in Non-Hepatic Cells

In order to investigate whether the interaction of NS5B with β-tubulin could be detected in other mammalian cells, pulldown assays were performed using NS5B or LacZ bound to beads and Huh7 or 293T cell lysates. Captured proteins were subjected to SDS-PAGE and stained with coomassie blue stain (Fig. 6.5, A) or subjected to WB for detection of NS5B and LacZ using α-His antibodies or for detection of β-tubulin by anti-β-tubulin (Fig. 6.5, B).

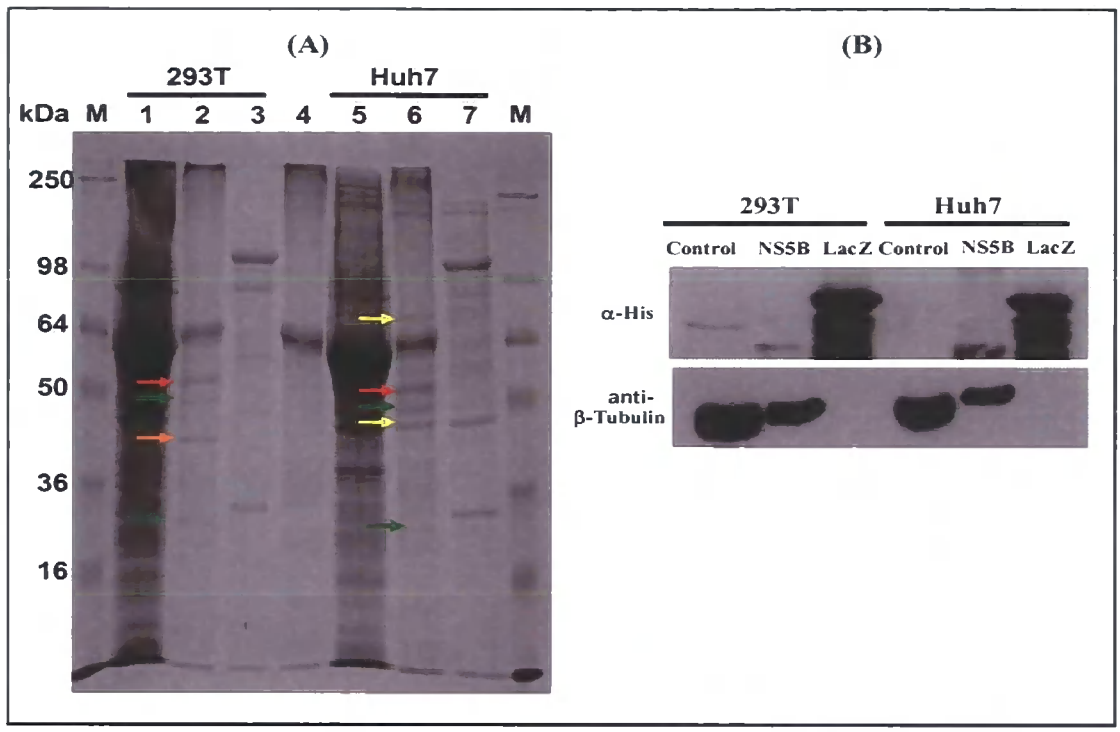

Fig. 6.5: Pull-down assay for detection of NS5B-associated proteins. (A) (1-3) 293T cell lysate, with NS5B, or with LacZ, respectively, (4) NS5B-beads, (5-7) Huh7 cell lysate, with NS5B-beads, or with LacZ-beads, respectively. Coloured arrows indicate proteins pulldown in both cell lines (see text) (B) Detection of the interaction of NS5B with β-tubulin in Huh7 and 293T cells. Cell lysate control, plus NS5B-beads or plus LacZ-beads were subjected to WB for detection of NS5B, LacZ, or β-tubulin.

As β-tubulin was detected in both cell lines (indicated by red arrows in Fig. 6.5, A), it was suggested that the interaction was not specific for Huh7 cells. Interestingly, different protein profiles were obtained for NS5B-associated proteins from Huh7 and 293T cells. Three proteins were detected in both cell lines (red and green arrows), two proteins were pulled down in Huh7 but not in 293T cells (yellow arrows), and a unique protein was observed in 293T cells (orange arrow).

6.6 Interaction of NS5B with β-tubulin in Huh7

To investigate if the interaction of NS5B with β-tubulin can be demonstrated *in vivo*, Huh7 cells were transfected with pcDNA4-5B (Appendix 9.11) or pcDNA4-LacZ (Invitrogen) for expression of full length NS5B or LacZ proteins in fusion with His-tag at the N-termini, respectively. Expression was allowed to proceed for 48 hrs before harvesting and preparing cell lysates. Lysates were incubated with Ni-beads for 3 hrs at 4°C to capture NS5B or LacZ and any associated cellular proteins. Beads were washed and subjected to SDS-PAGE followed by WB analysis for detection of NS5B, LacZ, or β-tubulin. As in the *in vitro* pull-down, β-tubulin was only observed with NS5B (Fig. 6.6).

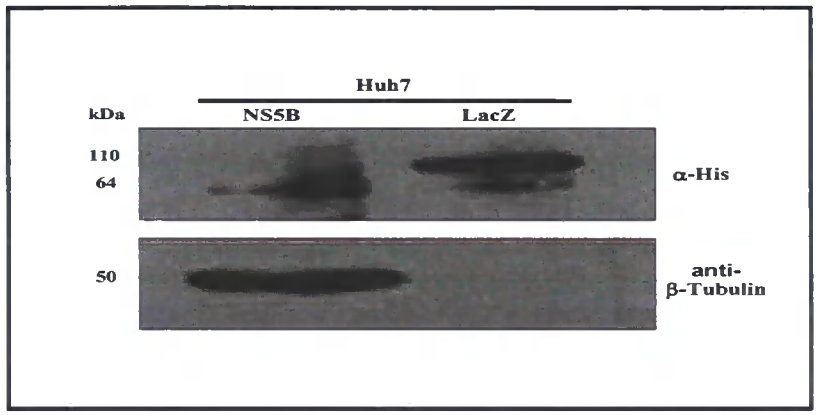

Fig. 6.6: Interaction of NS5B with β-tubulin *in vivo* in Huh7. Huh7 cells were transfected with pcDNA4-5B or pcDNA4-LacZ. Cell lysates were prepared and incubated with Ni-beads for 3 hrs at 4°C. Washed beads were mixed with SDS sample buffer and then subjected to SDS-PAGE and WB for detection of β-tubulin.

6.7 Interaction of NS5B with β-tubulin is an Indirect Interaction

The next step was to determine whether the interaction of NS5B with β-tubulin was a direct interaction or it was bridged by some intermediatory. The β-tubulin ORF sequence (1338 bp, Appendix 9.7.4) (Accession Number BC020946) was successfully amplified from total Huh7 RNA using RT-PCR and cloned into pGEX-6P-1 using *EcoRI* and *XhoI* restriction sites for expression of β-tubulin in fusion with GST (Appendix 9.11). GST-β-tubulin was successfully

overexpressed in *E. coli* BL21 (DE3) cells and purified using Glutathioine-Sepharose-4B beads. Expression of full-length β-tubulin in fusion with GST was confirmed by SDS-PAGE and Western blotting analysis using α-GST-HRP antibody (not shown).

A pull-down assay was performed to test for a direct interaction between NS5B and β–tubulin. β-tubulin bound to beads was incubated with soluble tNS5B or LacZ for 3 hrs at 4°C. Washed beads were subjected to SDS-PAGE and WB analysis for detection of the proteins using anti-β-tubulin and α-His antibodies to probe for β-tubulin and NS5B or LacZ, respectively. β-tubulin-beads failed to pull down NS5B that suggested that the interaction of NS5B with β-tubulin was an indirect interaction (data not shown) or that the fusion of β-tubulin with GST-tag altered the protein's ability to interact with NS5B. As the *in vitro* GST-pull-down assay failed to confirm a direct interaction, the yeast two-hybrid system was employed to test for a possible direct interaction *in vivo*. β-tubulin was cloned into pGADT7 to create pGAD-TubDF (Appendix 9.11) that was employed in the yeast two-hybrid assay with pGBK-5B. However, the yeast two-hybrid assay was also unable to detect a direct interaction between NS5B and β-tubulin (not shown).

6.8 Mapping NS5B-Domain(S) That Mediates the Association with β-Tubulin

In order to determine the NS5B-domain responsible for the association with β-tubulin or rather the domain needed by the intermediatory, co-carboxy deletion mutants, NS5BD1 ($NS5B^{\Delta 154-591}$) and NS5BD2 ($NS5B^{\Delta 302-591}$), were generated and cloned into pET21d. pET21-5B, -5BD1, and -5BD2 were introduced into *E. coli* BL21 (DE3) and protein expression and purification were performed as described before for tNS5B (Section 4.2).

tNS5B, NS5B domains or LacZ bound to beads were incubated with Huh7 cell lysates for 3 hrs at 4°C. Beads were washed extensively and subjected to SDS-PAGE and WB for analysis of the expression of NS5B and the domain

mutants and any associated β-tubulin. β-Tubulin was detected with all NS5B domains but not with LacZ suggesting that NS5BD1 (1-153 a.a.) was sufficient for the interaction that led, indirectly, to the pull-down of β-tubulin (Fig. 6.7). Interestingly, the strength of interaction was proportional with the increase in size of the NS5B deletion where the amount of β-tubulin associated with NS5BD1 and NS5BD2 was greater than that with tNS5B. Possibly the β-tubulin binding site on NS5B was more accessible on the deletion mutants.

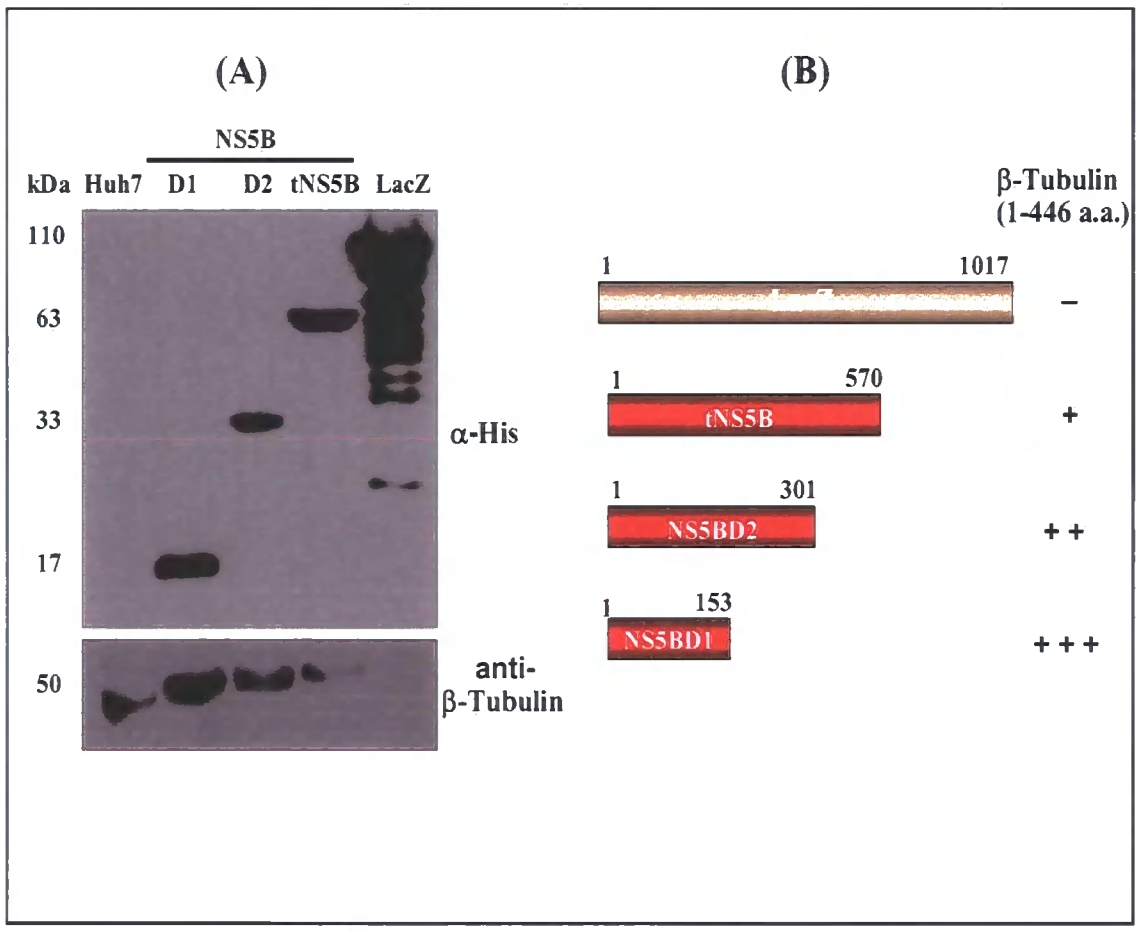

Fig. 6.7: Mapping the β-tubulin-interacting domain of NS5B. (A) NS5BD1, NS5BD2, tNS5B, and LacZ were used to pulldown β-tubulin from Huh7 cell lysate. Washed beads were subjected to SDS-PAGE and WB analysis for detection of NS5B domains and LacZ using α-His and for β-tubulin using anti-β-tubulin antibodies. Huh7 cell lysate was used as a positive control for β-tubulin. (B) A schematic diagram for mapping the β-tubulin-interacting domain on NS5B. All NS5B domains were able to pull-down β-tubulin from Huh7 cells lysates with different strengths (+).

6.9 Interaction of NS5B with α-Tubulin *In Vitro*

As NS5B indirectly associated with β-tubulin, we proposed that it could be another protein(s) that mediated this association. α-tubulin, β-tubulin's partner, which together formed the microtubule network, could be a potential candidate for mediating this association.

In order to evaluate our hypothesis and investigate whether NS5B was able to pull-down α-tubulin or not, His-tagged NS5B or LacZ proteins bound to Ni-beads were incubated with Huh7 cells lysates for 3 hrs at 4°C. Washed beads were subjected to SDS-PAGE and WB analysis for the detection of α-tubulin using an anti-α-tubulin antibody (Calbiochem) and α–His antibody for NS5B or LacZ. Only NS5B was able to pull-down α-tubulin from a Huh7 cell lysate demonstrating that NS5B could pull-down both α- and β-tubulin (Fig. 6.8).

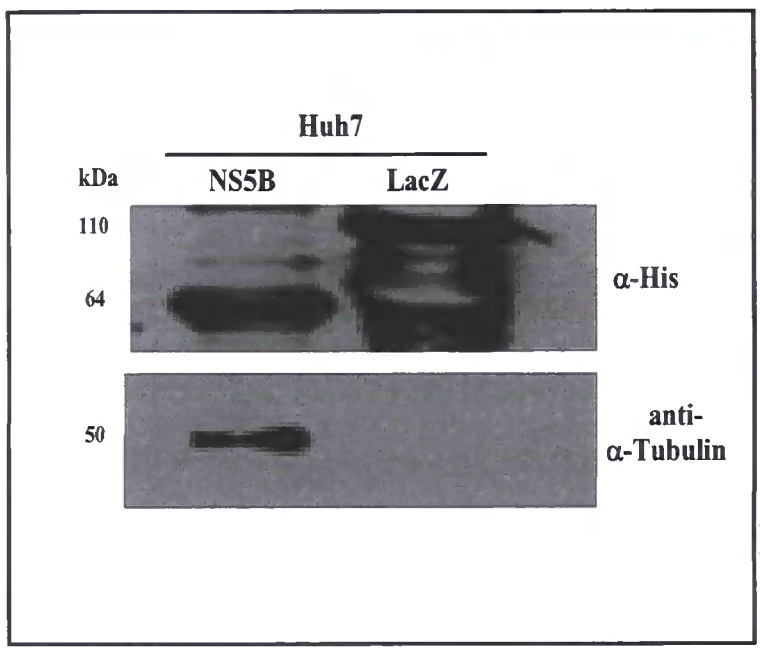

Fig. 6.8: Interaction of NS5B with α-tubulin. NS5B and LacZ beads were incubated with Huh7 cell lysate for 3 hrs at 4°C. Washed beads were subjected to SDS-PAGE and WB. Detection of NS5B and lacZ was performed using α-His, while α-tubulin detection was carried out by anti-α-tubulin.

6.10 Interaction of NS5B with α-Tubulin is an Indirect Interaction

In order to investigate whether or not, the association of NS5B with α-tubulin was a direct interaction we used a different approach from the normal approaches, yeast two-hybrid assay or *in vitro* pull-down assay using recombinant protein expressed in bacteria. Nocodazole, an inhibitor of microtubule formation that blocks the interaction of α- with β-tubulin, was used to examine the interaction of α- and β-tubulin with NS5B.

NS5B was tested for its ability to pull-down α- and β-tubulin *in vitro* in the presence of different concentrations of nocodazole. NS5B bound to beads was incubated with Huh7 cell lysate in the presence of 10 or 40 μM nocodazole, or with DMSO as a control. Cells were incubated with beads for 3 hrs at 4°C. Beads were collected and washed extensively before analysis by SDS-PAGE and WB for detection of associated α- and β-tubulin proteins.

Nocodazole had no significant effect on the interaction of NS5B with β-tubulin (Fig. 6.9), while the interaction with α-tubulin was markedly decreased in a concentration-dependent manner. This suggested that, by the addition of nocodazole, the α- and β-tubulin complex dissociated leaving β-tubulin associated with NS5B and releasing α-tubulin. From these results, we can conclude that the interaction of NS5B with α-tubulin is also an indirect interaction that is possibly mediated via β-tubulin with the α- and β-tubulin complex associated with NS5B via an unknown cellular partner, or partners.

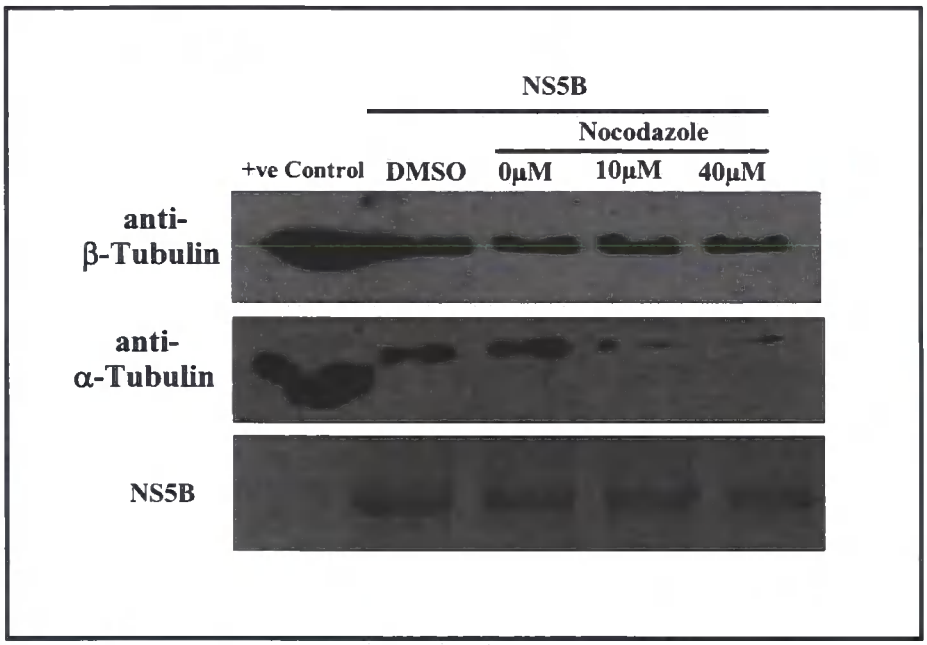

Fig. 6.9: Effect of nocodazole on the interaction of NS5B with α- and β-tubulin *in vitro*. Huh7 cells lysate was incubated with NS5B-beads in absence or presence of DMSO, 10 μM, and 40 μM nocodazole for 3 hrs at 4°C. Washed beads were subjected to SDS-PAGE and WB for detection of α- and β-tubulin using specific antibodies. SDS-PAGE was used to indicate that the same amount of NS5B-beads was used for the pulldown assays. Huh7 cell lysate was used as +ve control for α- and β-tubulin.

In order to exclude the possibility that the addition of nocodazole to Huh7 cells negatively influenced the intracellular level of α-tubulin, two flasks of Huh7 cells were cultured with an equivalent number of cells. One flask was left as a non-treated control and the other was treated with 10 μM nocodazole for 24 hrs. Cells lysates were prepared and subjected to SDS-PAGE and WB for detection of α-tubulin. As expected, nocodazole had no significant effect on the intracellular level of α-tubulin compared with non-treated cells which confirmed that nocodazole had a direct effect on the association of α-tubulin with NS5B.

6.11 Effect of Nocodazole on the Interaction of NS5B with α- and β-Tubulin *In Vivo*

In order to determine whether nocodazole had the same effect on the interaction of NS5B with α- and β-tubulin *in vivo*, Huh7 cells were transfected with the same amount of pcDNA-5B in two separate flasks cultured with an equivalent number of cells. One flask was subsequently treated with 10 μM nocodazole, 24 hrs post-transfection, for 24 hrs. Pulldown assays were carried out as described before. Washed beads were subjected to WB analysis for detection of NS5B, α- tubulin or β-tubulin.

The effect of nocodazole addition on the interaction of NS5B with α- and β-tubulin *in vivo* was the same as *in vitro* studies where no significant effect was observed on the interaction with β-tubulin. The association with α-tubulin was inhibited by the addition of nocodazole supporting the hypothesis that the interaction with NS5B was through β-tubulin (Fig. 6.10).

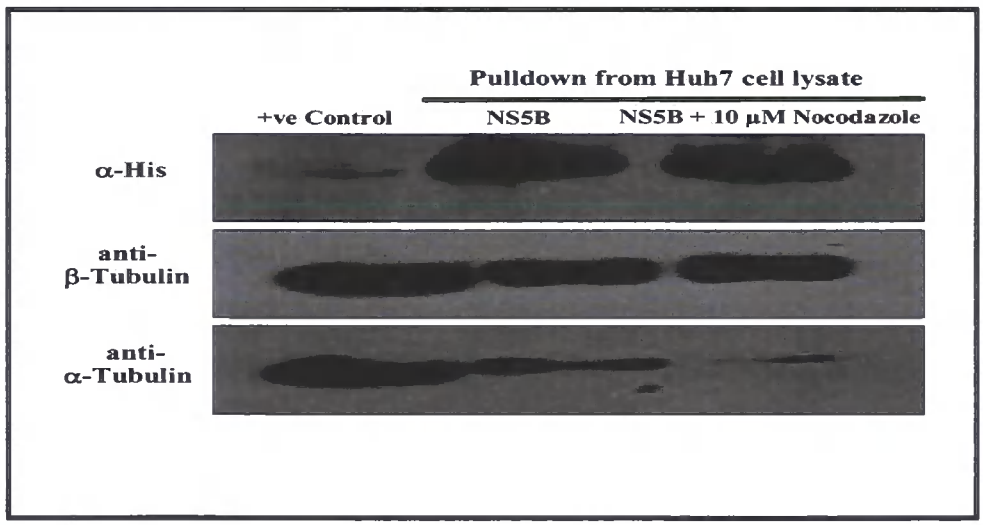

Fig. 6.10: Effect of nocodazole on the interaction of NS5B with - α- and β-tubulin *in vivo*. Huh7 cells transfected with pcDNA-5B were treated with 10 μM nocodazole. Lysates from treated and non-treated cells were incubated with Ni-beads. Beads were subjected to SDS-PAGE and WB analysis for detecting the expression of NS5B using α-His antibody or anti- α- and β-tubulin antibodies for detection of α- and β-tubulin. Huh7 cell lysate was used as +ve control for α- and β-tubulin.

6.12 Effect of Nocodazole on the HCV Sub-genomic Replicon Replication

The effect of nocodazole on the replication of the pFK-Luc sub-genomic replicon was tested. Huh7 cells harbouring replicon RNA were incubated 24 hrs post-transfection with various concentrations of nocodazole for a further 24 hrs. Luciferase activity was determined 48 hrs post-transfection using One-Glo Luciferase Assay System (Promega).

The effect of increasing the nocodazole concentration was to reduce luciferase activity in a concentration dependent manner. The addition of 0.3125 µM nocodazole reduced *luc* activity by 30 %, while 20 µM nocodazole reduced *luc* activity by 70% (Fig. 6.11). These results confirmed the previously reported suppressive effect of nocodazole on HCV sub-genomic replicon replication (Bost *et al.*, 2003).

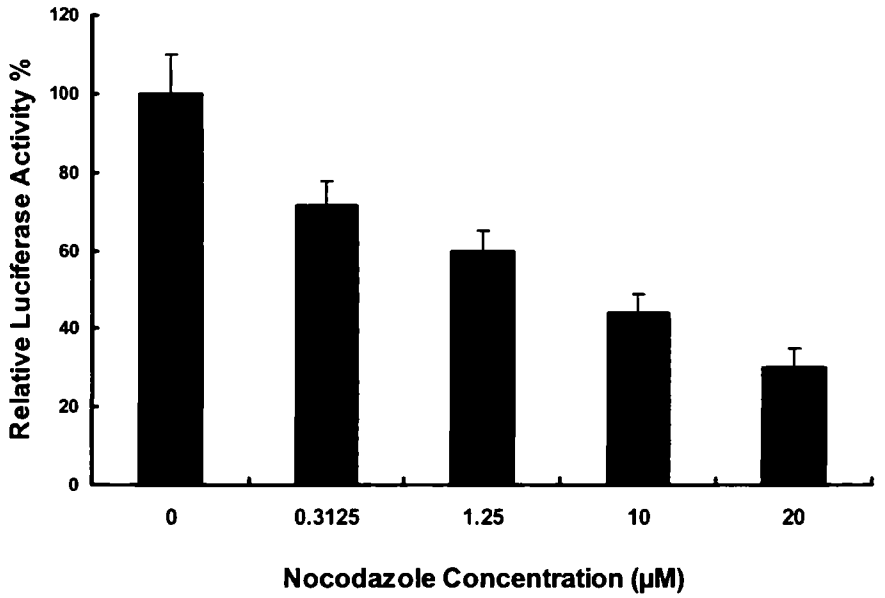

Fig. 6.11: Effect of nocodazole on HCV sub-genomic replicon replication. Huh7 cells transfected with RNA *in vitro* transcribed using pFK-Luc as a template were treated with different concentrations of nocodazole for 24 hrs. Luciferase activity was measured 48 hrs post-transfection and expressed as a percentage relative to that of the non-treated control cells. Each sample was measured in triplicate in three separate experiments Error bars represent the SEM of three separate experiments.

6.13 Discussion

In addition to the yeast two-hybrid system, a second approach was used to identify host cell proteins that can interact with NS5B. A pulldown methodology coupled with mass spectrometry was employed for this purpose (Fig. 6.1). This method is simple and characterized by its ability to identify direct and indirect interactions that form multi-protein complexes. The same methodology was successfully used to identify cellular proteins that were able to interact with NS5B such as the RNA-binding protein, hnRNP A1 (Kim *et al.*, 2007).

Mass spectrum (Fig. 6.2) produced by MALDI-TOF MS analysis of the major NS5B-associated protein band identified it as β-tubulin (Fig. 6.3). The ability of NS5B to capture β-tubulin was confirmed *in vitro* (Figs. 6.4). The interaction of NS5B with β-tubulin was further investigated in a non-hepatic cell line (293T) in addition to Huh7 (Fig. 6.5). Interestingly, the pattern of proteins associated with NS5B was different in each cell line as some bands were present in both cell lines while some were found in one but not in the other. This difference could be the basis for future work on HCV hepatic cell tropism. However, the β-tubulin pull-down was consistent in both lines.

The association of β-tubulin with NS5B was confirmed *in vivo* in Huh7 cells (Fig. 6.6). A pull-down assay, using soluble purified tNS5B and β-tubulin-GST-fusion previously bound to beads, was unable to detect a direct interaction *in vitro* that suggested that the interaction of NS5B with β-tubulin was indirect. The yeast two-hybrid assay also failed to demonstrate a direct interaction between NS5B and β-tubulin (not shown) which implied an indirect association of NS5B with β-tubulin (Section 6.7). The interacting domain of NS5B that mediated the pull-down of β-tubulin was determined and data implicated $NS5B^{\Delta 154\text{-}591}$ (NS5BD1) domain to be largely responsible for the pulldown (Fig. 6.7).

As the association with β-tubulin was determined to be indirect, it was proposed that another protein(s) could mediate this association. It was postulated that α-tubulin may mediate this association as α-tubulin interacts with β-tubulin to form a polymer that is required for HCV RNA replication (Bost *et al.*, 2003). The association of α-tubulin with NS5B was subsequently demonstrated *in vitro* (Fig. 6.8).

In order to investigate the effect of nocodazole on the association of NS5B with both α- and β-tubulin and whether it can affect this association, different concentrations of nocodazole were tested *in vitro* and *in vivo* (Fig. 6.9 and 6.10, respectively). Results showed that there was no significant effect on the amount of β-tubulin pulled down by NS5B in the presence of nocodazole. However, the amount of α-tubulin pulled down by NS5B was markedly decreased by nocodazole in a dose dependent manner. The decrease was not due to instability in the presence of nocodazole, but rather to a knock off effect on the association of α-tubulin with β-tubulin. These results indicated that the association of NS5B with α-tubulin is an indirect interaction that is mediated by β-tubulin.

HCV replicon replication was markedly reduced by the addition of nocodazole (Fig. 6.11) that suggested that the association of α/β tubulin with NS5B is required for HCV RNA replication. Notably α/β tubulin polymerization was shown to be essential for HCV as treatment with nocodazole markedly reduced RNA replication (Bost *et al.*, 2003). Moreover, the induction of lipid droplet re-distribution by HCV core was shown to be mediated by microtubules that may facilitate the interaction of HCV RNA replication with virion assembly complexes (Boulant *et al.*, 2008b). Recently, it has been reported that the HCV replication complex was associated with microtubules through an interaction with NS3 and NS5A and that this interaction may provide the basis for the movement of the viral replication complex to other regions within the cell (Lai *et al.*, 2008).

CHAPTER 7

General Discussion

The interaction between virus and host is critical in determining the outcome of an infection. Study of HCV-host interaction would improve our knowledge about how the virus replicates in the host and develops long-term complications such as liver fibrosis, cirrhosis, and HCC. It would also lead to a greater understanding of the mechanisms by which the virus negates the host immune system to facilitate persistence.

At the beginning of this project, we aimed to establish a new area of HCV research focusing on identifying cellular proteins that interact with a HCV protein target. Thus, time was dedicated to generate reagents of use within this study and to establish and develop suitable protocols for this project. In order to achieve this goal, NS5B was used in this study as bait as it is the key component of the viral replication complex that interacts with viral and cellular proteins to drive viral replication and persistence (Gosert *et al.*, 2003). Therefore, identification of host cell proteins involved in viral replication and persistence could serve as targets for future anti-HCV drug development.

The main outcomes achieved in this study may be summarized as follows:

- Identification of new cellular partners for NS5B that may contribute to viral replication and/or persistence using two different approaches, (1) yeast two-hybrid system and (2) pull-down assay accompanied by mass spectrometry to identify protein species.

- The interaction of PLSCR1, ZNF143 and RTN3 with NS5B were confirmed *in vitro*. The interacting domains of NS5B and PLSCR1 were successfully determined (Sections 4.9 and 4.10).

- It was observed that NS5B had a suppressive effect on the ability of PLSCR1 and ZNF143 to amplify IFN activity on ISRE-driven *luc* expression in Huh7 and VERO cells (Section 5.2).

- In the absence of IFN, PLSCR1 over-expression had no significant effect on HCV sub-genomic replicon replication; while down-regulating its expression had an enhancing effect on replication. ZNF143 over-expression had a suppressive effect on replicon replication but decreasing its expression had no effect on replication (Section 5.4).

- Using *in vitro* pull-down assay with NS5B as bait and mass spectrometry, α- and β-tubulin were identified to associate with NS5B indirectly in hepatic (Huh7) and non-hepatic cells (293T) (Chapter 6). The effect of nocodazole on the association of NS5B with α- and β-tubulin was examined *in vitro* and *in vivo* in Huh7. This association was shown to be essential for HCV sub-genomic replicon replication in Huh7 (Section 6.12).

7.1 Identification of Cellular Proteins That Can Interact with NS5B

The development of the yeast two-hybrid system has improved our knowledge about protein-protein interactions that occur in many cellular pathways. In this project, the MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) was used to identify cellular proteins that can interact with NS5B. This system is highly sensitive for weak and transient interactions and also provides high-stringency selection conditions that reduce the possibility of false positive.

Yeast two-hybrid screening successfully identified seven novel potential partners for NS5B (PLSCR1, ZNF143, RTN3, OSBP8, HAX1, TIMM50, and SFRS10, Section 3.4). Significantly, these proteins are involved in many important cellular pathways such as IFN signalling, lipid metabolism and

transport, protein trafficking, transcription, cellular proliferation and apoptosis. Recently, a proteome-wide mapping of interactions between HCV and cellular proteins showed that PLSCR1 could interact with HCV core protein. In this screen, PLSCR1 was suggested to be necessary for the full functionality of Jak/Stat and insulin signalling pathways. However, the interaction of core with PLSCR1 may interfere with these pathways (de Chassey *et al.*, 2008). RTN3 was shown to interact with NS4B, which is part of the viral RNA replication complex (Liu *et al.*, 2005; Piccininni *et al.*, 2002). None of other proteins identified in this study have been previously reported to interact with HCV proteins.

The cellular location of proteins may have significance in any presumptive role of an interaction with NS5B (Table 7.1). PLSCR1 is found normally at the plasma membrane but migrates to the nucleus after induction with interferon (Wiedmer *et al.*, 2003). Interestingly, a truncated form of NS5B, with no nuclear localization signal (NLS), has been shown to be localized to the nucleus, suggesting a potential to interact with nuclear factors (Yamashita *et al.*, 1998). Indeed, nucleolin changed its nuclear localization to the cytoplasm by an interaction with full-length NS5B (Hirano *et al.*, 2003). Similarly, the human RNA helicase, p68, was redistributed from the nucleus to the cytoplasm by an interaction with NS5B (Goh *et al.*, 2004). It is probable that the interaction of NS5B with normally nuclear localizing proteins, such as PLSCR1, ZNF143 and SFRS10, alters the trafficking of these proteins away from the nucleus thereby affecting their function. These interactions may also change the location of NS5B for some function.

Table 7.1: Location of cellular proteins that identified to interact with NS5B

Protein	Location
PLSCR1	Plasma membrane and nucleus
ZNF143	Nuclear
RTN3	ER and Golgi
OSBP	ER
SFRS10	Nuclear
HAX1	ER and mitochondria
TIMM50	Mitochondria

7.2 Characterization of the Interaction of NS5B with PLSCR1 or ZNF143

PLSCR1 was selected as the main target for this project as it had an important role in IFN and EGFR signalling pathways (Dong *et al.*, 2004; Nanjundan *et al.*, 2003; Sun *et al.*, 2002). It was also responsible for transporting phosphatidylserine and phosphatidylethanolamine to the outer surface of plasma membrane in response to apoptotic signals that subsequently led to clearance of apoptotic cells by macrophages (Sims and Wiedmer, 2001). Interestingly, externalization of phosphatidylserine, a marker for apoptosis, has been observed in JFH1-infected cells, the sole model for HCV replication (Sekine-Osajima *et al.*, 2008). This process involved ER-stress and was correlated to particular amino acids substitutions within NS5B (Sekine-Osajima *et al.*, 2008). Consistent with the putative role of NS5B in viral persistence, it has been shown that NS5B down-regulated the surface expression of cell surface proteins such as Major Histocompatibility Class-I (MHC-I) complexes preventing elimination of HCV-infected cells by cytotoxic T-cell lymphocytes (CTLs) which contributes to viral persistence (Pavio and Lai, 2003). ZNF143's suitability for further study was its potential role in RNA polymerase II and III transcription activities, cell survival and IRF3 transcription control (Grossman *et al.*, 2004; Ishiguchi *et al.*, 2004; Mach *et al.*, 2002). Interestingly, escape of innate immunity by HCV involved a stringent control of the IRF3 response (Binder *et al.*, 2007).

NS5B, PLSCR1 and ZNF143 were successfully expressed in bacteria and purified in soluble forms (Sections 4.2 and 4.3) for *in vitro* analysis which subsequently confirmed the ability of NS5B to bind PLSCR1 and ZNF143 (Sections 4.4, 4.5, 4.7 and 4.8). The NS5B-interacting domain of PLSCR1 was mapped to amino acids 61-137 (Section 4.9). Interestingly, this domain encompassed a DNA-binding domain and a SH3-like domain that can mediate recruitment and activation of cellular signalling molecules such as EGFR, the Shc adaptor protein and Src kinases (Nanjundan *et al.*, 2003; Sun *et al.*, 2002; Sun *et al.*, 2001; Zhou *et al.*, 2005).

Both PLSCR1 and ZNF143 were shown to interact with a single NS5B motif within the N-terminus (amino acids 1 to 153, Sections 4.10). This motif occupies most of the NS5B finger domain (amino acids 1-187) that with the NS5B thumb domain encircles the enzymatic active site (Kang *et al.*, 2008; Lesburg *et al.*, 1999; Lohmann *et al.*, 1997). Importantly, the finger domain of NS5B mediated the interaction of NS5B with PRK2 resulting in the regulation of RNA replication (Kim *et al.*, 2004). This motif also interacted with α-actinin that was proposed to be a part of the viral RNA replication complex (Lan *et al.*, 2003). The NS5B finger domain was also shown to have a sphingolipid-binding motif that mediated the association of NS5B with sphingomyelin in lipid rafts (Sakamoto *et al.*, 2005). Therefore it is not a surprising result that both PLSCR1 and ZNF143 bind to what already has been demonstrated to be a domain involved in multiple interactions.

Unfortunately, our analysis was unable to demonstrate the interactions in mammalian cells which suggested that the interactions, if true, were weak and may be disrupted under our protocol conditions. It is probable that the interactions only occurred under certain conditions such as IFN or EGF stimulation that create a favourable environment for interactions. For example, in the presence of IFN, PLSCR1 was de-palmitoylated and mainly localized to the nucleus whilst, in the absence of IFN, the PLSCR1 palmitoylated form is dominant and localized to the plasma membrane (Wiedmer *et al.*, 2003).

7.3 Effect of NS5B Interactions on IFN Signalling Pathway

PLSCR1 and ZNF143 significantly enhanced the effect of IFN on an ISRE-driven *luc* expression in Huh7 and VERO cells (Section 5.2). Interestingly, co-expression of NS5B negated this activity which suggested that the interaction of NS5B with PLSCR1 or ZNF143 may block their roles in IFN signalling. Absence of a significant effect for NS5B on ISRE-driven expression, when expressed alone, implied a degree of specificity for NS5B effect on PLSCR1 and ZNF143 activities. These results suggested that NS5B reduced the activity of IFN on ISRE by an interaction with PLSCR1 or ZNF143 which could contribute to viral persistence by suppressing the development of an antiviral state in cell.

Expression of NS5B, PLSCR1 or ZNF143 had no effect on the expression of ISG15, ISG54, OAS2 and IRF7 in Huh7 (Section 5.3). However, a more sensitive technique such as real-time RT-PCR may be required for detection of significant effects. The effect of NS5B, PLSCR1 or ZNF143 on ISRE activity (Section 5.2) could involve other ISGs that were not tested in this study.

HCV can interfere with the host innate immune response and counteract the antiviral effect of IFN by many strategies that create an environment suitable for its replication and persistence (Foy *et al.*, 2005). These strategies involve the interaction of HCV proteins with IFN-signalling pathways molecules interfering with their function in delivering IFN antiviral downstream effects (Weber, 2007). NS3/4A was shown to modulate IFN signalling by disrupting the retinoic acid-inducible gene (RIG-1) pathway preventing the activation of IRF3 and NF-κB (Foy *et al.*, 2005; Foy *et al.*, 2003). Similarly NS5A interfered with IFN signalling by the disruption of the contribution of the MAPK pathway to JAK-STAT signalling pathway, by the interaction with Grb2 (He *et al.*, 2002; Katze *et al.*, 2002). NS5A, in addition to E2, was also shown to bind to and inhibit PKR antiviral kinase activity (Gale *et al.*, 1997; Sato, 2001). Similarly, NS5A interacted with and inhibited the ability of 2'-5' OAS to produce 2'-5' oligoadenylate that is needed for the activation of endoribonuclease L, RNAse L, which degrades viral and cellular RNA (Chevaliez and Pawlotsky, 2007; Taguchi *et al.*, 2004).

A previous study showed that NS5B had a role in interfering with IFN signalling pathways by suppressing the IRF7-mediated IFN-α promoter activation (Zhang *et al.*, 2005). Using RT-PCR, we could not detect a significant change in IRF7 expression at the transcriptional level in cells expressing NS5B (Section 5.3). Taken together, NS5B may affect IRF7 activation at a posttranscriptional level. NS5B also demonstrated a potential mechanism for HCV pathogenicity by modulating TNF-α signalling pathways via the interaction with IKKα, an inhibitor of NF-κB (I-κB) kinase α (Choi *et al.*, 2006). IKKα is not only essential for NF-kB activation but also for Toll-like receptors 7/9 (TLR7/9)-induced IFN-α production (Hoshino *et al.*, 2006).

Other studies have demonstrated that NS5B induced IFN-β via the TLR3 signalling pathway (Naka *et al.*, 2006). NS5B was also shown to activate 2'-5'-OAS gene promoter activity through ISRE although we could not replicate this effect using RT-PCR (Section 5.3) (Dansako *et al.*, 2005). These conflicting effects of NS5B on different stages of IFN signalling pathways suggested that NS5B, in addition to its role in viral replication, has a regulatory role in subverting IFN signalling pathway(s) that may lead to viral persistence.

7.4 Effect of PLSCR1 and ZNF143 on HCV Sub-Genomic Replicon Replication

Preliminary results showed that while over-expression of PLSCR1 had no significant effect on the replicon replication, down-regulating its expression significantly increased replication (Section 5.4). Over-expression of ZNF143 had a suppressive effect on replication but reducing its expression by shRNA had no observable effect on replicon replication. From these results we hypothesized that PLSCR1 and ZNF143 may have a role in creating an antiviral state in cell. PLSCR1, a known ISG, had been shown to have an antiviral activity against VSV, EMCV and DENV (Dong *et al.*, 2004; Sariol *et al.*, 2007). Notably, PLSCR1 expression was up-regulated in chimpanzee infected with HCV (Bigger *et al.*, 2004). It was also observed that expression of NS5A down-regulated PLSCR1 expression levels in Huh7 cells (Girard *et al.*, 2002). In addition, Huh7 cells that harbouring a HCV sub-genomic replicon had lower levels of PLSCR1 expression (Sumpter *et al.*, 2004). These data suggested that down-regulation of PLSCR1 expression during HCV infection may have a role in the development of a persistent infection.

Reportedly, ZNF143 stimulated the expression of IRF3 which mediated activation of IFN-α and -β (Mach *et al.*, 2002). Therefore the interaction of HCV with ZNF143 could interfere with its role in the activation of IRF3 or other host gene(s) expression to suppress the antiviral state and facilitate persistence. The data in Chapter 5 suggested that NS5B may participate in negating the response to IFN, in part, by an interaction with these proteins.

7.5 Interaction of NS5B with α- and β-Tubulin

Using a pulldown methodology coupled with mass spectrometry, β-tubulin was identified as a potential NS5B-associated protein in Huh7 cells (Section 6.2). Subsequently, it was apparent that it was the α/β tubulin complex that had been isolated. This interaction could be demonstrated in hepatic (Huh7) and non-hepatic (293T) cell lines. Interestingly, it was observed that there were different patterns of NS5B-associated proteins between the cell-lines (Fig. 6.5); possibly this will be a subject for further investigation in the future to identify factors involved in viral replication and hepatic tropism. The physical association with tubulin was subsequently shown to be an indirect interaction (Section 6.7 and 6.10). Investigation of the NS5B domain that mediated the tubulin pulldown determined that amino acid residues 1-153 were responsible for the interaction (Section 6.8).

In vitro and *in vivo* analysis demonstrated that nocodazole reduced the association of NS5B with α-tubulin in a concentration-dependent manner but had no effect on the association with β-tubulin that suggested that β-tubulin was responsible for the association of α-tubulin with NS5B (Section 6.10 and 6.11). Our results showed that nocodazole had a suppressive effect on HCV sub-genomic replicon replication (Section 6.12). These results indicated that the role of α- and β-tubulin in the viral replication was probably not only required for providing microtubules as a track for the movement of the viral replication complex (Lai *et al.*, 2008) but also could be essential for constructing the RNA replication complex by an association with NS5B.

This work supported the observations that α/β tubulin polymerization is essential for HCV RNA synthesis and may be required for constructing the RNA replication complex (Bost *et al.*, 2003). β-tubulin was not only essential for RNA synthesis of other viruses such as Sendai virus and VSV *in vivo* but also seemed to have a functional role in interacting with the VSV polymerase as it stimulated the virus polymerase activity *in vitro* (Hill *et al.*, 1986; Mizumoto *et al.*, 1995; Moyer *et al.*, 1986). Furthermore, tubulin has been suggested to be a

subunit of the measles virus RNA polymerase (Berghall *et al.*, 2004; Moyer *et al.*, 1990).

7.6 Conclusion

HCV is a major cause of chronic liver disease that can lead to cirrhosis and HCC. Up to now, the current treatment for HCV infection using IFN and ribavirin is not efficient as 60 % of patients (genotype 1, Section 1.6) do not respond to treatment (Pawlotsky, 2003). The mechanisms of IFN treatment failure remain partly unknown. One of these mechanisms could be the blocking of IFN signalling pathways by HCV proteins via the interaction with host cell proteins in order to establish an infection (Weber, 2007). Little is known about the host factors that have a role in establishment of chronic infection. Therefore, there is a need to identify host cell proteins involved in viral replication and persistence.

Work presented in this thesis aimed to identify cellular proteins that can interact with NS5B and could have a role in the viral replication, persistence and/or pathogenesis. The thesis work has made a number of observations that form the basis for future work on new interactions for NS5B. These interactions and the results obtained from the work on two of them (PLSCR1 and ZNF143) have suggested that NS5B, in addition to its role in RNA replication, may have an important role in virus persistence and pathogenesis by modulation of cellular functions of these proteins. The results in this study have suggested that NS5B may be involved in interfering with IFN signalling pathways in part by the interaction with PLSCR1 and ZNF143. Results have indicated for the first time that NS5B also associates with tubulin, indirectly, and that this association is important for viral replication.

7.7 Future Work

As the identification of novel interactions for NS5B represents an early stage of the study, a significant work is required to confirm and determine the biological consequences of these interactions. Some focus for future investigations could include:

1. The interaction of NS5B with PLSCR1 or ZNF143 may change the cellular localization of these proteins; indeed the cellular proteins may influence the cellular distribution of NS5B. The co-localization of NS5B with PLSCR1 and ZNF143 under normal, IFN or EGF stimulation conditions could be studied using confocal microscopy.
2. The interaction of NS5B with ZNF143 may affect the binding of ZNF143 to the IRF3 promoter. Using the electrophoretic mobility shift assay (EMSA) the binding of ZNF143 to the IRF3 promoter in the presence or absence of NS5B could be investigated. The effect of NS5B on the transcriptional activity of the IRF3 promoter could be studied with a reporter construct containing the IRF3 promoter which drives the expression of luciferase.
3. To further the biological significance, the effect of PLSCR1 and ZNF143 on the lifecycle of JFH1 virus system i.e whole virus, should be considered. These investigations would determine whether they have any role in the virus life cycle such as entry, maturation and release and on subsequent infectivity.
4. An examination of the effect of the NS5B/PLSCR1 interaction on the activation of EGFR, Shc and c-Src kinase is important to determine if NS5B affects the role of PLSCR1 in the EGFR signalling pathway. This could be carried out by investigating the phosphorylation status of EGFR, Shc, and c-Src in the presence of NS5B and whether this is mediated by PLSCR1 using knockout or shRNA studies.
5. An investigation of the effect of α- and β-tubulin on NS5B polymerase activity could be performed by using an *in vitro* polymerase assay. It is possible that the addition of tubulin have the effect of increasing enzyme activity which can confirm that tubulin is part of the viral RNA replication

complex. Work, using further proteomic studies, is needed to identify the cellular partner(s) that mediates the interaction with tubulin.

6. As it was observed in Section 6.5 there were different protein profiles between cell lines. This should be extended to other cell lines/types and possibly to primary cell lines to identify hepatic specific proteins that may be involved in the disease process.

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APPENDICES

9.1 Bacterial Culture Media

MEDIA	COMPOSITION	APPLICATION
Luria-Bertani (LB)	10 g/L NaCl 5 g/L Yeast Extract 10 g/L Bacto-tryptone (20 g/L Bacto-agar for plates)	*E. coli* culture
2x YT	5 g/L NaCl 10 g/L Yeast Extract 16 g/L Bacto-tryptone (20 g/L Bacto-agar for plates)	*E. coli* culture
NZY^+ Broth	10 g/L NZ amine (Casein Hydrolysate), 5 g/LYeast Extract, 5 g/L NaCl, NaOH to pH 7.5. Then the following filer-sterilized supplements were added prior to use 12.5 ml of 1 M MgCl2, 12.5 ml of 1 M MgSO4, and 20 ml of 20% (w/v) glucose.	*E. coli XL1-Blue* culture in Mutagenesis Protocol

9.2 DNA Manipulation

TAE (Tris/Acetate/EDTA) Buffer (1X)

COMPONENTS	CONCENTRATION
Tris Base	40 mM
EDTA	2 mM
Glacial Acetic Acid	0.1%

DNA Loading Buffer (6X)

COMPONENTS	CONCENTRATION
Bromophenol blue	0.25 g
100 % glycerol	3 ml
TAE (1X)	7 ml

1% Agarrose Gel

COMPONENTS	CONCENTRATION
Agarose	1 g
Ethidium Bromide	0.5 µg/ml
TAE (1X)	100 ml

Sodium Acetate (3M)

COMPONENTS	CONCENTRATION
Sodium Acetate Trihydrate (NaC2H3O2.3H2O)	408 g
dH_2O	To 1000 ml
Acetic Acid (3M)	To pH 5

9.3 RNA Working Solutions

DEPC-Treated Water

COMPONENTS	CONCENTRATION
DEPC	0.5 ml
dH_2O	1000 ml

The solution was shaked vigorously to dissolve the DEPC and incubated O/N at 37°C then autoclaved for 45 min at 121 °C to inactivate the remaining DEPC.

MOPS Buffer (10X)

COMPONENTS	CONCENTRATION
MOPS	0.2 M
Sodium Acetate	50 mM
EDTA	5 mM
dH_2O	To 1000 ml
NaOH (1M)	To pH 7.0

1% Denatured Agarose Gel (50 ml)

COMPONENTS	CONCENTRATION
Agarose	0.5 g
dH_2O	37 ml
MOPS (10X)	5 ml
Formaldehyde (40%)	8.75 ml

9.4 Protein Manipulation Solutions

9.4.1 SDS-PAGE Buffers

All reagents used in SDS-PAGE were purchased from Flowgen except the lysis buffer.

Bacterial Lysis Buffer

COMPONENTS	CONCENTRATION
Tris-HCl (pH 8.0)	20 mM
NaCl	500 mM
Glycerol	10 % (v/v)
Triton-X100	1 % (v/v)
β-Mercaptoethanol	1 mM
Imidazole	10 mM
Lysozyme	Flakes

Resolving Buffer (pH 8.8)

COMPONENTS	CONCENTRATION
Tris-HCl	1.5 M
SDS	0.384 %

Stacking Buffer (pH 6.8)

COMPONENTS	CONCENTRATION
Tris-HCl	0.5 M
SDS	0.4 %

30% Acrylamide

COMPONENTS	CONCENTRATION
Acrylamide	30 % (w/v)
Bis-Acrylamide	0.8 % (w/v)

Tris-Glycine Acrylamide Gel

A) 10% Acrylamide Separating Gel

COMPONENTS	CONCENTRATION
30% Acrylamide	3.33 ml
Resolving Buffer	2.6 ml
dH_2O	4.1 ml
Ammonium Persulphate (APS) (10%)	100 µl
TEMED	10 µl

B) 5% Acrylamide Stacking Gel

COMPONENTS	CONCENTRATION
30 % Acrylamide	0.65 ml
Stacking Buffer	1.25 ml
dH_2O	3 ml
Ammonium Persulfate (APS) (10 %)	25 µl
TEMED	5 µl

SDS Running Buffer (10X)

COMPONENTS	CONCENTRATION
Tris Base	0.25 M
Glycine	1.92 M
SDS	1 %
dH_2O	1000 ml

SDS Gel Loading Buffer (2X)

COMPONENTS	CONCENTRATION
Tris-HCl (pH 6.8)	0.125 M
SDS	4 %
β-Mercaptoethanol	10 %
Glycerol	20 %
Bromophenol Blue	20 mg

Coomassie Blue Stain

COMPONENTS	CONCENTRATION
Coomassie Brilliant Blue R-250	0.25 % (w/v)
Acetic Acid	10 % (v/v)
Methanol	10 % (v/v)

De-Stain Solution

COMPONENTS	CONCENTRATION
Acetic Acid	10% (v/v)
Methanol	10% (v/v)
dH_2O	1000 ml

9.4.2 Western Blot Buffers

Western Blot Transfer Buffer (pH 8.3)

COMPONENTS	CONCENTRATION
Tris Base	3 g
Methanol	200 ml
Glycine	14.5 g
HCl (Conc)	0.3 ml
dH_2O	1000 ml

TBS (Tris-Buffered Saline)

COMPONENTS	CONCENTRATION
Tris-HCl (pH 7.5)	100 mM
NaCl	0.9 % (w/v)
dH_2O	1000 ml

ECL (Enhanced Chemiluminescence) Reagents

Solution (A)

COMPONENTS	CONCENTRATION
Luminol*	2.5 mM
Coumaric Acid"	0.4 mM
Tris-HCl (pH 8.5)	0.1 M

*Luminol (3-aminophthalhydrazide) 250 mM stock in DMSO
"p-Coumaric Acid 90 mM stock in DMSO

Solution (B)

COMPONENTS	CONCENTRATION
30 % Hydrogen Peroxide (H_2O_2)	0.02 % (v/v)
Tris-HCl (pH 8.5)	0.1 M

9.4.3 ELISA Solutions

Bicarbonate/Carbonate Coating Buffer (pH 9.6):

COMPONENTS	CONCENTRATION
Na_2CO_3	3.03 g
$NaHCO_3$	6 g
dH_2O	1000 ml

PBS (pH 7.4)

COMPONENTS	CONCENTRATION
Na_2HPO_4	1.16 g
KCl	0.1 g
K_3PO_4	0.1 g
NaCl	4 g
dH_2O	500 ml

9.5 Yeast Two-Hybrid Screening Solutions

YPDA Medium

COMPONENTS	CONCENTRATION
Difco Peptone	20 g/L
Yeast Extract	10 g/L
Adenine Hemisulfate (0.2 %)	15 ml
dH_2O	To 950 ml

The pH was adjusted to 6.5, and then autoclaved at 121°C for 15 min. Medium was allowed to cool to ~50°C then 50 ml of a sterile 40 % glucose stock solution. For YPDA plates, 20 g/L of agar was added to the above components before autoclaving.

Dropout (DO) Solution (10X)

The following amino acids were combined and dissolved in 1000 ml dH_2O and then autoclaved.

COMPONENTS	CONCENTRATION
L-Adenine Hemisulfate salt	200 mg/L
L-Arginine HCl	200 mg/L
L-Histidine HCl Monohydrate	200 mg/L
L-Isoleucine	300 mg/L
L-Leucine	1000 mg/L
L-Lysine HCl	300 mg/L
L-Methionine	200 mg/L
L-Phenylalanine	500 mg/L
L-Threonine	2000 mg/L
L-Tryptophan	200 mg/L
L-Tyrosine	300 mg/L
L-Uracil	200 mg/L
L-Valine	1500 mg/L

For preparation of 10X -Leu DO solution, all the above amino acids were combined except L-Leucine and dissolved in 1L dH_2O and then autoclaved.

SD Medium

COMPONENTS	CONCENTRATION
Yeast Nitrogen Base without amino acids	6.7 g
10X Dropout Solution	100 ml
dH_2O	850 ml

The pH was adjusted to 5.8 and autoclaved then cooled down to 50°C before adding glucose to a final concentration of 2 % as a carbon source.

10X TE (Tris/HCl)

COMPONENTS	CONCENTRATION
Tris-HCl (pH 7.5)	0.1 M
EDTA	10 mM

10X LiAc (Lithium Acetate)

COMPONENTS	CONCENTRATION
Lithium Acetate	1 M
Acetic Acid	To pH 7.5

PEG/LiAc Solution

COMPONENTS	CONCENTRATION
50 % PEG 3350 (Sigma)	8 ml
TE Buffer	1X
LiAc	1X

Cracking Buffer Stock Solution

COMPONENTS	CONCENTRATION
Tris-HCl (pH 6.8)	40 mM
Urea	8 M
SDS	5 % (w/v)
EDTA	0.1 mM
Bromophenol Blue	0.4 mg/ml
dH_2O	Variable

Working Solution of Cracking Buffer (1.3 ml)

COMPONENTS	CONCENTRATION
Cracking Stock Solution	1 ml
β-Mercaptoethanol	10 µl
Protease Inhibitor Solution	70 µl

X-α-Gal (5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside)

X-α-Gal stock solution of 20 mg/ml in dimethylformamide was prepared and stored at -20°C in the dark.

9.6 Tissue Culture Solutions

DMEM (pH 7.0)

COMPONENTS	CONCENTRATION
DMEM Powder (GIBCO)(Low Glucose)	10 g
$NaHCO_3$	3.7 g
Glucose	3.5 g
dH_2O	To 1000 ml

The medium was filter sterilized using 0.22 µm filter and stored at 4°C in 500 ml flasks.

Calcium Phosphate Transfection Reagents

HBS (2X) (pH 7.11)

COMPONENTS	CONCENTRATION
NaCl	280 mM
HEPES	100 mM
Na_2HPO_4	1.5 mM

TE (0.1X)

COMPONENTS	CONCENTRATION
Tris-HCl (pH 8.8)	1mM
EDTA	0.1 mM

9.7 ORFs Sequences Used in This Study

9.7.1 Full Length HCV NS5B ORF Sequence (pCV-H77c genotype 1a, AF011751)

TCAATGTCTTATTCCTGGACAGGCGCACTCGTCACCCCGTGCGCTGCGGAAGAACAAAA
ACTGCCCATCAACGCACTGAGCAACTCGTTGCTACGCCATCACAATCTGGTGTATTCCA
CCACTTCACGCAGTGCTTGCCAAAGGCAGAAGAAAGTCACATTTGACAGACTGCAAGTT
CTGGACAGCCATTACCAGGACGTGCTCAAGGAGGTCAAAGCAGCGGCGTCAAAAGTGAA
GGCTAACTTGCTATCCGTAGAGGAAGCTTGCAGCCTGACGCCCCCACATTCAGCCAAAT
CCAAGTTTGGCTATGGGGCAAAAGACGTCCGTTGCCATGCCAGAAAGGCCGTAGCCCAC
ATCAACTCCGTGTGGAAAGACCTTCTGGAAGACAGTGTAACACCAATAGACACTACCAT
CATGGCCAAGAACGAGGTTTTCTGCGTTCAGCCTGAGAAGGGGGGTCGTAAGCCAGCTC
GTCTCATCGTGTTCCCCGACCTGGGCGTGCGCGTGTGCGAGAAGATGGCCCTGTACGAC
GTGGTTAGCAAGCTCCCCCTGGCCGTGATGGGAAGCTCCTACGGATTCCAATACTCACC
AGGACAGCGGGTTGAATTCCTCGTGCAAGCGTGGAAGTCCAAGAAGACCCCGATGGGGT
TCTCGTATGATACCCGCTGTTTTGACTCCACAGTCACTGAGAGCGACATCCGTACGGAG
GAGGCAATTTACCAATGTTGTGACCTGGACCCCCAAGCCCGCGTGGCCATCAAGTCCCT
CACTGAGAGGCTTTATGTTGGGGGCCCTCTTACCAATTCAAGGGGGGAAAACTGCGGCT
ACCGCAGGTGCCGCGCGAGCGGCGTACTGACAACTAGCTGTGGTAACACCCTCACTTGC
TACATCAAGGCCCGGGCAGCCTGTCGAGCCGCAGGGCTCCAGGACTGCACCATGCTCGT
GTGTGGCGACGACTTAGTCGTTATCTGTGAAAGTGCGGGGGTCCAGGAGGACGCGGCGA
GCCTGAGAGCCTTCACGGAGGCTATGACCAGGTACTCCGCCCCCCCCGGGGACCCCCCA
CAACCAGAATACGACTTGGAGCTTATAACATCATGCTCCTCCAACGTGTCAGTCGCCCA
CGACGGCGCTGGAAAGAGGGTCTACTACCTTACCCGTGACCCTACAACCCCCCTCGCGA
GAGCCGCGTGGGAGACAGCAAGACACACTCCAGTCAATTCCTGGCTAGGCAACATAATC
ATGTTTGCCCCCACACTGTGGGCGAGGATGATACTGATGACCCATTTCTTTAGCGTCCT
CATAGCCAGGGATCAGCTTGAACAGGCTCTTAACTGTGAGATCTACGGAGCCTGCTACT
CCATAGAACCACTGGATCTACCTCCAATCATTCAAAGACTCCATGGCCTCAGCGCATTT
TCACTCCACAGTTACTCTCCAGGTGAAATCAATAGGGTGGCCGCATGCCTCAGAAAACT
TGGGGTCCCGCCCTTGCGAGCTTGGAGACACCGGGCCCGGAGCGTCCGCGCTAGGCTTC
TGTCCAGAGGAGGCAGGGCTGCCATATGTGGCAAGTACCTCTTCAACTGGGCAGTAAGA
ACAAAGCTCAAACTCACTCCAATAGCGGCCGCTGGCCGGCTGGACTTGTCCGGTTGGTT
CACGGCTGGCTACAGCGGGGGAGACATTTATCACAGCGTGTCTCATGCCCGGCCCCGCT
GGTTCTGGTTTTGCCTACTCCTGCTCGCTGCAGGGGTAGGCATCTACCTCCTCCCCAAC
CGATGA

9.7.2 Full-Length PLSCR1 ORF Sequence (AF008445)

ATGGACAAACAAAACTCACAGATGAATGCTTCTCACCCGGAAACAAACTTGCCAGTTGG
GTATCCTCCTCAGTATCCACCGACAGCATTCCAAGGACCTCCAGGATATAGTGGCTACC
CTGGGCCCCAGGTCAGCTACCCACCCCCACCAGCCGGCCATTCAGGTCCTGGCCCAGCT
GGCTTTCCTGTCCCAAATCAGCCAGTGTATAATCAGCCAGTATATAATCAGCCAGTTGG
AGCTGCAGGGGTACCATGGATGCCAGCGCCACAGCCTCCATTAAACTGTCCACCTGGAT
TAGAATATTTAAGTCAGATAGATCAGATACTGATTCATCAGCAAATTGAACTTCTGGAA
GTTTTAACAGGTTTTGAAACTAATAACAAATATGAAATTAAGAACAGCTTTGGACAGAG
GGTTTACTTTGCAGCGGAAGATACTGATTGCTGTACCCGAAATTGCTGTGGGCCATCTA
GACCTTTTACCTTGAGGATTATTGATAATATGGGTCAAGAAGTCATAACTCTGGAGAGA
CCACTAAGATGTAGCAGCTGTTGTTGTCCCTGCTGCCTTCAGGAGATAGAAATCCAAGC
TCCTCCTGGTGTACCAATAGGTTATGTTATTCAGACTTGGCACCCATGTCTACCAAAGT
TTACAATTCAAAATGAGAAAAGAGAGGATGTACTAAAAATAAGTGGTCCATGTGTTGTG
TGCAGCTGTTGTGGAGATGTTGATTTTGAGATTAAATCTCTTGATGAACAGTGTGTGGT
TGGCAAAATTTCCAAGCACTGGACTGGAATTTTGAGAGAGGCATTTACAGACGCTGATA
ACTTTGGAATCCAGTTCCCTTTAGACCTTGATGTTAAAATGAAAGCTGTAATGATTGGT
GCCTGTTTCCTCATTGACTTCATGTTTTTTGAAAGCACTGGCAGCCAGGAACAAAAATC
AGGAGTGTGGTAG

9.7.3 Full-length ZNF143 ORF Sequence (NM_003442)

ATGTTGTTAGCCCAAATAAATCGAGATTCTCAGGGAATGACAGAGTTTCCTGGAGGAGG
GATGGAGGCGCAACATGTTACGCTGTGCTTGACAGAGGCAGTCACCGTGGCAGATGGTG
ACAACTTAGAAAATATGGAAGGCGTAAGCTTGCAAGCAGTAACACTTGCAGATGGTTCT
ACTGCTTACATACAACACAATTCTAAAGATGCAAAACTCATAGATGGCCAGGTCATTCA
GTTGGAAGATGGTTCTGCGGCCTATGTTCAACATGTACCCATACCTAAAAGTACAGGGG
ACAGTTTGCGTCTAGAGGATGGTCAAGCAGTACAGTTAGAAGATGGTACCACAGCATTT
ATTCACCACACCTCCAAAGATAGTTATGACCAGAGTGCATTACAGGCGGTTCAGCTGGA
AGATGGTACCACAGCTTATATCCACCATGCAGTGCAAGTCCCGCAGTCTGACACCATCT
TGGCAATTCAGGCTGATGGGACAGTGGCAGGTCTGCACACTGGGGATGCTACAATTGAC
CCTGACACCATCAGTGCTTTGGAACAGTATGCAGCAAAGGTGTCCATTGATGGAAGTGA
AAGTGTAGCAGGTACTGGAATGATTGGAGAAAATGAGCAAGAGAAAAAAATGCAGATTG
TTTTACAAGGACATGCTACAAGAGTAACTGCTAAATCTCAACAGAGTGGAGAGAAGGCA
TTTCGATGTGAATATGATGGATGTGGAAAATTATATACAACAGCTCATCATCTCAAGGT
CCATGAGAGGTCACACACAGGAGATCGGCCTTATCAGTGTGAGCATGCAGGCTGTGGGA
AGGCATTTGCAACAGGTTATGGATTAAAAAGTCACGTCAGAACTCATACAGGAGAAAAG
CCATATCGGTGTTCGGAAGATAATTGTACTAAATCTTTCAAAACTTCAGGAGATCTACA
GAAACACATCAGAACTCATACAGGAGAAAGGCCCTTTAAGTGTCCCTTCGAAGGCTGCG
GTCGGTCCTTTACAACATCAAATATCAGAAAAGTGCACGTTAGGACACACACAGGAGAA
AGACCTTATTACTGCACAGAGCCAGGATGTGGGAGGGCATTTGCCAGTGCAACAAATTA
TAAAAACCATGTGAGGATACACACAGGAGAAAAGCCATATGTTTGTACAGTTCCTGGGT
GTGACAAAAGGTTTACAGAATATTCCAGTTTGTACAAACATCATGTTGTCCACACTCAT
TCCAAACCTTACAACTGTAACCACTGTGGGAAGACATACAAGCAGATCTCCACGCTGGC
CATGCACAAACGGACAGCCCACAACGACACTGAGCCCATCGAGGAGGAGCAGGAAGCCT
TCTTTGAGCCGCCCCCAGGTCAAGGTGAAGATGTTCTTAAAGGGTCCCAGATTACGTAT
GTTACAGGTGTAGAAGGGGACGACGTTGTTTCTACACAAGTAGCCACAGTAACCCAATC
TGGACTGAGTCAACAAGTTACACTCATATCCCAGGATGGGACTCAGCATGTCAACATAT
CTCAAGCTGACATGCAGGCCATTGGCAACACCATCACAATGGTAACGCAGGATGGCACG
CCCATCACAGTCCCCGCCCATGATGCAGTCATCTCCTCAGCAGGAACGCACTCTGTTGC
TATGGTTACTGCTGAGGGTACAGAAGGGGAACAGGTTGCAATTGTAGCTCAAGACTTGG
CAGCATTCCATACTGCCTCATCAGAAATGGGGCACCAGCAGCATAGCCATCACTTAGTA
ACCACAGAAACCAGACCTCTGACCTTAGTAGCAACATCCAATGGCACCCAGATTGCAGT
TCAGCTTGGAGAACAGCCATCTCTGGAAGAAGCCATCAGAATAGCGTCTAGAATCCAAC
AAGGAGAAACGCCAGGGTTGGATGATTAA

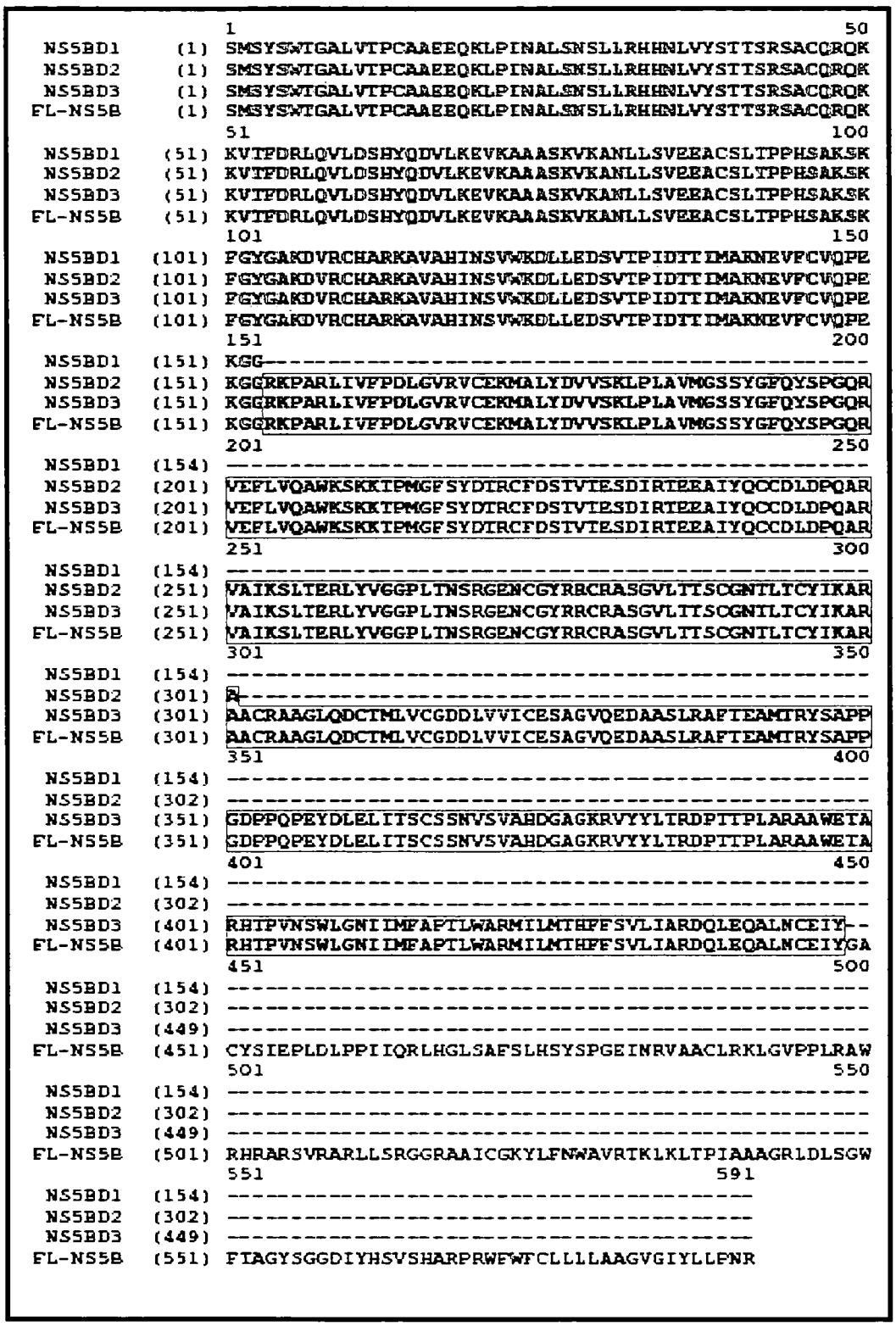
9.7.4 Full-length β-Tubulin ORF Sequence (BC020946)

ACCATGAGGGAAATCGTGCACATCCAGGCTGGTCAGTGTGGCAACCAGATCGGTGCCAA
GTTCTGGGAGGTGATCAGTGATGAACATGGCATCGACCCCACCGGCACCTACCACGGGG
ACAGCGACCTGCAGCTGGACCGCATCTCTGTGTACTACAATGAAGCCACAGGTGGCAAA
TATGTTCCTCGTGCCATCCTGGTGGATCTAGAACCTGGGACCATGGACTCTGTTCGCTC
AGGTCCTTTTGGCCAGATCTTTAGACCAGACAACTTTGTATTTGGTCAGTCTGGGGCAG
GTAACAACTGGGCCAAAGGCCACTACACAGAGGGCGCCGAGCTGGTTGATTCTGTCCTG
GATGTGGTACGGAAGGAGGCAGAGAGCTGTGACTGCCTGCAGGGCTTCCAGCTGACCCA
CTCACTGGGCGGGGGCACAGGCTCTGGAATGGGCACTCTCCTTATCAGCAAGATCCGAG
AAGAATACCCTGATCGCATCATGAATACCTTCAGTGTGGTGCCTTCACCCAAAGTGTCT
GACACCGTGGTCGAGCCCTACAATGCCACCCTCTCCGTCCATCAGTTGGTAGAGAATAC
TGATGAGACCTATTGCATTGACAACGAGGCCCTCTATGATATCTGCTTCCGCACTCTGA
AGCTGACCACACCAACCTACGGGGATCTGAACCACCTTGTCTCAGCCACCATGAGTGGT
GTCACCACCTGCCTCCGTTTCCCTGGCCAGCTCAATGCTGACCTCCGCAAGTTGGCAGT
CAACATGGTCCCCTTCCCACGTCTCCATTTCTTTATGCCTGGCTTTGCCCCTCTCACCA
GCCGTGGAAGCCAGCAGTATCGAGCTCTCACAGTGCCGGAACTCACCCAGCAGGTCTTC
GATGCCAAGGACATGATGGCTGCCTGTGACCCCCGCCACGGCCGATACCTCACCGTGGC
TGCTGTCTTCCGTGGTCGGATGTCCATGAAGGAGGTCGATGAGCAGATGCTTAACGTGC
AGAACAAGAACAGCAGCTACTTTGTGGAATGGATCCCCAACAATGTCAAGACAGCCGTC
TGTGACATCCCACCTCGTGGCCTCAAGATGGCAGTCACCTTCATTGGCAATAGCACAGC
CATCCAGGAGCTCTTCAAGCGCATCTCGGAGCAGTTCACTGCCATGTTCCGCCGGAAGG
CCTTCCTCCACTGGTACACAGGCGAGGGCATGGACGAGATGGAGTTCACCGAGGCTGAG
AGCAACATGAACGACCTCGTCTCTGAGTATCAGCAGTACCAGGATGCCACCGCAGAAGA
GGAGGAGGATTTCGGTGAGGAGGCCGAAGAGGAGGCCTAA

9.8 PLSCR1 Domains' Protein Sequences

```
                  1                                                  50
  PLSCRD1     (1) MDKQNSQMNASHPETNLPVGYPPQYPPTAFQGPPGYSGYPGPQVSYPPPP
  PLSCRD2     (1) MDKQNSQMNASHPETNLPVGYPPQYPPTAFQGPPGYSGYPGPQVSYPPPP
  PLSCRD3     (1) MDKQNSQMNASHPETNLPVGYPPQYPPTAFQGPPGYSGYPGPQVSYPPPP
FL-PLSCR1     (1) MDKQNSQMNASHPETNLPVGYPPQYPPTAFQGPPGYSGYPGPQVSYPPPP
                  51                                                 100
  PLSCRD1    (51) AGHSGPGPAG----------------------------------------
  PLSCRD2    (51) AGHSGPGPAGFPVPNQPVYNQPVYNQPVGAAGVPWMPAPQPPLNCPPGLE
  PLSCRD3    (51) AGHSGPGPAGFPVPNQPVYNQPVYNQPVGAAGVPWMPAPQPPLNCPPGLE
FL-PLSCR1    (51) AGHSGPGPAGFPVPNQPVYNQPVYNQPVGAAGVPWMPAPQPPLNCPPGLE
                 101                                                 150
  PLSCRD1    (61) ----------------------------------------------------
  PLSCRD2   (101) YLSQIDQILIHQQIELLEVLTGFETNNKYEIKNSFGQ----------
  PLSCRD3   (101) YLSQIDQILIHQQIELLEVLTGFETNNKYEIKNSFGQRVYFAAEDTDCCT
FL-PLSCR1   (101) YLSQIDQILIHQQIELLEVLTGFETNNKYEIKNSFGQRVYFAAEDTDCCT
                 151                                                 200
  PLSCRD1    (61) ----------------------------------------------------
  PLSCRD2   (138) ----------------------------------------------------
  PLSCRD3   (151) RNCCGPSRPFTLRIIDNMGQEVITLERPLRCSSCCCPCCLQE--------
FL-PLSCR1   (151) RNCCGPSRPFTLRIIDNMGQEVITLERPLRCSSCCCPCCLQEIEIQAPPG
                 201                                                 250
  PLSCRD1    (61) ----------------------------------------------------
  PLSCRD2   (138) ----------------------------------------------------
  PLSCRD3   (193) ----------------------------------------------------
FL-PLSCR1   (201) VPIGYVIQTWHPCLPKFTIQNEKREDVLKISGPCVVCSCCGDVDFEIKSL
                 251                                                 300
  PLSCRD1    (61) ----------------------------------------------------
  PLSCRD2   (138) ----------------------------------------------------
  PLSCRD3   (193) ----------------------------------------------------
FL-PLSCR1   (251) DEQCVVGKISKHWIGILREAFTDADNFGIQFPLDLDVKMKAVMIGACFLI
                 301            318
  PLSCRD1    (61) -----------------
  PLSCRD2   (138) -----------------
  PLSCRD3   (193) -----------------
FL-PLSCR1   (301) DFMFFESTGSQEQKSGVW
```

9.9 NS5B domains' Protein Sequences



9.10 V5H Sequence Used to Build pWPXL-V5H Construct

Poly-His tag

```
5′ ga tct acc atg ggt cat cac cat cac cat cac cat cac
3′       a tgg tac cca gta gtg gta gtg gta gtg gta gtg
```

V5 epitope

```
cat cac ggt aag cct atc cct aac cct ctc ctc ggt ctc gat
gta gtg cca ttc gga tag gga ttg gga gag gag cca gag cta
```

(MCS)

BamHI ***SmaI*** ***EcoRI***

```
Tct acg gat ccc cgg gaa ttc a       3′
Aga tgc cta ggg gcc ctt aag tgg cc  5′
```

Two oligonucleotides were annealed together to form a dsDNA insert with start codon, His-tag, and V5 epitope-tag at the N-terminus of multiple cloning sites (MCS). Start codon is presented in green colour. Poly-His tag sequence is presented in bold black. V5 epitop sequence is presented in blue and the multiple cloning sites are presented in red.

9.11 Oligonucleotides and Primers

Oligonucleotides and primers used in this study were synthesized by Sigma. Primers were reconstituted in distilled water to a stock concentration of 100 µM. Oligonucleotides and primers used in this thesis are listed in Table 9.1 and Table 9.2.

Table 9.1: Primers and Oligonucleotides used in this study

CONSTRUCT NAME	INSERT	AMINO ACID	TAG AND ITS LOCATION	CLONING SITES	PRIMERS SEQUENCES
pGBK-5B (PCR Cloning)	NS5B	1-591	DNA-BD N-terminus	*BamHI* *SalI*	CACGGATCCGGACCATGTCAATGTCTTATTCCTGG CAGCGTCGACTTATCATCGGTTGGGGAGGAGGTAG
pGBK-5BD1 (PCR Cloning)	NS5BD1	1-153	DNA-BD N-terminus	*BamHI* *SalI*	CACGGATCCGGACCATGTCAATGTCTTATTCCTGG GTAGTCGACACCCCCCTTCTCAGGCTGAACGCAGA
pGBK-5BD2 (PCR Cloning)	NS5BD2	1-301	DNA-BD N-terminus	*BamHI* *SalI*	CACGGATCCGGACCATGTCAATGTCTTATTCCTGG GTAGTCGACTGCCCGGGCCTTGATGTAGCAAGTGA
pGBK-5BD3 (PCR Cloning)	NS5BD3	1-448	DNA-BD N-terminus	*BamHI* *SalI*	CACGGATCCGGACCATGTCAATGTCTTATTCCTGG GTAGTCGACGTAGATCTCACAGTTAAGAGCCTGTT
pET21-5B (PCR Cloning)	tNS5B	1-570	His-tag C-terminus	*BamHI* *XhoI*	CACGGATCCGGACCATGTCAATGTCTTATTCCTGG CAGCCTCGAGTTATCATCGGTTGGGGAGGAGGTAG
pET21-5BD1 (PCR Cloning)	NS5BD1	1-153	His-tag C-terminus	*BamHI* *SalI*	CACGGATCCGGACCATGTCAATGTCTTATTCCTGG GTAGTCGACACCCCCCTTCTCAGGCTGAACGCAGA
pET21-5BD2 (PCR Cloning)	NS5BD2	1-301	His-tag C-terminus	*BamHI* *SalI*	CACGGATCCGGACCATGTCAATGTCTTATTCCTGG GTAGTCGACTGCCCGGGCCTTGATGTAGCAAGTGA
pNTAP-5B (PCR Cloning)	NS5B	1-591	Streptavidin N-terminus	*BamHI* *BamHI*	CTACGGATCCTACCATGTCAATGTCTTATTCCTGG CAGCGGATCCTTATCATCGGTTGGGGAGGAGGTAG
pcDNA4-5B (PCR Cloning)	NS5B	1-591	His-tag N-terminus	*BamHI* *BamHI*	CTACGGATCCTACCATGTCAATGTCTTATTCCTGG CAGCGGATCCTTATCATCGGTTGGGGAGGAGGTAG

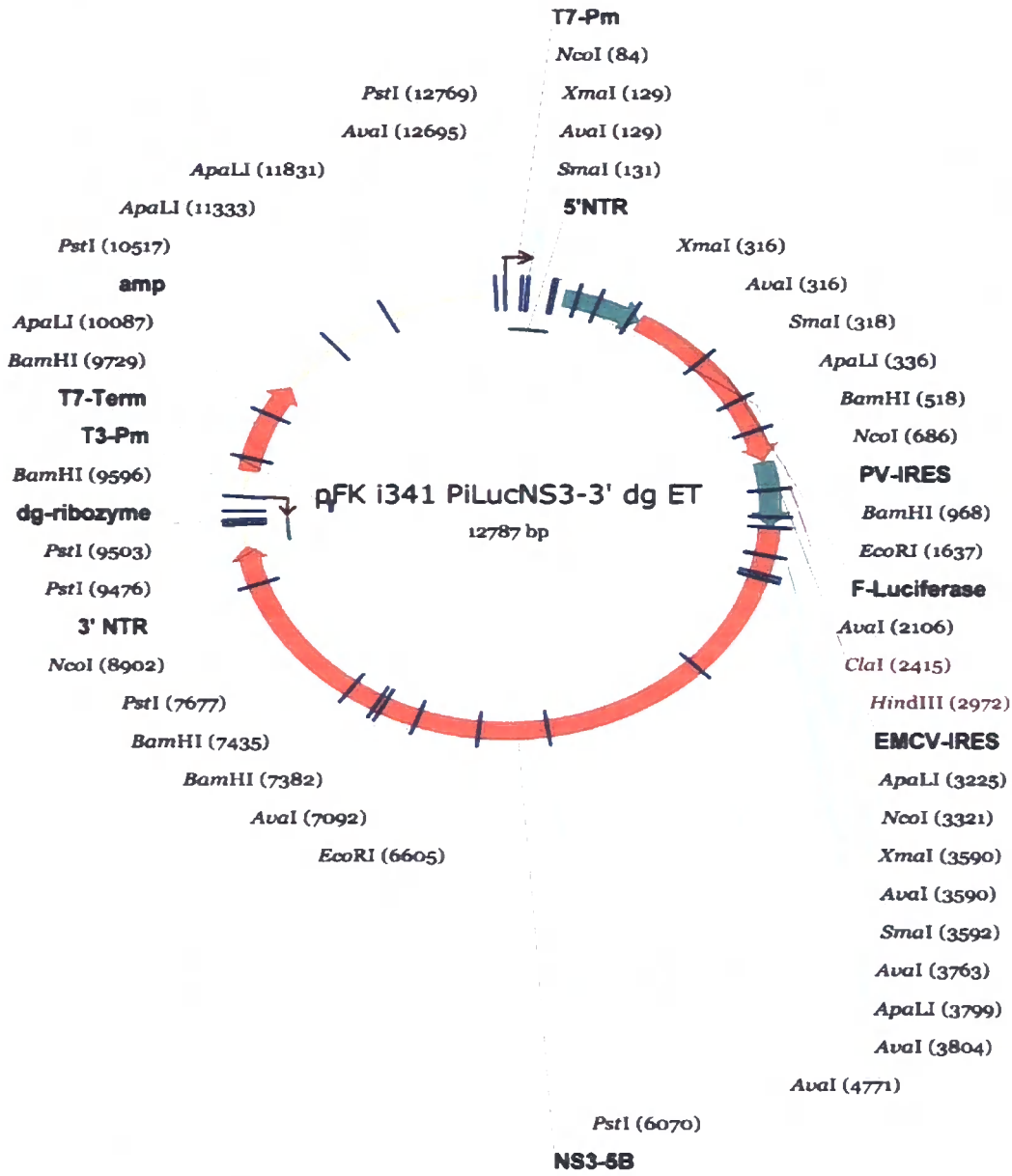
pWPXL-5B (PCR Cloning)	NS5B	1-591	–	*BamHI* *SpeI*	CTACGGATCCTACCATGTCAATGTCTTATTCCTGG CAGCACTAGTTTATCATCGGTTGGGGAGGAGGTAG
pGAD-SCR1 (Nested RT-PCR Cloning)	PLSCR1	1-318	DNA-AD N-terminus	*Nested* *EcoRI* *XhoI*	**F:** GACCGAAACCAGGAGCCGCGG **R:** CAACCAGAGCTACAGGCCTTA CACGAATTCCGGGAGCGGAAACAGCGGCAGCCAGA GGCCTCGAGCTGAGGAGACTTTCACTAATCCACTA
pGAD-SCRD1 (Mutagenesis)	PLSCRD1	1-60	DNA-AD N-terminus	*Muta-genesis*	**F:**CCCAGCTGGCTAATAAGAGCTCAATCAGCCAG **R:**CTGGCTGATTGAGCTCTTATTAGCCAGCTGGG
pGAD-SCRD2 (Mutagenesis)	PLDCRD2	1-137	DNA-AD N-terminus	*Muta-genesis*	**F:**CTTTGGACAGTAATAAGAGCTCGCAGCGGAAG **R:**CTTCCGCTGCGAGCTCTTATTACTGTCCAAAG
pGAD-SCRD3 (Mutagenesis)	PLSCRD3	1-192	DNA-AD N-terminus	*Muta-genesis*	**F:**AGAGGATGTATAATAAGAGCTCGGTCCATGTG **R:**CACATGGACCGAGCTCTTATTATACATCCTCT
pGAD-ZNF143 (Nested RT-PCR Cloning)	ZNF143	1-639	DNA-AD N-terminus	*Nested* *EcoRI* *XhoI*	**F:**CCTGGTGCATGGTGGTCGGAC **R:**TCCTGGGCCCGGGCTTCATGG CGATGAATTCATGTTGTTAGCCCAAATAAATCGAG CGATCTCGAGTTAATCATCCAACCCTGGCGTTTCT
pGEX-SCR1 (PCR Cloning)	PLSCR1	1-318	GST-tag N-terminus	*EcoRI* *XhoI*	CACGAATTCCGGGAGCGGAAACAGCGGCAGCCAGA GGCCTCGAGCTGAGGAGACTTTCACTAATCCACTA
pGEX-ZNF143 (PCR Cloning)	ZNF143	1-639	GST-tag N-terminus	*BamHI* *EcoRI*	CGATGGATCCATGTTGTTAGCCCAAATAAATCGAG CGATGAATTCTTAATCATCCAACCCTGGCGTTTCT
pcDNA4-SCR1 (PCR Cloning)	PLSCR1	1-318	His-tag N-terminus	*EcoRI* *XhoI*	CACGAATTCCGGGAGCGGAAACAGCGGCAGCCAGA GGCCTCGAGCTGAGGAGACTTTCACTAATCCACTA
pcDNA4-ZNF143 (PCR Cloning)	ZNF143	1-639	His-tag N-terminus	*BamHI* *EcoRI*	CGATGGATCCATGTTGTTAGCCCAAATAAATCGAG CGATGAATTCTTAATCATCCAACCCTGGCGTTTCT
pWPXL-V5H (Restriction Cloning)	V5H	1-32	–	*BamHI*	Sense: GATCTACCATGGGTCATCACCATCACCATCACCATCACCATC ACGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTA CGGATCCCCGGGAATTCA

				EcoRI	Anti-sense: CCGGTGAATTCCCGGGGATCCGTAGAATCGAGACCGAGGAGA GGGTTAGGGATAGGCTTACCGTGATGGTGATGGTGATGGTGA TGGTGATGACCCATGGTA
pWPXL-V5H-SCR1 (PCR Cloning)	PLSCR1	1-318	His-tag N-terminus	*BamHI* *EcoRI*	CACGGATCCCATGGACAAACAAACTCACAGATGA GGCGAATTCCTACCACACTCCTGATTTTTGTTCCT
pWPXL-V5H-ZNF143 (PCR Cloning)	ZNF143	1-639	His-tag N-terminus	*BamHI* *EcoRI*	CGATGGATCCCATGTTGTTAGCCCAAATAAATCGA CGATGAATTCTTAATCATCCAACCCTGGCGTTTCT
pLVTHM-shSCR1 (Restriction Cloning)	shPLSCR1	–	–	*MluI* *ClaI*	Sense CGCGTCCCCGGACCTCCAGGATATAGTGTTCAAGAGACACTA TATCCTGGAGGTCCTTTTTGGAAAT Anti-sense CGATTTCCAAAAAGGACCTCCAGGATATAGTGTCTCTTGAAC ACTATATCCTGGAGGTCCGGGGA
pLVTHM-shZNF143 (Restriction Cloning)	shZNF143	–	–	*MluI* *ClaI*	Sense CGCGTCCCCGCCATATCGGTGTTCGGAAGATTCAAGAGATCT TCCGAACACCGATATGGCTTTTTGGAAAT Anti-sense CGATTTCCAAAAAGCCATATCGGTGTTCGGAAGATCTCTTGA ATCTTCCGAACACCGATATGGCGGGGA
pGAD-Tub (Nested RT-PCR Cloning)	β-Tubulin	1-446	DNA-AD N-terminus	*Nested* *EcoRI* *XhoI*	F: TCCAGCCTGCGACCTGCGGAG R: CAAGATAGAGGCAGCAAACAC CGAGAATTCACCATGAGGGAAATCGTGCACATCCA CGACTCGAGTTAGGCCTCCTCTTCGGCCTCCTCAC
pGEX-Tub (PCR Cloning)	β-Tubulin	1-446	GST-tag N-terminus	*EcoRI* *XhoI*	CGAGAATTCACCATGAGGGAAATCGTGCACATCCA CGACTCGAGTTAGGCCTCCTCTTCGGCCTCCTCAC

Table 9.2: Sequencing and RT-PCR Screening Primers

PLASMID	SEQUENCE
NS5B (Sequencing)	NS5B-Mid-F: CCACATCAACTCCGTGTG NS5B-Mid-R: CCCTGGCTATGAGGACGC
pGBKT7 (Sequencing)	pGBKT7-Rev: TTTTCGTTTTAAAACCTAAGAGTC
pGADT7 (Sequencing)	AD-F: CTATTCGATGATGAAGATACCCCACCAAAC AD-R: GTGAACTTGCGGGGTTTTTCAGTATCTACG
pNTAP (Sequencing)	pNTAP-F: TGAGGTTTAAACAATTAACCCTCACTAAAGGGAAC pNTAP-R: GAAGTCATATGGTAATACGACTCACTATAGGGCGA
pGEX-6P-3 (Sequencing)	5' pGEX: GGGCTGGCAAGCCACGTTTGGTG 3' pGEX: CCGGGAGCTGCATGTGTCAGAGG
pWPXL or pWPXL-V5H (Sequencing)	pWPXL-F: CCGATCACGAGACTAGCCTCG pWPXL-R: CATAGTTAAGAATACCAGTC
pLVTHM (Sequencing)	pLVTHM-F: GTCGCTATGTGTTCTGG pLVTHM-R: AGAGACCCAGTACAAGC
(Sequencing)	T7 Promoter: CGAAATTAATACGACTCACTATAGG T7 Terminator: ATGCTAGTTATTGCTCAGCGGT
ISG15 (RT-PCR)	ATGGGCTGGGACCTGACGGTG CCTTAGCTCCGCCCGCCAGGC
ISG54 (RT-PCR)	ATGAGTGAGAACAATAAGAAT CCAGAGCCTTCTCAAAGCACA
OAS2 (RT-PCR)	ATGGGAAATGGGGAGTCCCAG AAGGATCTTTTGAGCTCTCGA
IRF7 (RT-PCR)	ATGGCCTTGGCTCCTGAGAGG TGGAGTCCAGCATGTGTGTGT
β-actin (RT-PCR)	GACAACGGCTCCGGCATGTG TGGCTGGGGTGTTGAAGGTC

9.12 HCV Sub-Genomic Replicon Used in This Study



9.13 pLVTHM Used in RNAi Work